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Stress induced lncRNA Lockd
inhibits proliferation and angiogenesis
of myocardial endothelial cells and promotes
maladaptive cardiac remodelling during pressure overload

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Abstract

Myocardial endothelial cells play a pivotal role in modulating the cardiac stress response and influencing heart growth and function. While past research has predominantly focused on endothelial-derived proteins, recent evidence points to the involvement of long non-coding RNAs (lncRNAs) in these processes. This study sought to investigate the significance of the lncRNA Lockd in angiogenesis, endothelial cell function, and cardiac responses during cardiac pressure overload.

Through a systematic examination of differentially regulated lncRNAs in cardiac endothelial cells under pressure overload conditions, Lockd, an intergenic lncRNA downstream of *Cdkn1b* was identified. Lockd expression was notably upregulated in heart tissue during cardiac pressure overload induced by transverse aortic constriction (TAC). It peaked one-week post-TAC in cardiomyocytes and endothelial cells, but not in fibroblasts, gradually returning to basal levels thereafter. This observation suggests Lockd's induction during the compensatory phase of cardiac remodelling. Functional studies of Lockd in endothelial cells revealed that its overexpression suppressed angiogenesis in a sprouting assay, while its knockdown enhanced sprouting activity. Moreover, these effects were more pronounced in the presence of the angiogenic cytokine Fgf2. Additionally, Lockd downregulation led to increased endothelial cell proliferation, whereas its overexpression reduced cell proliferation, as demonstrated by BrdU proliferation assay and phospho-histone-3 staining. RNA sequencing of Lockd knockdown endothelial cells unveiled reduced expression of genes associated with extracellular matrix organization and regulation of angiogenesis, while genes linked to cell cycle progression were upregulated. Analysis of single-cell sequencing data from isolated cardiac endothelial cells revealed Lockd's exclusive presence in a specific cluster associated with cell cycle and cell division. Protein-RNA pull-down assays demonstrated Lockd's binding to ribosomal proteins, possibly influencing the translation of select proteins. Furthermore, Chromatin Immuno RNA precipitation showed Lockd's association with chromatin regions, suggesting its potential direct involvement in gene expression regulation. To validate the functional relevance of Lockd *in vivo*, its upregulation post-TAC using antisense GapmeRs in mice was targeted. This intervention significantly improved systolic cardiac function and reduced hypertrophy compared to control GapmeR-treated mice. Staining of heart tissue post-TAC demonstrated increased capillary density and decreased cardiomyocyte hypertrophy in mice treated with Lockd GapmeRs. RNA sequencing of cardiac endothelial cells one-week post-TAC indicated substantial dysregulation of cell-cycle-related genes upon Lockd knockdown, emphasizing its role in cell cycle progression and proliferation *in vivo*.

In conclusion, these findings suggest that Lockd upregulation inhibits cell-cycle progression, thereby counteracting endothelial proliferation and angiogenesis after TAC. Consequently, Lockd upregulation in heart tissue appears to be detrimental during cardiac pressure overload and cardiac remodelling.

Zusammenfassung

Myokardiale Endothelzellen spielen eine entscheidende Rolle bei der Modulation der kardialen Stressantwort und beeinflussen das Wachstum und die Funktion des Herzens maßgeblich. Während frühere Forschungsarbeiten vor allem endotheliale Proteinen adressierten, gibt es zunehmende Hinweise auf die Beteiligung von langen nichtkodierenden RNAs (lncRNAs) an diesen Prozessen. Diese Studie untersuchte die Bedeutung der lncRNA Lockd für die Angiogenese, die Funktion von Endothelzellen und die kardialen Reaktionen während des kardialen Drucküberlastungszustands. Durch eine systematische Untersuchung differentiell regulierter lncRNAs in kardialen Endothelzellen unter Drucküberlastungsbedingungen identifizierten wir Lockd, eine intergenische lncRNA *downstream* von Cdkn1b. Die Expression von Lockd war während der durch transversale Aortenkonstriktion (TAC) induzierten kardialen Drucküberlastung signifikant erhöht. Sie erreichte ihren Höhepunkt eine Woche nach TAC in Kardiomyozyten und Endothelzellen, nicht jedoch in Fibroblasten, und kehrte anschließend graduell auf Basalniveau zurück. Diese Beobachtung legt nahe, dass Lockd während der kompensatorischen Phase der kardialen Remodellierung induziert wird. Funktionelle Studien an Endothelzellen zeigten, dass die Überexpression von Lockd die Angiogenese in einem „Aussprossungstest“ unterdrückte, während ihr Knockdown die „Aussprossungsaktivität“ verstärkte. Diese Effekte waren in Gegenwart des angiogenen Zytokins Fgf2 ausgeprägter. Zusätzlich führte die Herunterregulierung von Lockd zu einer erhöhten Proliferation der Endothelzellen, während die Überexpression die Zellproliferation reduzierte, wie durch den BrdU-Proliferationstest und die Färbung mit phospho-Histon-3 gezeigt wurde. Die RNA-Sequenzierung von Lockd-Knockdown-Endothelzellen ergab eine verminderte Expression von Genen, die mit der extrazellulären Matrixorganisation und der Regulation der Angiogenese assoziiert sind, während Gene, die mit dem Zellzyklus assoziiert sind, hochreguliert wurden. Die Analyse von Single-Cell-Sequenzierungsdaten von isolierten kardialen Endothelzellen zeigte, dass Lockd ausschließlich in einem spezifischen Cluster vorhanden war, das mit dem Zellzyklus und der Zellteilung assoziiert ist. Protein-RNA-Pull-Down-Assays zeigten, dass Lockd an ribosomale Proteine bindet und möglicherweise die Translation bestimmter Proteine beeinflusst. Darüber hinaus wurde durch Chromatin-Immuno RNA-Präzipitierung festgestellt, dass Lockd mit Chromatin assoziiert ist, was auf eine potenzielle direkte Beteiligung an der Regulation der Genexpression hindeutet. Um die funktionelle Relevanz von Lockd *in vivo* zu validieren, wurde dessen Hochregulierung nach TAC mithilfe von Antisense GapmeRs in Mäusen gezielt inhibiert. Diese Intervention führte zu einer signifikanten Verbesserung der systolischen Herzfunktion und einer Reduktion der Hypertrophie im Vergleich zu mit Kontroll-GapmeRs behandelten Mäusen. Die Färbung des Herzgewebes nach TAC zeigte eine erhöhte Kapillardichte und eine verminderte Hypertrophie der Kardiomyozyten bei den mit Lockd GapmeRs

behandelten Mäusen. Die RNA-Sequenzierung von kardialen Endothelzellen eine Woche nach TAC ergab eine deutliche Dysregulation von Genen, die mit dem Zellzyklus zusammenhängen, nachdem Lockd herunterreguliert wurde, was auf seine Rolle bei der Zellzyklusprogression und Proliferation *in vivo* hinweist.

Zusammenfassend legen unsere Ergebnisse nahe, dass die Lockd-Hochregulierung das Fortschreiten des Zellzyklus hemmt und dadurch der Endothelproliferation und Angiogenese entgegenwirkt. Folglich scheint die Lockd-Hochregulation im Herzgewebe bei Herzdrucküberlastung und Herzumbau schädlich zu sein.

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Abbreviations

Abbreviation	Full form
4E-BP1	4E-binding protein 1
ACE	Angiotensin-converting-enzyme
Akt	Protein kinase B
Ang II	Angiotensin II
Angptl4	Angiopoietin-like 4 ANP
ANP	Atrial Natriuretic Peptide
ANRIL	Antisense RNA In The INK4 Locus
ARB	Angiotensin receptor blocker
AT1R	angiotensin II type 1 receptor
ATP	Adenosine triphosphate
Aurkb	Aurora kinase b
BrdU	Bromodeoxyuridine
BRG1	Brahma-related gene 1
BSA	Bovine serum albumin
Bvht	Braveheart
CaMKII	Calcium/ calmodulin dependent protein kinase II
Ccnd3	Cyclin D3
Cdc25b	Cell Division Cycle 25B
Cdk1	Cyclin-dependent kinase 1
Cdkn1b	Cyclin-dependent kinase inhibitor 1B
CKI	cyclin-dependent kinase inhibitor
CM	Cardiomyocytes
Col1a1	Collagen type I
Col4a1	Collagen type IV
CRT	Cardiac resynchronization therapy device
Ctrl	Control
CVD	Cardiovascular diseases
Cyp26b1	Cytochrome P450 Family 26 Subfamily B Member 1
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate

dGTP	Deoxyguanine 5'-triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dTTP	Deoxythymidine 5'-triphosphate
Dusp6	Dual Specificity Phosphatase 6
Dynll1	Dynein Light Chain LC8-Type 1
EC	Endothelial cell
Echo	Echocardiographie
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
eIF4E	Eukaryotic initiation factor 4E
EndoMT	Endothelial to mesenchymal transition
EV	Extracellular vesicles
FB	Fibroblast
FC	Fold Change
FCS	Foetal Calf Serum
Fgf2	Fibroblast growth factor 2
g	Gram
G1	Growth phase 1
G2	Growth phase 2
Gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA4	GATA transcription factor 4
GPCRs	G protein-coupled receptor
h	Hour(s)
HAT	Histone acetyltransferase
HCMEC	Human Cardiac Microvascular Endothelial Cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HF	Heart failure
HMT	Histone methyltransferase
Hotair	HOX Antisense Intergenic RNA
Hprt1	Hypoxanthine Phosphoribosyltransferase 1

Hrp	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
IB4	Isolectin B4
ICD	Implantable cardioverter-defibrillators
ICH	Immunohistochemistry
IGF-1	Insulin-like growth factor 1
IgG	Immunglobulin G
IL-1 β	Interleukin 1-beta
IP	Immunoprecipitation
iPSC-CM	Induced pluripotent stem cell-derived cardiomyocytes
KCL	Potassium Chloride
kDa	Kilodalton
Kif	Kinesin Family Member
KIS	Kinase Interacting with Stathmin
LCCA	Left common carotid artery
Lipg	Lipase G
LNA	Locked Nucleic Acid
lncRNA	Long non-coding RNA
Lockd	Long non-coding RNA downstream of Cdkn1b
LV	Left ventricle
LVAD	Left Ventricular Assist Devices
LVPW	Left ventricle posterior wall
M	Molar
M-phase	Mitotic phase
Malat1	Metastasis Associated Lung Adenocarcinoma Transcript 1
MAPK	Mitogen-activated protein kinases
MEF	Myocyte enhancer factor
Mettl13	Methyltransferase-like protein 13
MgCl ₂	Magnesium Chloride
MHC	Myosin Heavy Chain
Mhrt	Myosin Heavy Chain Associated RNA Transcript
MI	Myocardial infarction
min	Minutes

miRNA	Micro RNA
miRNA	Micro Ribonucleic Acid
mL	Mililiter
MMP	Matrix metalloproteinase
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
mTor	Mechanistic Target of Rapamycin
Myc	MYC Proto-Oncogene
Myh6	Myosin heavy chain 6
Myh7	Myosin heavy chain 7
NaCl	Sodium Chloride
NASH	Non-alcoholic steatohepatitis
NCBI	National Center for Biotechnology Information
NF-KB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
ng	Nanogram
Nppa	Natriuretic Peptide B
Nppb	Natriuretic Peptide A
nt	Nucleotides
Nras	Neuroblastoma RAS Viral Oncogene Homolog
Nuf2	NUF2 Component Of NDC80 Kinetochore Complex
Oip5os1	Opa Interacting Protein 5 Antisense RNA 1
Opti-MEM	Minimum Essential Medium
ORF	Open Reading Frame
PBS	Phosphate buffered saline
Pbx1	Pre-B-cell leukemia transcription factor 1
PCR	Polymerase chain reaction
Pdgfb	Platelet Derived Growth Factor Subunit B
PFA	Paraformaldehyd
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
Pik3ca	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PKC	Protein kinase C
PVDF	Polyvinylidendifluorid

Pvt1	Plasmacytoma Variant Translocation 1
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
Rack1	Receptor for activated C kinase 1
RAP-MS	RNA antisense purification coupled - Mass spectroscopy
Rb	Retinoblastoma protein
RCCA	Right common carotid artery
rG4	RNA G-quadruplex
RIP	RNA Immunoprecipitation
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute Medium
Rps6	Ribosomal Protein S6
rRNA	ribosomal Ribonucleic Acid
RT	Room Temperature
s	seconds
S-phase	DNA snythesis phase
S6K1	Ribosomal protein S6 kinase beta-1
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecylsulfate -polyacrylamide gel electrophoresis
SEM	Standard Error of Mean
siRNA	Small interfering Ribonucleic Acid
sm22	Transgelin
SRF	Serum response factor
TAC	Transverse aortic constriction
Taok2	Thousand and one amino acid protein kinase 2
TBS-T	Tris-buffered saline and Tween 20
Tgf- β	Transforming growth factor beta
Tnf- α	Tumour necrosis factor alpha
tRNA	Transfer RNA
TSS	Transcription Start Site
TTS	Transcription Termination Site
Uhmk1	U2AF Homology Motif Kinase 1
UTR	Untranslated region

UV	Ultraviolet
Vegf	Vascular endothelial growth factor
WGA	Wheat germ agglutinin
WT	Wild type
x g	Gravitational force
Xist	X-Inactive Specific Transcript
β -AR	β -adrenergic receptors
β -MHC	the β -myosin heavy chain
μ g	Microgram
μ L	Microliter
μ M	Micromolar

1. Introduction

1.1 Heart failure and cardiac hypertrophy

Cardiac hypertrophy and heart failure are two intricately related conditions that are of significant interest to medical research due to insufficient treatment options. Cardiac hypertrophy, characterized by the thickening of the heart muscle (myocardium), can be classified into two main types: pathological and physiological hypertrophy [1]. Pathological hypertrophy occurs in response to chronic pressure or volume overload, often resulting from conditions like hypertension or valvular heart disease [2-5]. In this type of hypertrophy, the heart muscle undergoes abnormal growth and remodelling, leading to an enlarged heart with increased muscle mass [6]. Pathological hypertrophy is more likely to progress to heart failure if left untreated compared to physiological hypertrophy. This is primarily due to the underlying molecular and cellular changes that occur in pathological hypertrophy [7, 8].

On the other hand, physiological hypertrophy is typically observed in athletes or in women during pregnancy. It is a normal adaptation of the heart to increased physiological demands, such as regular exercise or the need to support a growing foetus [6, 9]. During physiological hypertrophy the heart undergoes controlled growth and remodelling to meet increased demands [6, 10]. The hypertrophic response is accompanied by beneficial changes in gene expression, enhanced contractile function, and maintenance of normal cardiac structure and function. Importantly, physiological hypertrophy does not compromise overall heart function, and the heart remains able to effectively pump blood [1, 10]. However, it is important to note that prolonged and excessive physiological hypertrophy, such as in elite athletes or individuals with prolonged high-intensity exercise, may have some overlap with pathological hypertrophy. In these cases, the continuous and extreme cardiac remodelling associated with physiological hypertrophy can potentially lead to maladaptive changes and an increased risk of heart failure [11, 12].

Furthermore, cardiac hypertrophy can be characterized by the pattern of ventricular remodelling, resulting in eccentric or concentric hypertrophy. Eccentric hypertrophy is primarily associated with adaptations to increased volume overload (e.g. due to valvular leakage) or certain forms of dilated cardiomyopathy [9]. In these conditions, the heart chambers enlarge in response to increased blood volume, allowing the heart to accommodate and pump a higher volume of blood [13, 14]. This type of hypertrophy is often also observed in physiological hypertrophy where the heart needs to work harder to meet the body's demands. Therefore, in physiological settings eccentric hypertrophy is often considered a beneficial adaptation that helps maintain cardiac function and meet increased

hemodynamic demands [15, 16]. However, in certain pathological conditions, such as chronic volume overload or dilated cardiomyopathy, eccentric hypertrophy is maladaptive and contributes to the progression of heart failure. In these cases, eccentric hypertrophy can lead to maladaptive ventricular dilation, impaired contractility, and altered chamber geometry. These changes can disrupt normal cardiac function, decrease cardiac output, and ultimately contribute to the development of heart failure [15, 17].

Concentric hypertrophy, on the other hand, is characterized by an increase in the thickness of the ventricular walls without a significant enlargement of the chamber size [15, 18]. In contrast to eccentric hypertrophy, concentric hypertrophy is generally considered a pathological response to increased pressure overload on the heart [19, 20]. The thickening of the ventricular walls helps to counteract the increased pressure, but it also leads to a reduced chamber size and impaired diastolic function. As a result, the heart may have difficulty filling with blood during relaxation, leading to increased stiffness and reduced cardiac output. The increased wall thickness can impair the heart's ability to adequately pump blood [19, 21, 22]. Additionally, the altered chamber geometry and increased wall stress associated with concentric hypertrophy can promote adverse remodelling processes, including fibrosis and cellular dysfunction, which further contribute to the progression of heart failure [23, 24]. It is important to note that while concentric hypertrophy is primarily considered a pathological response, there may be cases where a certain degree of concentric remodelling can be a compensatory adaptation to pressure overload without progressing to heart failure [13, 15, 25].

Pathological hypertrophy often can progress to heart failure if left untreated. Heart failure occurs when the heart is unable to effectively pump blood to meet the body's demands [26, 27]. In pathological hypertrophy, the abnormal growth and remodelling of the heart muscle can impair its contractile function, leading to reduced cardiac output [23, 24]. This results in symptoms such as fatigue, shortness of breath, fluid accumulation in the lungs (pulmonary edema), and fluid retention in other tissues (peripheral edema) [28, 29]. Ultimately, if heart failure is left uncontrolled, it can lead to severe complications and ultimately to death [30].

Heart failure is a significant health concern in Europe, affecting millions of people and leading to a high number of hospitalizations annually [31-33]. The prevalence of heart failure is projected to rise due to factors like an aging population and the increasing incidence of cardiovascular risk factors such as obesity, diabetes, and hypertension [28, 34, 35]. Despite notable progress in the treatment of cardiac hypertrophy and heart failure, there is still a lack of comprehensive understanding regarding the underlying mechanisms and pathways involved in these conditions [36].

Pharmacological therapies play a pivotal role in managing cardiac hypertrophy and heart failure. Ongoing research efforts aim to develop new medications and drug combinations that target specific molecular pathways. Established drug classes to treat heart failure include beta-blockers, angiotensin-converting enzyme inhibitors (ACE inhibitors), angiotensin receptor blockers (ARBs), aldosterone antagonists, and neprilysin inhibitors [37-40]. These medications have demonstrated efficacy in alleviating symptoms, enhancing cardiac function, and prolonging survival rates. ACE inhibitors for example, are a class of medications commonly used in the treatment of cardiovascular conditions such as heart failure. They work by blocking the activity of the enzyme ACE, which is responsible for converting angiotensin I to angiotensin II. By inhibiting this enzyme, ACE inhibitors reduce the production of angiotensin II, a potent vasoconstrictor that also stimulates the release of aldosterone. This leads to a dilatation in blood vessels, reduction in peripheral vascular resistance, and in lower blood pressure. This vasodilation helps improve blood flow and reduces the workload on the heart. Thereby ACE inhibitors can help prevent adverse cardiac remodelling by reducing the growth and thickening of the heart muscle [41-43].

Device therapies have also emerged as valuable treatment options. Implantable devices such as cardiac resynchronization therapy (CRT) devices, implantable cardioverter-defibrillators (ICDs), and left ventricular assist devices (LVADs) are employed in selected patients to improve heart function and manage heart failure symptoms [44-47]. A LVAD is a mechanical pump that is implanted in the body to help the heart pump blood in patients with severe heart failure. The LVAD is typically placed in the chest and connected to the left ventricle and aorta. The LVAD works by drawing blood from the left ventricle and pumping it into the aorta, which then distributes the oxygenated blood to the rest of the body. It assists or takes over the pumping function of the weakened left ventricle, thereby increasing the overall blood flow and improving circulation [46, 48, 49].

Furthermore, early diagnosis and accurate prognosis of cardiac hypertrophy and heart failure are crucial for improving patient outcomes. There is ongoing scientific research aiming to advance the development of novel diagnostic tools and biomarkers capable of identifying individuals at risk of developing cardiac hypertrophy and heart failure, while also providing prognostic insights into disease progression and treatment response [50, 51]. Current therapies for cardiac hypertrophy and heart failure, such as medications and surgical interventions, have limitations and are not effective for all patients. Researchers are working to develop new therapies and interventions that target the underlying mechanisms of these conditions, such as gene therapy, stem cell therapy, and immunotherapy [52, 53].

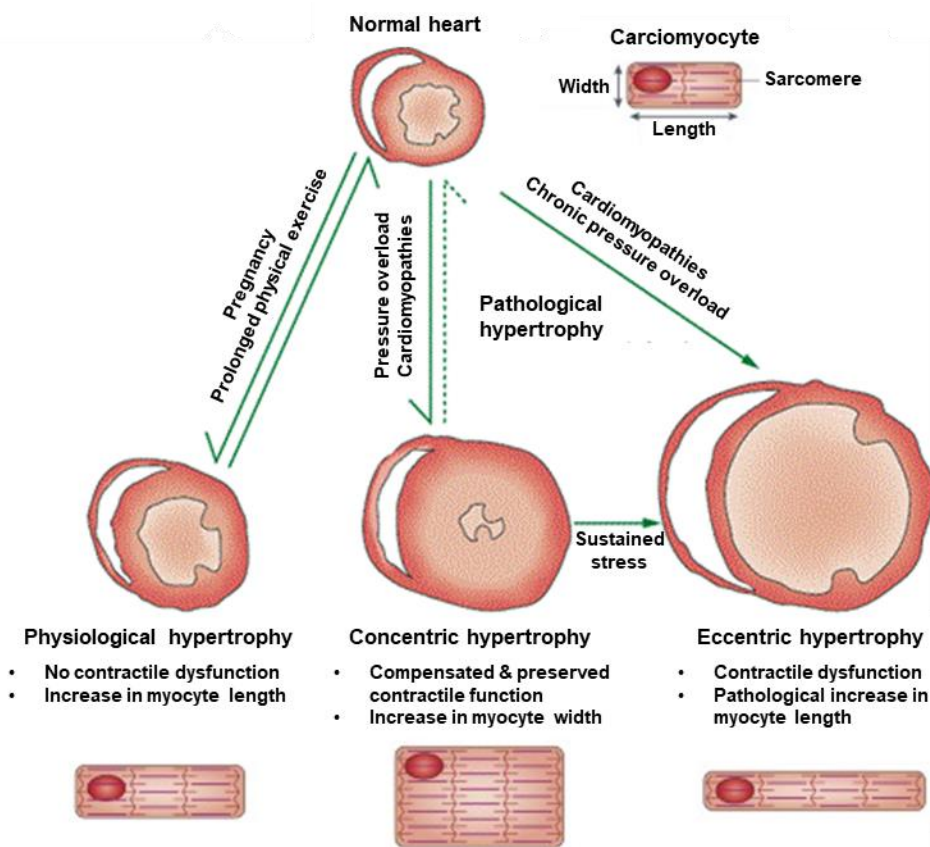


Figure 1: Different modes of hypertrophic remodelling and their general features

The figure illustrates the process of cardiac remodelling under different conditions of cardiac hypertrophy induced by physiological or pathological stimuli. In normal adult hearts, the adaptation to stress during pregnancy or physical training leads to a balanced form of remodelling known as physiological hypertrophy, which can be reversible. During this process, the heart maintains or slightly improves its cardiac function via a physiological eccentric hypertrophy. Conversely, in response to pathological stimuli, the heart typically undergoes concentric hypertrophy, characterized by increased wall thickness along with reduced chamber dimensions. This type of hypertrophy can eventually lead to heart failure. Pathological hypertrophic growth may be followed by pathological eccentric hypertrophy, resulting in dilated chambers and cardiac contractile dysfunction. Additionally, certain genetic diseases can directly lead to cardiomyopathies. Image adapted from Fuster V, Walsh RA, Harrington RA: *Hurst's The Heart* 13th Edition and Nakamura, Michinari, and Junichi Sadoshima. "Mechanisms of physiological and pathological cardiac hypertrophy." *Nature reviews. Cardiology* (2018)

1.2 Cardiac pressure overload

Cardiac pressure overload is a condition characterized by increased pressure on the heart, often resulting from factors such as hypertension or aortic stenosis [54]. For instance, hypertension leads to elevated systemic blood pressure and puts increased resistance on the heart, forcing it to work harder

to pump blood throughout the body. Over time, this increased workload can lead to cardiac pressure overload [14, 55, 56].

Aortic stenosis is a condition characterized by the narrowing of the aortic valve, which restricts blood flow from the left ventricle to the aorta. The heart must generate greater pressure to overcome the obstruction, leading to pressure overload on the left ventricle [57, 58]. The increased pressure load on the left ventricle triggers a series of adaptive responses at cellular and molecular levels. Over time, this chronic pressure overload can lead to changes in the structure and function of the heart, ultimately resulting in cardiac hypertrophy [15, 19].

Ultimately this pathological state of pressure overload places a significant burden on the heart and leads to adverse structural and functional changes, ultimately culminating in heart failure [23]. Understanding the mechanisms and consequences of cardiac pressure overload is of paramount importance in cardiovascular research, as it has profound clinical implications.

The pathophysiology of cardiac pressure overload involves intricate molecular and cellular processes, including cardiac hypertrophy, fibrosis, inflammation, and alterations in gene expression [4, 59]. Initially, these responses are adaptive, aimed at maintaining cardiac function in the face of increased workload. For instance, in response to chronic hypertension, the heart undergoes adaptive hypertrophic remodelling to cope with the elevated blood pressure. The myocardium thickens, enabling the heart to generate greater force and pump blood against the increased resistance. This initially helps maintain cardiac output and preserve organ perfusion.

However, if the pressure overload persists over an extended period, these adaptive responses can become maladaptive. Prolonged pressure overload leads to maladaptive remodelling characterized by increased fibrosis, inflammation, and alterations in gene expression. The myocardium becomes stiffer, impairing diastolic filling, and the heart's contractile function may decline. These maladaptive changes further compromise cardiac function and increase the risk of heart failure [23].

Research in pathophysiological cardiac remodelling aims to unravel the underlying molecular and cellular mechanisms driving the development and progression of this condition. By elucidating the intricate signalling pathways, molecular interactions, and cellular changes associated with cardiac pressure overload, research strives to identify potential therapeutic targets and interventions to mitigate or prevent adverse cardiac remodelling and improve patient outcomes [4, 23].

1.3 Molecular mechanisms of cardiac hypertrophy following cardiac pressure overload

Cardiac hypertrophy is a complex process involving a multitude of molecular mechanisms that are activated in response to cardiac pressure overload. For example, during cardiac pressure overload, G protein-coupled receptors (GPCRs), such as angiotensin II type 1 receptor (AT1R) and β -adrenergic receptors (β -ARs) are stimulated, leading to the activation of intracellular signalling cascades [59, 60]. Activation of AT1R by angiotensin II (Ang II) promotes hypertrophic signalling through pathways involving protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs) [61, 62]. Stimulation of β -ARs increases cyclic adenosine monophosphate (cAMP) levels and activates downstream effectors, including protein kinase A (PKA). PKA signalling pathways promote cardiomyocyte hypertrophy by activating various downstream targets involved in cellular growth, protein synthesis, and gene expression, leading to an increase in cardiomyocyte size and mass, which contributes to cardiac hypertrophy [63].

In addition, growth factors, such as insulin-like growth factor 1 (IGF-1), fibroblast growth factors (FGFs), and transforming growth factor-beta (TGF- β), play critical roles in cardiac hypertrophy [64-66]. Activation of their receptors initiates intracellular signalling cascades, including phosphoinositide 3-kinase (PI3K)/Akt and MAPK pathways, which promote cardiomyocyte growth and hypertrophy [67, 68].

Furthermore, epigenetic modifications, including DNA methylation and histone modifications, play crucial roles in regulating gene expression during cardiac hypertrophy. DNA methylation patterns can be dynamically altered, leading to changes in gene expression profiles associated with hypertrophy [69]. Histone modifications, such as acetylation and methylation, controlled by histone acetyltransferases (HATs) and histone methyltransferases (HMTs), respectively, also modulate hypertrophic gene expression [70, 71].

Moreover, the activation of transcription factors can have an important impact on the development of cardiac hypertrophy. Multiple transcription factors are implicated in cardiac hypertrophy during pressure overload. For example, GATA4, a zinc finger transcription factor, plays a central role in regulating cardiac gene expression and hypertrophic responses [72]. It interacts with other transcription factors, such as MEF2 and serum response factor (SRF), to promote the expression of genes involved in hypertrophy [73] [74]. Nuclear factor kappa B (NF- κ B), a key transcription factor involved in inflammatory responses, also contributes to hypertrophic signalling by modulating gene expression and promoting cardiomyocyte growth [75].

Cardiac hypertrophy is characterized by an increase in the size of cardiomyocytes, and this process requires enhanced protein synthesis to support the growth and structural remodelling of the heart.

Cardiomyocytes primarily undergo growth rather than proliferation in response to cardiac stress, because they have limited regenerative capacity. Unlike other cells in the body, adult cardiomyocytes have a low ability to divide and regenerate. Therefore, in order to adapt to increased workload or stress, cardiomyocytes mainly increase in size rather than increasing in number (proliferation). This hypertrophic response allows the individual cardiomyocytes to generate more force and contractility to meet the demands placed on the heart [76, 77]. The mammalian target of rapamycin (mTOR) pathway is a critical regulator of protein synthesis and cell growth, playing a significant role in cardiac hypertrophy [78, 79].

Activation of the mTOR signalling pathway occurs in response to various stimuli, including growth factors, nutrient availability, and mechanical stress. Upon activation, mTOR forms complexes, within mTOR complex 1 (mTORC1) being particularly important in the context of cardiac hypertrophy. mTORC1 regulates protein synthesis by promoting ribosomal biogenesis, translation initiation, and elongation [80].

One key downstream effector of mTORC1 is ribosomal protein S6 kinase 1 (S6K1). S6K1 phosphorylates ribosomal protein S6, leading to increased ribosomal biogenesis and enhanced translation of proteins involved in hypertrophic growth [81]. Another critical effector of mTORC1 is eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Phosphorylation of 4E-BP1 by mTORC1 releases its inhibitory effects on eukaryotic translation initiation factor 4E (eIF4E), allowing eIF4E to promote translation initiation and subsequent protein synthesis [82, 83].

In addition to its role in protein synthesis, the mTOR pathway also influences other aspects of cardiac hypertrophy, including cell growth, metabolism, and autophagy. mTOR promotes the activation of anabolic pathways involved in cell growth and hypertrophy, such as protein and lipid synthesis, while inhibiting catabolic processes like autophagy [78, 84]. These effects contribute to the overall hypertrophic response and remodelling of the heart.

Several studies have demonstrated the involvement of the mTOR pathway in cardiac hypertrophy. For example, genetic manipulation of key components of the mTOR pathway, such as mTOR itself or its downstream effectors S6K1 and 4E-BP1, has been shown to impact hypertrophic growth and cardiac function in animal models [67, 85]. Furthermore, pharmacological agents targeting mTOR signalling, such as rapamycin and its derivatives, have been investigated as potential therapeutic interventions for pathological cardiac hypertrophy [78, 85].

Activation of fibrosis-related pathways is a key aspect of cardiac remodelling in response to pressure overload. In addition to cardiomyocyte hypertrophy, the excessive deposition of extracellular matrix

components contributes to the development of fibrotic remodelling. Transforming growth factor-beta (TGF- β) signalling pathway has been identified as a central player in promoting fibrosis during hypertrophy [86].

TGF- β is a multifunctional cytokine that regulates various cellular processes, including fibroblast activation, extracellular matrix synthesis, and immune responses. Upon activation, TGF- β binds to its receptors and initiates intracellular signalling cascades, particularly the Smad-dependent pathway [87]. Activation of the TGF- β pathway leads to the phosphorylation and nuclear translocation of Smad proteins, which act as transcription factors regulating the expression of fibrogenic genes and extracellular matrix components [88].

The downstream effects of TGF- β signalling in cardiac fibroblasts include the stimulation of fibroblast proliferation, differentiation into myofibroblasts, and increased production of collagens and other extracellular matrix proteins. These processes contribute to the excessive accumulation of fibrotic tissue in the heart, impairing normal cardiac function [89, 90].

Experimental models and clinical studies have demonstrated increased TGF- β expression and activation in hypertrophic hearts and fibrotic cardiac tissues [86]. Additionally, genetic manipulation or pharmacological targeting of TGF- β signalling components has shown promising results in attenuating cardiac fibrosis and improving cardiac function [91].

It is crucial to acknowledge that the molecular mechanisms involved in cardiac hypertrophy do not act independently but instead exhibit crosstalk and interconnectedness. Multiple signalling pathways interact and converge to regulate hypertrophic gene expression and cardiomyocyte growth. Among these pathways, MAPKs (mitogen-activated protein kinases), AKT, TGF- β (transforming growth factor-beta), FGFs (fibroblast growth factors), mTOR (mammalian target of rapamycin) signalling, and calcium signalling play significant roles, to name a few [2-4, 92].

Calcium signalling, an essential process for cardiac contraction, also participates in hypertrophic signalling through pathways involving calcium/calmodulin-dependent protein kinases (CaMKs) and calcineurin [93, 94].

These molecular mechanisms collectively contribute to the development and progression of cardiac hypertrophy in response to cardiac pressure overload.

1.4 The role of the endothelium in cardiac pressure overload

The endothelium, a monolayer of endothelial cells lining the inner surface of blood vessels, serves as a dynamic interface between circulating blood and the surrounding tissues, thereby regulating the transport of molecules and cells in and out of the blood vessels [95]. Different types of endothelial cells exist in different blood vessels within the cardiovascular system. Arterial endothelial cells are located in arteries, which experience higher blood pressure. Arterial endothelial cells are responsible for regulating blood flow and vessel tone to ensure proper distribution of oxygenated blood to tissues [96, 97]. Capillaries are the smallest blood vessels and facilitate the exchange of oxygen, nutrients, and waste products between the blood and surrounding tissues [98]. Capillary endothelial cells have thin walls that enhance efficient exchange processes [95]. Venous endothelial cells play a role in returning deoxygenated blood back to the heart and regulating venous tone, assisting in the efficient circulation of blood throughout the body [96]. Each type of endothelial cell performs specific functions based on its location within the cardiovascular system, contributing to the overall regulation of cardiac physiology and maintaining vascular homeostasis [99, 100].

The endothelium plays a crucial role in the pathophysiology during cardiac pressure overload. Investigating the endothelium in the context of cardiac pressure overload is of significant importance in cardiovascular research, as it offers valuable insights into the mechanisms underlying adverse cardiovascular outcomes and provides potential therapeutic targets for intervention [101, 102].

The endothelium is intimately involved in cardiac remodelling processes during pressure overload. It interacts with cardiomyocytes, fibroblasts, and other cells within the cardiac tissue, influencing their behaviour and the overall structural and functional changes that occur in the heart. Endothelial cells secrete factors such as vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF- β) that can directly impact cardiac hypertrophy, fibrosis, and angiogenesis [103-105]. VEGF promotes the growth of new blood vessels (angiogenesis) and can influence cardiac hypertrophy [106]. TGF- β is involved in fibrosis, contributing to the excessive deposition of extracellular matrix components, and remodelling of the heart tissue in conditions like cardiac hypertrophy [107, 108].

In the context of cardiac pressure overload, the endothelium undergoes functional and structural changes, often leading to endothelial dysfunction. This dysfunction may manifest as impaired vasodilation, increased production of vasoconstrictors, enhanced oxidative stress, inflammation, and altered angiogenic responses [109, 110].

For example, studies have shown that endothelial dysfunction, characterized by reduced nitric oxide (NO) bioavailability and impaired endothelium-dependent vasodilation, is commonly observed in

patients with cardiac pressure overload [111]. This dysfunction contributes to increased vascular resistance, impaired coronary flow, and compromised myocardial perfusion [112, 113].

Furthermore, the vasculature, including arteries, arterioles, and capillaries, undergoes remodelling in response to cardiac pressure overload. These structural changes involve alterations in vessel structure, such as, hyperplasia, and fibrosis, along with changes in vessel wall stiffness and vascular resistance [114, 115]. These vascular adaptations contribute to the increased pressure overload and myocardial workload, further exacerbating cardiac dysfunction and heart failure progression [116-118].

Research focusing on the endothelium and vasculature in cardiac pressure overload aims to elucidate the underlying molecular and cellular mechanisms governing these adaptations. Understanding the significance of studying the endothelium and vasculature in cardiac pressure overload is essential, as endothelial dysfunction and vascular remodelling play critical roles in the development and progression of heart failure. By targeting these processes, researchers aim to improve myocardial perfusion, reduce myocardial workload, and attenuate adverse cardiac remodelling in patients with chronic pressure overload. This comprehensive approach holds promise for preventing or reversing adverse cardiovascular outcomes and improving patient outcomes.

1.5 Impaired angiogenesis in cardiac pressure overload

Impaired angiogenesis, the inadequate formation of new blood vessels, plays a critical role in the pathophysiology of cardiac pressure overload. Investigating the mechanisms behind impaired angiogenesis in cardiac pressure overload is crucial in cardiovascular research. It provides valuable insights into the development of adverse cardiac outcomes and offers potential therapeutic strategies for intervention. Understanding these mechanisms can pave the way for restoring sufficient blood supply to the heart, enhancing cardiac function, and potentially arresting or reversing the progression of heart failure [119, 120]. Interventions may be developed that promote the formation of new blood vessels, improve myocardial perfusion, and mitigate the adverse consequences of cardiac pressure overload [121].

Angiogenesis, the process of blood vessel formation, is tightly regulated by a complex interplay of pro-angiogenic and anti-angiogenic factors. In the context of cardiac pressure overload, the balance between these factors is disrupted, resulting in impaired angiogenesis and compromised blood supply to the myocardium [122, 123].

For instance, studies have demonstrated that in conditions of cardiac pressure overload, such as hypertrophic cardiomyopathy or chronic hypertension, there is a decreased density of capillaries in

the myocardium, indicating insufficient angiogenesis [124, 125]. This insufficient vascularization leads to inadequate oxygen and nutrient supply to the heart, contributing to myocardial ischemia and progressive cardiac dysfunction [124, 126].

At the molecular level, several key factors and signalling pathways involved in angiogenesis regulation are affected in cardiac pressure overload. For example, dysregulation of vascular endothelial growth factor (VEGF) and its receptor signalling has been observed in animal models and human samples of cardiac pressure overload. Reduced VEGF expression, altered VEGF receptor activity, or impaired downstream signalling can disrupt the angiogenic process and hinder blood vessel formation in the heart [11, 127, 128],[129, 130].

Additionally, other molecular players, such as angiopoietins, fibroblast growth factors (FGFs), and matrix metalloproteinases (MMPs), are also implicated in angiogenesis [131]. Altered expression or dysregulated activity of these molecules can disrupt endothelial cell function, extracellular matrix remodelling, and vessel stabilization, further contributing to the inadequate angiogenic response [132, 133].

Taken together, inadequate angiogenesis and impaired myocardial perfusion play significant roles in the progression of heart failure, a prevalent and debilitating cardiovascular condition [134, 135].

Targeting angiogenesis represents a promising strategy to enhance myocardial blood flow, preserve cardiac function, and improve clinical outcomes in patients with cardiac pressure overload. By addressing the molecular and cellular factors that contribute to impaired angiogenesis, research aims to develop innovative interventions that can promote the formation of new blood vessels in the myocardium [136]. These interventions hold the potential to restore adequate blood supply, improve oxygen and nutrient delivery to cardiac tissues, and counteract the detrimental effects of chronic pressure overload on cardiac function such as ischemia for example [137, 138].

1.6 Endothelial cell proliferation

Endothelial cell proliferation is a fundamental process involved in angiogenesis, the formation of new blood vessels from pre-existing ones. It plays a critical role in various physiological and pathological conditions, including tissue growth and repair; wound healing, and tumour progression [139]. Understanding the mechanisms that regulate endothelial cell proliferation is of great importance in the context of cardiovascular biology and disease.

Endothelial cell proliferation is tightly regulated by a complex interplay of signalling pathways, growth factors, and cellular interactions. Vascular endothelial growth factors (VEGFs) for example, are key

regulators of endothelial cell proliferation and are essential for angiogenesis [140]. Binding of VEGFs to their receptors on endothelial cells activates downstream signalling pathways, including the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways, which promote cell proliferation [141, 142].

In addition to VEGFs, other growth factors and cytokines, such as fibroblast growth factors (FGFs) and platelet-derived growth factor (PDGF), also contribute to endothelial cell proliferation [143]. These factors activate their respective receptors on endothelial cells, initiating intracellular signalling cascades that stimulate cell division and proliferation [144, 145].

Furthermore, cell-cell and cell-matrix interactions play a crucial role in endothelial cell proliferation. The extracellular matrix (ECM), consisting of various proteins and proteoglycans, provides structural support and signalling cues for endothelial cells. Integrins, a family of cell adhesion molecules, mediate the interaction between endothelial cells and the ECM, transmitting signals that regulate cell proliferation and survival [146].

Moreover, endothelial cell proliferation is influenced by the surrounding microenvironment and cellular interactions. Paracrine signalling from neighbouring cells, such as smooth muscle cells and pericytes, can modulate endothelial cell proliferation through the secretion of growth factors and cytokines [147]. Additionally, endothelial cell proliferation can be influenced by mechanical forces, such as shear stress and stretch, which are important in blood vessels exposed to hemodynamic forces [148].

Cardiomyocytes also play an important role in regulating endothelial cell proliferation in the heart through paracrine signalling and direct cell-cell interactions [103, 149].

Paracrine signalling involves the release of soluble factors, such as growth factors and cytokines, from cardiomyocytes that act on endothelial cells to stimulate their proliferation. Such factors include, vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), and angiopoietins [150, 151].

Direct cell-cell interactions between cardiomyocytes and endothelial cells also contribute to endothelial cell proliferation. Cardiomyocytes can physically interact with neighbouring endothelial cells through specialized junctions called intercalated discs [152, 153]. These junctions facilitate cell communication and the exchange of signalling molecules, which can promote endothelial cell proliferation [154].

In addition, cardiomyocytes can produce extracellular matrix (ECM) proteins that provide structural support and signalling cues for endothelial cells. The ECM can modulate endothelial cell behaviour

and promote proliferation through interactions with specific integrins and other cell surface receptors [155-157].

Cardiac fibroblasts can also regulate the proliferation of endothelial cells. They secrete various growth factors, cytokines, and extracellular matrix components that can influence neighbouring cells, including endothelial cells.

Fibroblasts can secrete pro-angiogenic factors such as fibroblast growth factor (FGF) for example, which stimulate endothelial cell proliferation and angiogenesis.

Additionally, fibroblasts as well as myeloid cells can interact with endothelial cells through direct cell-cell contact and the secretion of paracrine factors. These interactions can modulate endothelial cell behaviour, including proliferation, migration, and tube formation, which are essential processes in angiogenesis.

Overall, the regulation of endothelial cell proliferation by cardiomyocytes and other cell types such as fibroblasts involves a complex interplay of intercellular communication. This crosstalk between the various cardiac cell types and endothelial cells is essential for maintaining vascular homeostasis, angiogenesis, and overall cardiac function [149, 158].

1.7 Cell cycle re-entry and proliferation

Endothelial cell cycle entry or re-entry refers to the process by which quiescent endothelial cells transition from a resting state to an active proliferative state [159]. The cell cycle is a highly regulated process that consists of a series of events leading to cell division and subsequent growth. In the context of endothelial cells, cell cycle entry/re-entry is a critical step in angiogenesis [160, 161].

The cell cycle is regulated by a complex interplay of CDKs, cyclins, CKIs, and other regulatory proteins. Dysregulation of these controls can lead to abnormal cell proliferation and contribute to various diseases, including cancer [162, 163]. The cell cycle consists of several stages, including G1 (growth phase 1), S (DNA synthesis), G2 (growth phase 2), and M (mitosis). During G1, cells prepare for DNA replication. In the S phase, DNA replication occurs, resulting in the duplication of the genetic material. G2 phase follows, during which the cell prepares for cell division. Finally, the M phase involves mitosis, where the replicated chromosomes are separated and distributed into two daughter cells [164, 165].

The progression of the cell cycle is tightly regulated by a complex network of molecular factors. Key regulators include cyclin-dependent kinases (CDKs) and cyclins. CDKs are enzymes that control cell cycle transitions, while cyclins bind to CDKs, activating their kinase activity [162, 163].

The G1 to S transition is regulated by the cyclin D-CDK4/6 complex, which phosphorylates proteins involved in promoting cell cycle progression [166]. The S phase is regulated by the cyclin E-CDK2 complex, which initiates DNA replication [167]. The G2 to M transition is regulated by the cyclin B-CDK1 complex, which triggers entry into mitosis [168]. CDK activity is further regulated by cyclin-dependent kinase inhibitors (CKIs), such as p21 and p27 (Cdkn1b), which inhibit CDKs and halt cell cycle progression [169].

Other key regulators include the retinoblastoma protein (Rb), which acts as a checkpoint in the G1 phase. Rb is phosphorylated by cyclin D-CDK4/6, releasing transcription factors that promote cell cycle progression [170]. Additionally, the tumour suppressor protein p53 plays a crucial role in cell cycle regulation by activating CKIs and inducing cell cycle arrest or apoptosis in response to DNA damage [171].

Under normal physiological conditions, endothelial cells are predominantly in a quiescent state, maintaining vascular integrity and homeostasis. However, during various physiological and pathological processes such as tissue growth, wound healing, and angiogenic sprouting, endothelial cells need to enter the cell cycle to support vessel growth and remodelling [172, 173].

The entry of endothelial cells into the cell cycle is tightly regulated by a complex interplay of various signalling pathways, growth factors, and extracellular cues. Key regulators involved in promoting cell cycle entry/re-entry include cyclins, cyclin-dependent kinases (CDKs), and their inhibitors [174-176]. Cyclins and CDKs form complexes that drive cell cycle progression, while CDK inhibitors act as negative regulators, halting cell cycle progression [177, 178].

Growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) play crucial roles in promoting endothelial cell cycle entry/re-entry [179, 180]. These growth factors activate specific signalling pathways, including the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) pathways, which converge on the regulation of cyclins and CDKs [176, 181]. Activation of these pathways leads to the phosphorylation and inactivation of CDK inhibitors, allowing cyclin-CDK complexes to promote cell cycle progression [182, 183].

Cdkn1b, also known as p27^{Kip1}, is a cyclin-dependent kinase inhibitor that plays a crucial role in controlling cell cycle progression. It functions as a negative regulator of the cell cycle by inhibiting the activity of cyclin-dependent kinases (CDKs) and preventing the phosphorylation of their target

proteins. This inhibition leads to cell cycle arrest and the regulation of various cellular processes [184, 185].

Cdkn1b primarily controls the G1 phase of the cell cycle, which is a critical checkpoint for determining whether cells will continue dividing or exit the cell cycle and enter a quiescent state. It acts by binding to and inhibiting the activity of cyclin-CDK complexes, particularly cyclin D-CDK4/6 and cyclin E-CDK2 complexes, which are responsible for driving cell cycle progression [174, 186].

UHMK1 (U2AF Homology Motif Kinase 1), also known as KIS (Kinase Interacting with Stathmin), is a serine/threonine protein kinase that has been implicated in the regulation of cell cycle progression. UHMK1 can influence cell cycle progression by modulating the activity and stability of the cyclin-dependent kinase inhibitor Cdkn1b [187, 188].

UHMK1 influences Cdkn1b through direct phosphorylation. UHMK1 has been shown to phosphorylate Cdkn1b at specific serine residues, such as Ser10 [189]. Phosphorylation of Cdkn1b by UHMK1 at Ser10 has been associated with cytoplasmic localization of Cdkn1b. This phosphorylation event promotes the translocation of Cdkn1b from the nucleus to the cytoplasm, leading to reduced nuclear Cdkn1b levels [190]. The cytoplasmic sequestration of Cdkn1b prevents its inhibitory interaction with cyclin-CDK complexes, allowing cell cycle progression to proceed [191, 192].

It is worth noting that the role of UHMK1 in cell cycle regulation is not limited to its interaction with Cdkn1b, and it may also influence other cell cycle-related proteins and pathways [193]. Additionally, the impact of UHMK1 on cell cycle progression can vary depending on the cell type and cellular context.

Furthermore, extracellular matrix components and cell-cell interactions provide essential cues for endothelial cell cycle entry [194-196]. Integrins and cell adhesion molecules facilitate cell attachment and signalling, influencing endothelial cell behaviour and promoting cell cycle progression [197, 198].

The dysregulation of endothelial cell cycle entry can have detrimental consequences. Excessive or uncontrolled endothelial cell proliferation can contribute to pathological conditions such as tumour angiogenesis and vascular malformations [160, 199]. On the other hand, impaired endothelial cell cycle re-entry can lead to impaired angiogenesis and insufficient vessel growth.

Understanding the molecular mechanisms that govern endothelial cell cycle entry/re-entry is crucial for deciphering angiogenesis and its role in both physiological and pathological contexts. Targeting key regulators and pathways involved in endothelial cell cycle progression holds the potential for therapeutic interventions aimed at modulating angiogenesis and treating associated diseases.

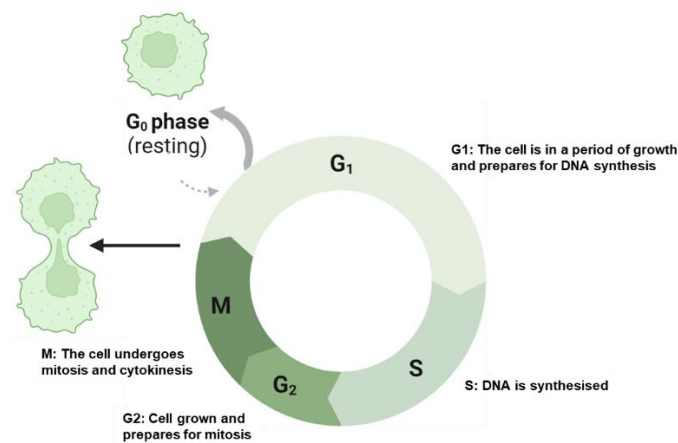


Figure 2: The cell cycle

Cell cycle begins with the G1 phase where the cell is in a period of growth and prepares for DNA synthesis, the S-phase is there the DNA is synthesised. In the G2 phase the cell grows and prepares for mitosis. In the M-phase the cell undergoes mitosis and cytokinesis.

Illustration created with BioRender

1.8 The role of ribosomes and translation during endothelial proliferation

Ribosomes and protein translation play a critical role in endothelial cell proliferation, contributing to the growth and expansion of blood vessels during angiogenesis. Ribosomes are cellular structures responsible for protein synthesis. They consist of large and small subunits that come together to form a functional ribosome complex. Each subunit is made up of a combination of ribosomal RNA (rRNA) molecules and numerous proteins (RPs) [200]. In eukaryotic cells, such as mammalian cells, the large subunit contains a 28S rRNA, 5.8S rRNA, and 5S rRNA, while the small subunit contains an 18S rRNA. The rRNA molecules provide the structural scaffold for ribosome assembly and also play a crucial role in catalyzing the formation of peptide bonds during protein synthesis [201]. The RPs are classified into two categories: the large subunit proteins, known as RPL proteins, and the small subunit proteins, known as RPS proteins [202]. The proteins as well as the rRNAs associated with the ribosome stabilize its structure and contribute to its functionality. The overall structure of the ribosome enables it to interact with messenger RNA (mRNA) and transfer RNA (tRNA) molecules to decode the genetic information and synthesize proteins.[203, 204].

In endothelial cells, ribosomes actively engage in protein translation to produce the proteins necessary for cell proliferation. Several factors and signalling pathways regulate ribosome activity and protein translation in endothelial cells. One of the important components involved in ribosomal function and protein synthesis is ribosomal protein S6 (Rps6). Rps6 is a ribosomal protein that is phosphorylated by

the ribosomal protein S6 kinase (S6K). Phosphorylation of Rps6 is a key step in the regulation of ribosome function and protein translation in response to growth factors and mitogens [205].

For example, the mammalian target of rapamycin (mTOR) signalling pathway is a central regulator of protein synthesis and cell growth. Activation of mTOR promotes ribosomal biogenesis, translation initiation, and protein synthesis, supporting endothelial cell proliferation and angiogenesis [80]. Furthermore, mTOR signalling promotes the phosphorylation of Rps6 by S6K, which enhances ribosome activity and protein translation [206].

In addition to general protein synthesis machinery, specific regulatory factors are involved in endothelial cell proliferation. For instance, the eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BPs) play a crucial role in translational control [207]. Phosphorylation of 4E-BPs by mTORC1 releases their inhibitory effect on the initiation factor eIF4E, promoting translation initiation and protein synthesis [208].

The role of ribosomes and protein translation in endothelial cell proliferation might be crucial for understanding the mechanisms of angiogenesis.

1.9 Long-non-coding RNAs

Long non-coding RNAs (lncRNAs) are by definition longer than 200 base pairs (bp). They are a diverse class of RNA molecules that do not encode proteins but play critical roles in various biological processes. They have emerged as key regulators of gene expression and are implicated in numerous cellular functions and disease pathogenesis. Investigating the roles and mechanisms of lncRNAs is of significant importance in molecular biology research, as it offers valuable insights into gene regulation, cellular processes, and disease development [209, 210].

In recent years, the significance of lncRNAs has gained increasing recognition, changing our understanding of the complexity of gene expression regulation. Unlike protein-coding genes, which comprise only a small fraction of the genome (3%), lncRNAs constitute a substantial portion of the transcriptome. They are involved in diverse biological processes, including chromatin remodelling, transcriptional regulation, RNA processing, and post-transcriptional modulation [211, 212].

One example highlighting the functional relevance of lncRNAs is the X-inactive specific transcript (XIST). XIST is crucial for X-chromosome inactivation, a process that ensures dosage compensation in female mammals by silencing one of the two X chromosomes. Through its interaction with chromatin modifiers and transcriptional machinery, XIST orchestrates the formation of a repressive nuclear compartment, contributing to gene silencing and X-chromosome inactivation [213, 214].

Moreover, lncRNAs are implicated in various human diseases, including cancer, cardiovascular disorders, neurodegenerative diseases, and immune-related conditions. For instance, the lncRNA HOTAIR (HOX transcript antisense RNA) has been found to be dysregulated in multiple cancer types and is associated with tumour progression and metastasis. HOTAIR functions as a molecular scaffold, recruiting chromatin-modifying complexes to specific genomic loci, thereby regulating gene expression programs involved in cancer development and metastatic spread [215, 216].

At the molecular level, lncRNAs exert their regulatory functions through diverse mechanisms. They can interact with DNA, RNA, or proteins, acting as guides, decoys, scaffolds, or enhancers, modulating the activity of transcription factors, chromatin modifiers, and other regulatory molecules. Additionally, lncRNAs can serve as precursors for small regulatory RNAs, such as microRNAs or small interfering RNAs, further expanding their functional repertoire [211, 217]. By influencing gene expression programs, lncRNAs can impact cellular processes, such as cell differentiation, proliferation, apoptosis, and immune responses [218, 219].

Research focused on lncRNAs aims to uncover the complex roles they play in gene regulation and cellular processes. By investigating their expression patterns, interactions, and functional mechanisms, scientists aim to decipher the intricate regulatory networks and molecular pathways involved. This knowledge has the potential to provide new therapeutic targets and strategies for various diseases, including cancer, cardiovascular disorders, and neurological conditions.

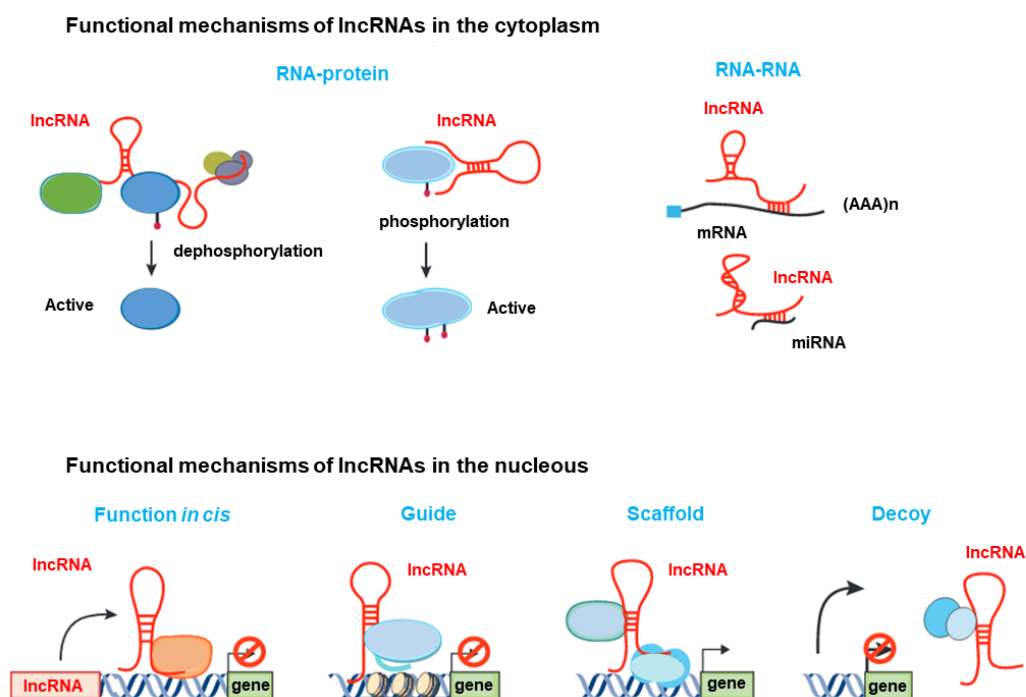


Figure 3: Mechanism of action for lncRNAs

lncRNAs mediate their molecular functions through a multitude of mechanisms in the cytoplasm or the nucleus. In the cytoplasm, lncRNAs act through RNA-protein or RNA-RNA interactions. lncRNAs can act as molecular scaffolds for the transcription factors. lncRNAs can interact with target mRNAs through base pairing to enhance their translation. In the nucleus, lncRNAs can act in cis or trans. lncRNAs can interact with factors to repress transcription of the neighboring gene. A lncRNA can interact with its protein partner as a guide, scaffold, or decoy molecule to mediate its molecular functions. Image adapted from Atianand MK, Cafferey DR, Fitzgerald KA. *"Immunobiology of Long Noncoding RNAs."* *Annu Rev Immunol* (2017)

1.10 lncRNAs in the heart

The significance of lncRNAs in cardiovascular research, particularly in the context of the heart, has garnered increasing attention. Investigating the roles and mechanisms of lncRNAs in the heart is of significant importance in molecular biology as it offers valuable insights into cardiac development, function, and disease pathogenesis [220, 221].

The heart is a highly specialized organ with complex regulatory networks governing its development, homeostasis, and response to pathological stimuli. Emerging evidence suggests that lncRNAs play integral roles in cardiac biology by modulating gene expression, chromatin organization, RNA processing, and protein-protein interactions in the heart [222-224].

For example, the lncRNA Braveheart (Bvht) is a well-studied cardiac-specific lncRNA that is crucial for cardiomyocyte differentiation during embryonic development. Bvht interacts with chromatin-modifying complexes and transcription factors to activate the expression of key cardiac genes, thereby promoting the formation of functional cardiomyocytes [225, 226].

Another well studied lncRNA is the lncRNA Mhrt (Myosin heavy-chain-associated RNA transcript). Mhrt has been identified as a key regulator of cardiac hypertrophy and heart failure. Mhrt is a cardiac-specific lncRNA that is transcribed from an antisense strand of the Myh7 gene, which encodes the β -myosin heavy chain (β -MHC), a critical contractile protein specific in the heart [227]. Studies have shown that Mhrt acts as a negative regulator of pathological cardiac hypertrophy. During cardiac hypertrophy, the expression of Mhrt is significantly reduced, contributing to the progression of the disease. Mhrt exerts its effects by modulating the activity of key cardiac transcription factors and signaling pathways involved in hypertrophic responses. One of the mechanisms through which Mhrt regulates cardiac hypertrophy is by interacting with the transcription factor BRG1 (Brahma-related gene 1) and preventing its binding to target gene promoters [228]. BRG1 is a component of the SWI/SNF chromatin remodelling complex that promotes the activation of hypertrophic genes, such as

Gata4 for example [229]. By sequestering BRG1, Mhrt inhibits its ability to activate pro-hypertrophic gene programs, thus attenuating hypertrophic growth [230].

Research focused on lncRNAs in the heart aims to unravel their functional roles, molecular mechanisms, and regulatory networks. By investigating the expression patterns, interactions, and functional mechanisms of lncRNAs, science aims to decipher their contributions to cardiac development, function, and disease.

The significance of studying lncRNAs in the heart lies in their potential implications for cardiovascular disease as they have emerged as important regulators of gene expression and cellular processes in the cardiovascular system. Dysregulation of gene expression and disrupted cellular processes underlie the pathogenesis of these diseases. By investigating the roles of lncRNAs in cardiac biology and disease, science can shed light on the molecular mechanisms contributing to cardiac dysfunction and identify new avenues for therapeutic intervention.

In summary, the exploration of lncRNAs in the heart is a rapidly growing field in molecular biology, providing valuable insights into gene regulation, cellular processes, and the pathogenesis of cardiovascular diseases. By investigating the functions and mechanisms of lncRNAs, scientists aim to uncover the intricate complexities of cardiac biology, gain a deeper understanding of the molecular foundations of cardiovascular disorders, and ultimately enhance clinical outcomes for individuals with heart diseases.

1.11 lncRNA in endothelial cells

Investigating the roles and molecular mechanisms of lncRNAs in endothelial cells is of significant importance in understanding vascular biology and exploring new avenues for therapeutic interventions [231, 232].

One lncRNA that has been identified as a regulator of endothelial cell angiogenesis is MALAT1 (metastasis-associated lung adenocarcinoma transcript 1). MALAT1 is highly expressed in endothelial cells and has been found to promote angiogenesis in both physiological and pathological conditions [233].

Studies have shown that MALAT1 enhances endothelial cell proliferation, migration, and tube formation, which are key steps in the angiogenic process [234]. It achieves this by interacting with and modulating the expression of various genes and signaling pathways involved in angiogenesis. For example, MALAT1 has been found to interact with VEGF (vascular endothelial growth factor) and

promote VEGF-induced angiogenesis [235]. It also interacts with miRNAs, such as miR-145, to regulate the expression of angiogenic factors [236].

Furthermore, MALAT1 can influence endothelial cell behavior by regulating endothelial cell apoptosis and inflammation, which are important processes in angiogenesis. It can inhibit endothelial cell apoptosis and suppress the production of pro-inflammatory molecules, thereby creating a favorable microenvironment for angiogenesis [237, 238].

Overall, lncRNA MALAT1 is a notable example of how lncRNAs can impact endothelial cell function and angiogenesis. By modulating gene expression, signaling pathways, and cellular processes, lncRNAs like MALAT1 can play crucial roles in endothelial cell behavior and contribute to angiogenesis in various physiological and pathological contexts.

Another well-studied lncRNA that affects endothelial cells is ANRIL (Antisense Non-Coding RNA in the INK4 Locus). ANRIL is transcribed from the INK4b-ARF-INK4a gene locus on chromosome 9p21 and has been associated with various cardiovascular diseases [239].

ANRIL has been shown to play a role in endothelial cell dysfunction and atherosclerosis, a chronic inflammatory condition characterized by the buildup of plaque in arterial walls [240]. It has been found to influence endothelial cell proliferation, apoptosis, senescence, and inflammation [241, 242].

One mechanism through which ANRIL affects endothelial cell function is by regulating the expression of key genes involved in cell cycle control and apoptosis. ANRIL has been shown to interact with chromatin-modifying complexes and epigenetically silence the expression of the CDKN2A/B locus, which encodes the tumour suppressor proteins p16INK4a and p15INK4b. Inhibition of these proteins can lead to increased proliferation and decreased apoptosis of endothelial cells, promoting pathological angiogenesis and atherosclerosis development [243, 244].

Additionally, ANRIL has been implicated in the regulation of endothelial cell senescence, a state of irreversible growth arrest associated with aging and vascular dysfunction [245]. ANRIL can modulate the activity of the p53 pathway, a key regulator of cellular senescence. By interacting with and inhibiting the activity of p53, ANRIL promotes endothelial cell senescence and contributes to vascular aging [246].

Furthermore, ANRIL has been linked to the regulation of endothelial cell inflammation. It can interact with NF- κ B (Nuclear Factor- κ B) and regulate its activity, leading to the modulation of inflammatory gene expression in endothelial cells. Dysregulation of ANRIL has been associated with increased inflammation in endothelial cells, promoting atherosclerosis development [247-249].

Overall, ANRIL is a well-studied lncRNA that influences endothelial cell function and is implicated in the pathogenesis of cardiovascular diseases. Its involvement in regulating cell cycle control, apoptosis, senescence, and inflammation highlights its importance in endothelial cell biology.

Understanding the functional significance of lncRNAs in endothelial cells has implications for vascular biology and cardiovascular diseases. Endothelial dysfunction is a hallmark of various cardiovascular disorders, and deciphering the roles of lncRNAs in endothelial cell function and dysfunction may lead to the identification of novel therapeutic targets for cardiovascular disease prevention and treatment. Therefore, studying lncRNAs in endothelial cells may provide an opportunity to gain fundamental insights into gene regulation and cellular processes relevant to vascular biology.

1.12 The long-non-coding RNA downstream of Cdkn1b, Lockd

Currently, the functional and mechanistic aspects of Lockd remain largely unknown in scientific literature. However, recent studies as of 2023 have shed some light on its role. It has been observed that the deletion of the 25-kb Lockd locus leads to a significant reduction of approximately 70% in Cdkn1b transcription in an erythroid cell line [250]. Conversely, the homozygous insertion of a polyadenylation cassette downstream of the Lockd transcription start site resulted in a drastic decrease of over 90% in the overall transcript level of the long noncoding RNA Lockd, without affecting Cdkn1b transcription. Through chromosomal conformation capture studies, it has been revealed that the Lockd promoter possesses a DNase-hypersensitive site, interacts with numerous transcription factors, and physically associates with the Cdkn1b promoter. Thus, it can be concluded that the Lockd gene exerts a positive regulatory effect on Cdkn1b transcription through an enhancer-like cis element, while the presence of the lncRNA itself is dispensable. These findings suggest a similar scenario may exist for other lncRNAs [250].

In a separate study conducted in 2022, researchers explored the effects of Lockd knockdown in NIH/3T3 cells using short hairpin RNA (shRNA). The findings revealed a reduction in the ability of the cells to form colonies, indicating a potential role of Lockd in cellular function, as its knockdown led to a significant impact. These observations suggest that Lockd may exert its effects through an RNA-dependent mechanism [251]. To further investigate this, they employed siRNAs and antisense oligos to knockdown Lockd. Interestingly, the researchers did not observe any changes in the RNA expression of Cdkn1b, which is known to be promoted by the Lockd loci [250].

To gain more insights into the molecular function of Lockd transcripts, the researchers conducted RNA sequencing analysis on stable shLockd and shControl cells. This analysis revealed significant alterations

in gene expression, with over 750 genes showing differential regulation in shLockd cells. Among these genes, 138 were identified as differentially regulated after rigorous filtering. Notably, several cell cycle regulators were included in this set of genes.

Of particular interest were three kinesins, namely Kif4, Kif11, and Kif14, which were found to be differentially regulated. Kinesins are a group of proteins involved in mitosis and emerged as key candidates in this context. To validate these findings, the researchers performed RT-qPCR analysis on Lockd knockdown induced by siRNA in NIH/3T3 and primary fibroblast cells. The effect on Kif11 and Kif14 expression was observed in both cell lines, while the effect on Kif4 expression was specifically observed in primary fibroblasts.

In summary, while it has been reported that the Lockd loci controls Cdkn1b transcription [250], this study revealed additional effects upon Lockd transcript knockdown that seem to function in the same pathway as Cdkn1b, exacerbating the negative impact on cell cycle progression. These findings provide further insights into the functional implications of Lockd and its association with Cdkn1b [251].

A third study aimed to elucidate the functional role of Lockd in the regulation of satellite cell activity and muscle regeneration. The researchers identified Lockd as a long noncoding RNA transcript that shows high induction upon satellite cell activation following injury. They provided evidence to demonstrate that Lockd plays a crucial role in promoting the proliferative expansion of myoblasts both *in vitro* and *in vivo* during the process of muscle regeneration [252].

Through detailed mechanistic investigations, they discovered that Lockd predominantly localizes in the cytoplasm of myoblasts and functions by binding with the DHX36 protein. This interaction enhances the stabilization of DHX36's interaction with EIF3B protein, forming a complex that facilitates the unwinding of the RNA G-quadruplexes (rG4) structure present in the 5' untranslated region (UTR) of Anp32e mRNA [252]. rG4s are RNA structures where guanine-rich sequences are folded into two or more layers of G-quartets, thereby playing an important regulatory motif [253]. The unwinding of this structure promotes the translation of Anp32e, leading to an increase in ANP32E protein levels. Subsequently, the increased ANP32E protein contributes to the facilitation of myoblast proliferation and the muscle regeneration process.

The absence of Lockd results in compromised DHX36-mediated unwinding of the rG4 structure, leading to decreased ANP32E protein levels. Consequently, this leads to a delay in myoblast proliferation and muscle regeneration. Overall, they claim that Lockd plays a vital role in enhancing the ability of satellite cells to regenerate damaged muscle tissue. Thus, it exerts a positive effect by promoting the proliferative expansion of myoblasts during the process of muscle regeneration [252].

In the most recent publication of Lockd, researchers showed maternal obesity leads to metabolic disorders and weight gain in offspring during early life. Analysis of liver samples revealed differential expression of long non-coding RNAs (lncRNAs) in the offspring of obese dams, including the lncRNA Lockd. Knockdown of Lockd using siRNA-lockd resulted in increased P62 mRNA levels, decreased LC3II mRNA levels, and inhibition of autophagy in an AML12 cell model of lipid accumulation. Furthermore, knockdown of Lockd promoted lipid accumulation in these AML12 cells [84].

The study also investigated the underlying mechanism by which Lockd regulates autophagy. The mTOR signalling pathway, known for its regulation of autophagy, was examined. Maternal obesity led to increased expression of p-mTOR protein in the livers of offspring, while treatment with rapamycin (an mTOR inhibitor) reduced lipid accumulation. Transfection of siRNA-lockd resulted in increased p-mTOR protein expression, indicating a potential role of Lockd in autophagy regulation through the mTOR pathway [84].

In summary, Lockd is a multifunctional lncRNA involved in cell cycle progression, muscle regeneration, and metabolic disorders associated with maternal obesity. Its regulatory effects on gene expression and its interactions with specific proteins contribute to its functional implications in various biological processes. Further research on Lockd holds promise for unravelling the molecular mechanisms underlying its diverse roles.

2. Aim of the study

Angiogenesis is a crucial process involved in various biological phenomena, including development, growth, wound healing, tissue repair, and cardiovascular health and disease. In severe cardiovascular conditions like myocardial infarction and pathological cardiac hypertrophy, neovascularization plays a significant role in disease progression.

The advancement of RNA sequencing has greatly contributed to the identification of genes, including long non-coding RNAs (lncRNAs) involved in the regulation of key processes in cardiovascular diseases. However, to date, there have been no reported studies investigating the role of lncRNAs in the early onset of pathophysiological cardiac remodelling, endothelial cell proliferation, and angiogenesis.

The aim of this thesis is to explore the functional role of the newly discovered lncRNA Lockd, which exhibits significant upregulation in cardiac tissue, specifically in endothelial cells, following the early phase of cardiac pressure overload. The focus of this research is to investigate the impact of Lockd on cardiac endothelial cells during the pathophysiological cardiac remodelling process induced by cardiac pressure overload. Furthermore, the study aims to unravel the molecular mechanisms underlying Lockd's involvement in endothelial cell proliferation and angiogenesis. By gaining a better understanding of Lockd's role, this research aims to contribute to the broader knowledge of cardiovascular diseases and the role of endothelial derived lncRNAs in this process.

3. Materials

3.1 Reagents and chemicals

Table 1. List of reagents and chemicals

Chemical / Reagent	Catalogue Number	Supplier
1,4-Dithiothreitol (DTT)	6908.1	Roth
2-Mercaptoethanol	4227.3	Roth
2-Propanol	9866.5	Roth
Acetic acid	3738.2	Roth
Actinomycin D	A1410-5MG	Sigma-Aldrich
Agar-Agar	6494.1	Roth
Ammoniumperoxodisulfat APS	9592.3	Roth
Atto 565 NHS Ester	AD 565-31	Atto Tec
Bepanthen eye ointment	/	Pharmaplace
Benzonase	E1014-5KU	Merck
Buprenovet	2540	WDT
Calcium chloride dihydrate	1023820500	Merck
Collagenase I	LS0004196	Worthington
Collagenase II	LS004177	Worthington
Collagen type I, Rat tail	08-115	Sigma Aldrich
Complete™, EDTA-free Protease Inhibitor	4693132001	Roche
D(+)-Biotin	8512090005	Merck
DAPI	62248	ThermoFisher
Deoxyribonuclease I	LS002007	Worthington
Dextran Sulfate 50% Solution	S4030	Merck
Direct Red 80 dye	365548	Sigma Aldrich
DMSO (Dimethyl Sulfoxide), Sterile	12611	Cell Signaling
DNA marker	SM0242	ThermoFisher
DMEM, w: 4.5 g/L Glucose, w/o: L-Glutamine, w/o: Sodium pyruvate, w: 3.7 g/L NaHCO ₃	P04-03500	Pan biotech
Dynabeads™ M-280 Sheep Anti-Rabbit IgG	11203D	ThermoFisher
Dynabeads™ MyOne™ Streptavidin C1	65002	ThermoFisher
ECL™ Prime Western-Blot-Detektion-reagent	12994780	Fisher Scientific

Endothelial Cell Growth Medium (Ready-to-use) - Includes Basal Medium and SupplementMix	C-22010	PromoCell
Gene Ruler 100bp DNA Ladder 5x 50 microg	SM0242	ThermoFisher
Gelatine	4582.1	Roth
Glycin	3790.2	Roth
GlycoBlue™ Coprecipitant (15 mg/mL)	AM9515	ThermoFisher
Go Taq G2 Flexi DNA Polymerase	M7808	Promega
Glycin	3790.4	Roth
Glutaraldehyde solution	G7526	Merck
Glycerol	C5516	Sigma-Aldrich
Heparin Natrium 25 000	3029843	Pharmaplace
HEPES	9105.4	Roth
HEPES solution (1M)	15630056	ThermoFischer
Histoacryl	1050052	Häberle
IGEPAL® CA-630	I8896	Sigma-Aldrich
ImmEdge Hydrophobic Barrier (PAP) Pen, 2-Pen Set	VEC-H-4000	Biozol
Isopropanol 2.5 l	9866.5	Roth
L-Glutamine	P04-80100	PAN
KCl	P4504	Sigma-Aldrich
KH ₂ PO ₄	P5379	Sigma-Aldrich
KHCO ₃	60339	Sigma-Aldrich
Liberase™ DH	5401089001	Merck
Lipofectamine-RNAiMAX	13778075	ThermoFischer
MACS BSA Stock Solution	130-091-376	Miltenyi Biotec
MACS Rinsing Solution	130-091-222	Miltenyi Biotec
Magnet® Protein A Beads	G8782	Promega
Magnesium sulfate heptahydrate	M5921	Sigma-Aldrich
Medium M199, 10x	M0650	Sigma-Aldrich
Methanol >99%	8388.6	Carl Roth
MG-132 ≥95%	133407-82-6	Hözel
MgSO ₄ ·7H ₂ O	M1880	Sigma-Aldrich
Methyl cellulose	M0512	Sigma-Aldrich
Midori green	MG04	Nippon

Mitomycin C	M4287	Sigma-Aldrich
MS columns	130-042-201	Miltenyi
Mycoplasma Off™	15-1000	Minerva Biolabs
NaCl	S9625	Sigma-Aldrich
Na ₂ HPO ₄	S0876	Sigma-Aldrich
NaHCO ₃	S5761	Sigma-Aldrich
N-Dodecyl-Beta-D-Maltoside (DDM, 85%)	250520P-1G	Sigma-Aldrich
N-Lauroylsarcosine sodium salt detergent	L9150-50G	Merck
N-Penatne	3747.1	Carl Roth
Nuklease-free Water (Nuclease-free water)	AM9937	Thermo
Opti-MEM™ I Reduced Serum Medium	31985062	Gibco
Phenol:Chloroform:Isoamyl Alcohol 25:24:1	P2069-100ML	Sigma-Aldrich
Pierce™ 16% Formaldehyd (w/v), methanolfrei	28906	ThermoFisher
Pierce™ BCA Protein Assay Kits	10678484	ThermoFisher
Phenylmethylsulfonyl fluoride (PMSF)	P7626	Sigma-Aldrich
Ponceau S solution	P7170-1l	Sigma-Aldrich
Potassium bicarbonate	60339	Sigma-Aldrich
Potassium chloride	1049360500	Merck
Potassium phosphate monobasic	P5379	Sigma-Aldrich
Powerup sybr	15360929	ThermoFisher
ProLong™ Gold Antifade Mountant	P10144	ThermoFisher
Proteinase K, Molecular Biology Grade	P8107S	NEB
ProSieve QuadColor Protein Marker	830537	Lonza
Puromycin	P7255	Sigma-Aldrich
Recombinant Human TGF-β1 (HEK293 derived)	100-21	Pepro tech
Recombinant Mouse FGF basic/FGF2/bFGF Protein	3139-FB-025/CF	R&D
Recombinant Mouse VEGF 164 Protein	493-MV-005/CF	R&D
Ribonucleoside vanadyl complex	94742-10ML	Sigma-Aldrich
RNase A	R6148-25ML	Sigma
RNase A/T1 Mix	EN0551	Thermo
rDNase set	740963	Macherey Nagel
RPMI 1640 Medium, no glutamine	31870025	ThermoFischer
RNA Clean & Concentrator-5	R1016	Zymo Research

RNAscope® Probe- Mm-Lockd	546621	acdbio
RNAscope® Probe- Mm-Cdh5-C2	312531-C2	acdbio
Roti®Histofix 4%	P087.5	Roth
Roticlear solution	A538.6	Roth
Roti®Mount FluorCare ohne DAPI	HP19.1	Roth
Serological pipette with filter, 25 ml	86.1685.001	Sarstedt
Serological pipette with filter, 50 ml	86.1256.001	Sarstedt
Sodium bicarbonate	S5761	Sigma-Aldrich
Sodium chloride	S9625	Sigma-Aldrich
Sodium deoxycholate	D6750	Sigma-Aldrich
Sodium dodecyl sulfate solution 20%	10607443	FisherScientific
Potassium phosphate monobasic	P5379	Sigma-Aldrich
Sodium hydroxide	30620	Sigma-Aldrich
SSC Buffer 20× Concentrate	S6639-1L	Merck
Stellaris RNA FISH Hybridization Buffer	SMF-HB1-10	Biosearch Technologies Cat
Stellaris RNA FISH Wash Buffer A	SMF-WA1-60	Biosearch Technologies Cat
Stellaris RNA FISH Wash Buffer B	SMF-WB1-20	Biosearch Technologies Cat
Streptavidin Magnetic Beads	S1420S	NEB
Streptavidin Magnetic Beads	S1420S	NEB
TCA, Trichloroacetic acid	T6399-250G	Sigma-Aldrich
Taurine	T0625	Sigma-Aldrich
TEMED	2367.3	Roth
TRIzol™ Reagenz	15596018	ThermoFisher
Triton X 100 reinst	3051.2	Roth
TRIS	5429.5	Roth
Tris-(2-carboxyethyl)-phosphin Hydrochlorid (TCEP)	HN95.2	Roth
Trypsin 2.5% no phenol red 100ml	15090-046	ThermoFisher
TURBO™ DNase kit (2 U/μL)	AM2238	ThermoFisher
UltraPure™ BSA	AM2618	ThermoFisher
UltraPure™ Salmon Sperm DNA Solution	15632011	ThermoFisher

Vanadyl Ribonucleoside Complex	S1402S	NEB
Vectashield Hardset with Dapi	H-1500	Vector Lab
VECTASHIELD HardSet(TM) Antifade Mounting Medium with DAPI	H-1500	Biozol
Xylene	1330-20-7	Merck
[5-(N-Ethyl-N-isopropyl) Amiloride HCl]	E8643	Sigma-Aldrich

3.2 Kits and consumables

3.2.1 Kits

Table 2. List of kits

Kit	Catalogue Number	Supplier
Apo-ONE® Homogeneous Caspase-3/7 Assay	G7790	Promega
Buffer Kit, RNase-free	AM9010	ThermoFisher
Cell Proliferation ELISA, BrdU	11647229001	Roche
Maxima H Minus First Strand cDNA Synthesis Kit	K1652	ThermoFisher
NucleoSpin RNA, Mini kit for RNA purification	740955.25	Macherey Nagel
Pierce™ BCA Protein Assay Kits	10678484	ThermoFisher
Propidium Iodide Flow Cytometry Kit	ab139418	Abcam
RNA Clean & Concentrator-5	R1016	Zymo Research
RNAscope Multiplex Fluorescent Reagent Kit v2	323100-USM	acdbio

3.2.2 Consumables

Table 3. List of consumables

Material	Catalogue Number	Supplier
7-0 ethilon	EB5133H	ethicon
10-0 ethilon	EH7467G	ethicon
1 ml Syringe	9161406	Buddeberg
5 ml Serological pipette with filter	861.253.001	Sarstedt
10 ml Serological pipette with filter	861.254.001	Sarstedt
25 ml Serological pipette with filter	86.1685.001	Sarstedt

50 ml Serological pipette with filter	86.1256.001	Sarstedt
15 ml Falcons	62.554.009	Sarstedt
50 ml Falcons	62.547.254	Sarstedt
6-well plate	83.3920	Sarstedt
12-well plate	83.3921	Sarstedt
24-well plate	83.3922	Sarstedt
96-well plate	83.3925	Sarstedt
2 mL Cryotubes	72.379	Sarstedt
Cell scraper	353085	BD Falcon
Cover glasses, round, 12 mm	631-0713	VWR
Cover glasses, round, 3 cm	631-1347	VWR
Animal hair trimmer	GT421	Aesculap
Parafilm	PF10	Häberle
Cell culture dish, 60 x 15 mm	83.3901	Sarstedt
Cell culture dish, 10 x 20 mm	83.3902	Sarstedt
Cell culture dish, 150 x 20 mm	83.3903	Sarstedt
Filter tip 1.250 µl	70.3060	Sarstedt
Filter tip 200 µl	70.3031	Sarstedt
Filter tip 100 µl	70.3030	Sarstedt
Filter tip 10 µl	F171203	Gilson
q-PCR plates	72.1980	Sarstedt
q-PCR Adhesive seal	95.1993	Sarstedt
Reagent reservoirs 25 ml	8093-11	Buddeberg
T75 flasks	833911002	Sarstedt
Syringe filter, pore size: 0.2 µm	831826001	Sarstedt

3.3 Buffers and solutions

Table 4. Buffers and solutions recipes

Buffer	Recipe
5X Laemmli buffer	60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue

10X ADS buffer	34 g NaCl, 23.8 g HEPES, 0.6 g NaH ₂ PO ₄ ·H ₂ O, 5 g Glucose, 3 g KCL, 0.5 g MgSO ₄ ·7H ₂ O in 500 mL sterile H ₂ O
0.5 % Gelatin	2,5g Gelatine in 500 mL sterile H ₂ O
Blocking buffer	3% BSA in TBS-T
ChIRP DNA Elution buffer	50 mM NaHCO ₃ , 1 % SDS in DNase-free H ₂ O
ChIRP Hybridization buffer	750 mM NaCl, 1% SDS, 50 mM Tris-Cl pH 7.0, 1 mM EDTA, 15% formamide, 1 mM PMSF, 1x protease inhibitors, 1X Rnase Inhibitors in RNase-free H ₂ O
ChIRP Lysis buffer	50 mM Tris-HCl pH 7.0, 10 mM EDTA, 1 % SDS, 1 mM PMSF, 1X protease inhibitors, 1X RNase inhibitors in RNase-free H ₂ O
ChIRP Proteinase K (pK) buffer	100 mM NaCl, 10 mM Tris-HCl (pH 7.0), 1 mM EDTA, 0.5 % SDS, 5 % v/v Proteinase K in RNase-free H ₂ O
ChIRP Wash buffer	2× SSC, 0.5% SDS, 1 mM PMSF in RNase-free H ₂ O
ChIRP-MS Biotin elution buffer	12.5 mM D-biotin, 7.5 mM HEPES (pH 7.5), 75 mM NaCl, 1.5 mM EDTA, 0.15% SDS, 0.075% sarkosyl, and 0.02% Na-Deoxycholate in distilled H ₂ O
dNTP mix	10 mM dATP, 10 mM dGTP, 10 mM dCTP, 10 mM dTTP
Endothelial Basal Medium	20% v/v FCS, 25mM HEPES, 1X Pen/Strep, 1X L-Glutamine in DMEM high glucose
Endothelial Cell Growth Medium	Supplement Mix, 5 mL 100X Penicillin-Streptomycin in Endothelial cell Growth Medium
Endothelial Cell Growth Medium MV	Supplement Mix, 5 mL 100X Penicillin-Streptomycin in Endothelial Cell Growth Medium MV Medium
Endothelial Full Medium	2 ml ECGS/H, 2.5 ml 100X Non-essential amino acids, 2.5 ml 100X sodium pyruvate in 250 mL Endothelial Basal medium
MACS buffer	0.5% BSA, 2 mM Na-EDTA in 1X PBS
Modified Eagle's Medium (DMEM)	5 mL 100X Penicillin-Streptomycin, 5 mL 100X L-Glutamin, 50 mL FCS in 500 mL DMEM high glucose
Langendorff Perfusion buffer	113 mM NaCl, 4.7 mM KCL, 0.6 mM KH ₂ PO ₄ , 0.6 mM Na ₂ HPO ₄ , 1.2 mM MgSO ₄ ·7H ₂ O, 12 mM NaHCO ₃ , 10 mM KHCO ₃ , 10 mM HEPES, 30 mM Taurine, 10 mM BDM, 55 mM glucose in sterile H ₂ O
Langendorff Digestion buffer	For 1 heart: 1 mL 5 mg/mL Liberase DH stock solution, 111,2 Trypsin stock-solution, 2.5 uL 100 mM CaCl ₂ in 19 mL perfusion buffer

Neonatal rat hearts digestion buffer	44 mg Collagenase II and 10 mg Pankreatin in 1X ADS buffer
Protein lysis buffer	10 mM Tris, 150 mM NaCl, 4% glycerol, 0.5 mM Natrium disulfit, 1% Triton, 0.1% Nadeoxycholate, 0.05% SDS, 1 mM DTT, and 1x protease inhibitor cocktail
Protein storage solution	100 mM HCl, 150 mM NaCl, 1 mM EDTA in sterile H ₂ O
RAP Hybridization buffer	10 mM Tris–HCl pH 7.5, 5 mM EDTA, 500 mM LiCl, 0.5% DDM, 0.2% SDS, 0.1% sodium deoxycholate, 4 M urea, and 2.5 mM TCEP in Rnase-free H ₂ O
RAP Benzonase elution buffer	20 mM Tris–HCl pH 8.0, 0.05% NLS, 2 mM MgCl ₂ , 0.5 mM TCEP in distilled H ₂ O
RAP Total cell lysis buffer	10 mM Tris–HCl pH 7.5, 500mM LiCl, 0.5% DDM, 0.2% SDS, 0.1% sodium deoxycholate, 1X protease inhibitor cocktail, 1000 U/mL Rnase inhibitor in Rnase-free H ₂ O
RIP 10X Polsome Lysis buffer	1000 mM KCl, 50 mM MgCl ₂ , 100 mM HEPES-NaOH (pH 7.0),5 % NP-40, 1mM DTT, 1X Rnase Inhibitors, 1x EDTA free protease inhibitors in Rnase-free H ₂ O
RIP 5X NT-2 buffer	250 mM Tris-HCl (pH 7.4), 750mM NaCl, 5 mM MgCl ₂ , 0.25% NP-40 in Rnase-free H ₂ O
RIP NET-2 buffer	20mM EDTA (pH 8.0), 1mM DTT, 1X Rnase inhibitors in 1X NT-2 buffer
RIP Proteinase K buffer	1% SDS, 5% v/v Proteinase K in 1X NT-2 buffer
RLN1 buffer	50 mM Tris-HCl pH 8.0, 140 mM NaCl, 1.5 mM MgCl ₂ , 0.5% Igepal, 2 mM Vanadyl Ribonucleosid Complex
RLN2 buffer	50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1.5 mM MgCl ₂ , 0.5% Igepal, 2 mM Vanadyl Ribonucleoside Complex
SDS-PAGE Running gel	for 1 gel: 3.3 mL 30% acrylamide mix, 2.5 mL 1.5M Tris (pH 8.8), 0.1 mL 10% SDS, 0.1 mL ammonium persulfate, 4 uL TEMED in 4 mL distilled H ₂ O
SDS-PAGE Stacking gel	for 1 gel: 0.5 mL 30% acrylamide mix, 0.38 mL 1.0M Tris (pH 6.8), 30 uL 10% SDS, 30 uL ammonium persulfate, 3 uL TEMED in 2.1 mL distilled H ₂ O
smFISH Hybridization buffer	880 uL 20x SSC, 880 uL Formamide in 7.04 mL Rnase-free H ₂ O

smFISH Wash buffer	23 μ L 20x SSC, 22 μ L Formamide, 2.2 μ M VRC, 2.2 μ L 10 mg/mL salmon sperm DNA, 44 μ L 50% Dextran sulfate, 0.88 μ L 50 mg/mL BSA in 126.72 RNase-free H ₂ O
Stop 1 solution	5 mL FCS, 62.5 μ L 10 mM CaCl ₂ in 45 mL Perfusion buffer
Stop 2 solution	5 mL FCS, 208 μ L 10 mM CaCl ₂ in 95 mL Perfusion buffer
TBE buffer	108 g TRIS, 55 g boric acid, 40 ml 0.5 M EDTA solution (pH 8.0) in 900 ml distilled H ₂ O
TBS-T	20 mM Tris, 150 mM NaCl, 0.1% (w/v) Tween 20 detergent in distilled H ₂ O
Western-Blot Running buffer	25 mM Tris, 192 mM glycine, 3.5 mM SDS in distilled H ₂ O
Western-Blot Transfer buffer	25 mM Tris, 192 mM glycine, 20% (v/v) methanol (pH 8.3) in distilled H ₂ O

3.4 Equipment and devices

Table 5. List of equipment and devices

Equipment / Devices	Model
Benchtop centrifuge	ThermoFisher Heraeus Megafuge 16R
Benchtop centrifuge	ThermoFisher Heraeus Fresco 17
Benchtop microscope	Olympus CKX53
Cell culture incubator	Binder Serie BD
echocardiogram	VisualSonics-Fujifilm Vevo 3100 device
End-to end rotor (4°C)	Phoenix Instrument RS-RD 5
End-to end rotor (RT)	Benchmark Roto-Bot
Fluorescent microscope	Leica DMI8
Mikroskop-camera	Leica DFC7000 GT
Mixing Block	Bioer Mixing Block MB-102
Nanodrop	DeNovix DS-11 Spectrophotometer
PCR machine	Bioer GeneTouch
Power supply	BioRad PowerPac Basic
qPCR Machine	Agilent Technologies AriaMX Real-Time PCR System MY16365264
Shaker	Benchmark Rocker 20
Slide scanner	ZEISS Axioscan 7

Sonicator	Omni-Ruptor 250
UV Crosslinker	analytikjena
UV Transilluminator	Syngene Ingenious 3
Wester-Blot Imager	Amersham 680

3.5 Nucleic acid sequences

3.5.1 Small interfering RNAs and single-stranded LNA oligonucleotides

Table 6. List of small interfering RNAs and single-stranded LNA oligonucleotides

Name	Sequence	Catalogue Number	Supplier
ON-TARGETplus Non-targeting Control Pool	/	D-001810-10-20	HorizonDharmacon
ON-TARGETplus Mouse Uhmk1 siRNA	/	L-041701-00-0010	HorizonDharmacon
Lincode Mouse Lockd siRNA SMARTPool	siLockd_5 GCAUCAAACAUUCACGGAU siLockd_6 ACAGAAUGCAGGCGGGAAG siLockd_7 UAGAAAAGUCCUUGACGGA siLockd_8 GCCCUGAGGUCAUGUGAAA	R-167458-00-0010	HorizonDharmacon
human_AI120476 duplex 1 siRNA	sihLockd_1 GAAAGAUACAGGAACUAUAUU	CTM-808759 (Custom siRNA)	HorizonDharmacon
human_AI120476 duplex 3 siRNA	sihLockd_3 CUGCCUAUGAGGUGUGUAAUU	CTM-808762 (Custom siRNA)	HorizonDharmacon
Lincode Mouse Lockd siRNA Individual	siLockd_5 GCAUCAAACAUUCACGGAU	N-167458-05-0005	HorizonDharmacon
NR_037955.1_1 Antisense LNA GapmeR	GGAGATAAGAGATGCA	LG00798903	Quiagen

Negative control A	AACACGTCTATACGC	LG00000002	Quiagen
Antisense LNA			
GapmeR			

3.5.2 RT - qPCR primer sequences

Table 7. List of qPCR primers

Sequence (5'-3')	Strand	Species	Name
GTAACCCGTTGAACCCCATTT	for	Mouse	18s rRNA
CCATCCAATCGGTAGTAGCG	rev	Mouse	18s rRNA
GCATCTATACGGACCAGGCT	fwd	Mouse	Ccnd3
CCCTGGCTCTGGAACTAGA	rev	Mouse	Ccnd3
GGTAGTACAGGCTGGGGTAG	rev	Mouse	Cdc25b
CTACAGCTTCTCCTCTCCG	fwd	Mouse	Cdc25b
TGAGAGTGTCTAACGGGAGC	for	Mouse Rat	Cdkn1b
CCAAGTCCCGGGTTAGTTCT	rev	Mouse Rat	Cdkn1b
AAGGGAGAGAGAGGAATGCC	fwd	Mouse	Col4a3
TACTCCCTTTCAAGCCTGGC	rev	Mouse	Col4a3
TTCCCTCCAGCCATAACCTC	fwd	Mouse	Col4a4
TACTCCTCCAGCCTTCTCCT	rev	Mouse	Col4a4
ATGCGGGCGAGTTCAAATAC	for	Mouse	Dusp6
CGTTCATGGACAGGTTGAGC	rev	Mouse	Dusp6
GAGATCTCAGCGAGTCCCTC	fwd	Mouse	Foxc2
TGGGGTTTGGTCCATAAGCT	rev	Mouse	Foxc2
ACCCAGAAGACTGTGGATGG	fwd	Mouse	Gapdh
CACATTGGGGGTAGGAACAC	rev	Mouse	Gapdh
ACCACCATGGAGAAGGCTGG	for	Rat	Gapdh
CTCAGTGTAGCCCAGGATGC	rev	Rat	Gapdh
ACCTGACCTGCCGTCTAGAA	for	Human	Gapdh
ACCCTGTTGCTGTAGCCAAA	rev	Human	Gapdh
CAGGGAGTGGAGCCAGAG	for	Mouse	Hotair
CTCTGGAAGGACTTAGCGCT	rev	Mouse	Hotair
AAGCTTGCTGGTGAAAAGGA	fwd	Mouse	Hprt1

TTGCGCTCATCTTAGGCTTT	rev	Mouse	Hprt1
GTGAAAGGAACAGTGGGCAG	fwd	Mouse	Lipg
CCACAAAGTCTGCATCGTCC	rev	Mouse	Lipg
AAGGTACAGGCGGGAGTTC	for	Mouse	Lockd
CAGAAGGACACAGGGCAAAC	rev	Mouse	Lockd
TAGGAAGGTACAGGCGGGAG	fwd	Mouse Rat	Lockd
GACAGCCAACAGGAGACAG	rev	Mouse Rat	Lockd
AGATGTAACCCAACTCGTTCAC	fwd	Human	Lockd
GCACTCCCCAGTTCTACCTT	rev	Human	Lockd
AGCAGGCATTGTGGAGAGGA	fwd	Mouse	Malat1
ATGTTGCCGACCTCAAGGAA	rev	Mouse	Malat1
CAATCCTTAACCTGCTGCCC	fwd	Mouse	Mtor
CCTTAAACTCCGACCTCACG	rev	Mouse	Mtor
GATTTCTTTGGGCGTTGGA	fwd	Mouse	Myc
GTGGGAAGCAGCTCGAATTT	rev	Mouse	Myc
GAGTGGGAGTTTATCGACTTCG	fwd	Mouse	Myh6
CCTTGACATTGCGAGGCTTC	rev	Mouse	Myh6
ACTGTCAACACTAAGAGGGTCA	fwd	Mouse	Myh7
TTGGATGATTTGATCTTCCAGGG	rev	Mouse	Myh7
TTCCTCGTCTTGGCCTTTTG	fwd	Mouse	Nppa
CCTCATCTTCTACCGGCATC	rev	Mouse	Nppa
GTCCAGCAGAGACCTCAAAA	fwd	Mouse	Nppb
AGGCAGAGTCAGAACTGGA	rev	Mouse	Nppb
AGAGAAACGTGCCTGGAGAA	for	Mouse	Nuf2
CAAAGCTTCCTGGTGACAT	rev	Mouse	Nuf2
CGTGAGAAGCGGAGTACTTG	fwd	Mouse	Oip5os1
CTATGGAGCCTTTCAAGCCG	rev	Mouse	Oip5os1
AGCATTCCGACTACAGAGCC	for	Mouse	Pbx1
CTGGATGGAGCTGAACTTGC	rev	Mouse	Pbx1
ATGAAATGCTGAGCGACCAC	fwd	Mouse	Pdgfb
TCGGGTCATGTTCAAGTCCA	rev	Mouse	Pdgfb
CGTTGCATCCGAGAACCATG	fwd	Mouse	Prg4
AGAGTACCCTTCCCCACATC	rev	Mouse	Prg4

CTCGGCCTTTTGGCTAAGAT	for	Mouse	U2
CGTTCCTGGAGGTAAGTCAA	rev	Mouse	U2
CTCGCTTCGGCAGCACA	for	Human Mouse Rat	U6
AACGCTTCACGAATTTGCGT	rev	Human Mouse Rat	U6
TGTTGATCTGTGGAGCCTCG	fwd	Mouse	Uhmk1
GAATTGCGGCATTCACCACT	rev	Mouse	Uhmk1
CCTTCACTTGGCTCCTGAGA	for	Mouse	Xist
ACTTTAGGGTCAGGCTTGCT	rev	Mouse	Xist

3.5.3 Sequences of oligos used for Fluorescence *in situ* hybridization

Table 8. List of oligos used for Fluorescence *in situ* hybridization experiments

Sequence (5'-3')	Species	Name
CATTCTGTCAACGGCACTTG	Mouse	Lockd-Fish-Probe1
TCTACCCAACTGAAGCTTC	Mouse	Lockd-Fish-Probe2
ATCCGTGAATGTTTGATGCT	Mouse	Lockd-Fish-Probe3
TCAGGGCTTAAAGAGTCCAG	Mouse	Lockd-Fish-Probe4
AGGGCAAACCTCTTTTCAACA	Mouse	Lockd-Fish-Probe5
TACAGACAAAGAGGACGCCT	Mouse	Lockd-Fish-Probe6
TCCACAATGGACTCTCTATC	Mouse	Lockd-Fish-Probe7
CCTATATAGTTCCTGTATCT	Mouse	Lockd-Fish-Probe8
AACTCCGTCAAGGACTTTTC	Mouse	Lockd-Fish-Probe9
ACAGATCCACCACTGCAGAG	Mouse	Lockd-Fish-Probe10

3.5.4 Sequences of biotinylated DNA oligos

Table 9. Sequences of biotinylated DNA oligos used for RAP-MS and ChIRP

Sequence (5'-3')	Species	Name
[Bt _n]CAGGATGCTCCACTCCCACAGATC CACCACTGCAGAGCCACTGAACAGGC **	Mouse	Lockd-RAP-MS Probe1
[Bt _n]GCATTTGGCAGCTAGTAACAGAGC CCAGAAACAGTAGTGGCTTAAGCCAG **	Mouse	Lockd-RAP-MS Probe2
[Bt _n]CTGTAAAAGAATTCTGGAGATAAG	Mouse	Lockd-RAP-MS Probe3

AGATGCAGTCCGGTAGAGCCTCGCCT **		
[Btn]TGTGGACAGCCAACAGGAGACAGA AGGACACAGGGCAAACCTCTTTTCAC **	Mouse	Lockd-RAP-MS Probe4
[Btn]TCTCTACCCAACTGAAGCTTCCCGC CTGCATTCTGTCAACGGCACTTGC **	Mouse	Lockd-RAP-MS Probe5
ATCCGTGAATGTTTGATGCT[BtnTg] *	Mouse	Lockd-ChIRP1
ACTTCTTTTCACATGACCTC[BtnTg] *	Mouse	Lockd-ChIRP2
TCCACAATGGACTCTCTATC[BtnTg] *	Mouse	Lockd-ChIRP3
AACTCCGTCAAGGACTTTTC[BtnTg] *	Mouse	Lockd-ChIRP4

* 3' [BtnTg] - Biotin TEG Tag

** 5' [Btn] - Biotin Tag

3.6 Biological material

3.6.1 Cell lines

Table 10. Cell line, origin and growth medium

Origin	Name	Catalogue number	Supplier	Growth Media
Heart (Human)	Human Cardiac Microvascular Endothelial Cells	C-12285	PromoCell	Endothelial cell growth medium MV
Umbilical Vein (Human)	Human Umbilical Vein Endothelial Cells	C-12200	PromoCell	Endothelial cell growth medium
Yolk sac (House mouse)	C166	CRL-2581	ATCC	DMEM

3.6.2 Mouse lines

Table 11. Mouse lines

Mice	Supplier
RjOrl:SWISS (CD-1)	Janvier Labs
C57BL/6NRj	Janvier Labs

3.6.3 Antibodies

Table 12. List of primary antibodies

Antibody	Catalogue Number	Supplier
Alexa Fluor® 555 Phalloidin	A34055	Life Technologies
Anti-Aurora B	A5102	Sigma
Anti-CD146(LSEC)	130-092-007	Miitenyi Biotec
Anti-Col1a1	sc-293182	Santa Cruz
Anti-Gapdh	10R-G109A	Fitzgerald / Biozol
Anti-Mouse CD102	553326	Bdbiosciences
Anti-Mouse CD31	553370	Bdbiosciences
Anti-Phospho-Histone H3 (Ser10) (9HCLC)	ab177961	abcam
Anti-Phospho-S6 Ribosomal Protein (Ser235/236)	2211	Cell Signaling Technology
Anti-RACK1 (D59D5)	5432	Cell Signaling Technology
Anti-RPS6	ab70227	abcam
Anti-S6 Ribosomal Protein	2217	Cell Signaling Technology
Anti-TAGLN/Transgelin	ab14106	abcam
Anti-Troponin T	ab8295	abcam
Anti-Vinculin	V9131-100UL	Sigma-Aldrich
Anti- Cdkn1b	ab215434	abcam
Anti-Phospho- Cdkn1b Kip1 (Ser10)	10536090	Invitrogen
Isolectin B4, Fluorescein	FL-1201	Vector labs-Biozol
Normal rabbit IgG	2729S	Cell Signaling Technology
Wheat germ agglutinin Texas Red	w21405	Invitrogen

Table 13. List of secondary antibodies

Antibody	Catalogue Number	Supplier
Anti-Mouse IgG-HRP konj.	62-6520	ThermoFisher
Anti-Mouse IgG Alexa 488	4408	Cell Signaling Technology
Anti-Mouse IgG Alexa 555	4409	Cell Signaling Technology
Anti-Mouse IgG2a, Fc gamma Specific-HRP konj.	33416	Cell Signaling Technology
Anti-Rabbit IgG Alexa 647	711-605-152	Jackson Immuno research

Anti-Rabbit IgG Alexa 488	4412	Cell Signaling Technology
Anti-Rabbit IgG Alexa 555	4413	Cell Signaling Technology
Anti-Rabbit IgG-HRP konj.	NA934-1ML	GE Healthcare/Amersham

3.7 Online webtools and softwares

Table 14. List of online

Name	Site
BioRender	biorender.com
ChatGPT	chat.openai.com
UCSC genome browser	genome.ucsc.edu
String	string-db.org
ORF Finder	genecorner.ugent.be/orf_find
Primer3	primer3.ut.ee
Coding Potential Assessment Tool	bio.tools/cpat
Metascape - A Gene Annotation & Analysis Resource	metascape.org
National Library of Medicine	ncbi.nlm.nih.gov
LALIGN/PLALIGN	fasta.bioch.virginia.edu

Table 15. List of softwares

Software	Provider
Adobe Illustrator	Adobe
Flow Jo	FlowJo, LLC
GraphPad Prism 8	GraphPad Software, San Diego, USA
Image J	National Institutes of Health, Bethesda, USA
MS Office	Microsoft
Knime	KNIME AG, Zürich
Vevo Lab	VisualSonics
ZEN (blue edition)	Carl Zeiss Microscopy

4. Methods

4.1 Cell culture

All primary cells and immortalised cell lines were cultured in a humidified incubator at 37°C and 5% CO₂.

4.1.1 Culture of immortalized cell lines

The C166 mouse embryonic yolk-sac endothelial cell line (ATCC: CRL-2581) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 8% Fetal Calf Serum (FCS), 1x L-glutamine, and 1x penicillin/streptomycin, under standard cell culture conditions. The cells were cultured in 3 ml of medium in a 6 cm Petri dish for maintenance. The cells were cultured up to a maximum of 20 passages. The HL-1 mouse cardiomyocyte cell line (Merck: SCC065) was cultured in Claycomb media (Sigma: 51800C) supplemented with 8% FCS, 0.1 mM norepinephrine, 2 mM L-glutamine, and 1x penicillin/streptomycin (P/S). HL-1 cells were grown on 0.02% gelatin-coated flasks, and cell culture media was exchanged daily. The cells were cultured in 3 ml of medium in a 6 cm Petri dish for maintenance. The cells were cultured up to 10 passages to maintain their cardiomyocyte characteristics.

4.1.2 Culture of primary cell lines

All primary cell lines were purchased from PromoCell.

HUVECs (Human Umbilical Vein Endothelial Cells) from PromoCell were cultured in Endothelial Cell Growth Medium (PromoCell: C- 22010) supplemented with the corresponding supplement mix (PromoCell: C-39215) and 1x penicillin/streptomycin (P/S). The culture medium was replaced every 2-3 days, and the cells were passaged when they reached 80-90% confluency using trypsin/EDTA solution. The cells were cultured up to 5 passages to maintain their endothelial characteristics.

Human Cardiac Microvascular Endothelial Cells (HCMEC) from PromoCell were typically cultured in Endothelial Cell Growth Medium MV (PromoCell: C-22020) supplemented with the corresponding supplement mix (PromoCell: C-39225) and 1x penicillin/streptomycin (P/S). The culture medium was replaced every 2-3 days, and the cells were passaged when they reached 80-90% confluency using trypsin/EDTA solution. The cells were cultured up to 4 passages to maintain their endothelial characteristics.

4.1.2 Culture of isolated primary juvenile cardiac endothelial cells

Primary juvenile cardiac endothelial cells were isolated (see 4.2.1) and cultured on dishes pre-coated with 0.5% gelatin. The cells were cultured in endothelial full medium, comprising ECGS/H, 1X Non-essential amino acids, 1X sodium pyruvate, 20% v/v FCS, 20mM HEPES, 1X Pen/Strep, and 1X L-Glutamine in DMEM high glucose. Media renewal was performed bi-weekly to ensure optimal cell growth and maintenance. To preserve their endothelial characteristics, the cells were cultured for up to a maximum of 4 passages.

4.1.3 Culture of isolated primary neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were isolated (see 4.2.2) and seeded on dishes previously coated with 0.5% gelatin. On the isolation day, the cells were resuspended and plated using a plating medium composed of 75% DMEM high glucose with 1X Pen/Strep, 15% M199, and 10% Fetal Calf Serum (FCS). The following day, non-adherent and non-viable cells were washed away with 1X Phosphate-Buffered Saline (PBS), and the culture medium was changed to M199 medium. Subsequently, the cardiomyocytes were maintained in M199 medium for a maximum of three days.

4.1.4 Passaging of cells

To passage cultured cells, the cells were first grown to confluence in a cell culture dish or flask. The culture medium was removed and cells were washed with phosphate-buffered saline (PBS). Next, a trypsin-EDTA solution was added to the cells and the cells were incubated at 37°C until they detached from the surface. The trypsin was then inactivated by adding culture medium containing serum to the cells. The cells were then centrifuged and the supernatant was removed. The cells were resuspended in fresh medium and plated in new culture dishes or flasks. The number of cells plated depended on the experimental design and the growth rate of the cells. The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ until they reached the desired confluence. The process of passaging cells was repeated as needed to maintain the cells in culture. The cells were checked regularly for any signs of contamination or changes in morphology or growth rate.

4.1.5 Freezing of cells

Cells were frozen at 80-90% confluency by detaching them from the cell culture plate using a trypsin/EDTA solution. Following detachment, cells were harvested, and the growth medium was removed by centrifugation. A cell culture medium containing 20% FCS and 5% DMSO, a cryoprotectant, was added to the cell pellet. The cells were then resuspended in the freezing medium at a concentration of 1-2 million cells/mL. The cell suspension was dispensed into cryovials, with 1 mL

per vial. Subsequently, the vials were gradually cooled down to -80°C in a freezing container. The following day, the cryovials were transferred to a liquid nitrogen freezer at -196°C.

4.1.6 Thawing of frozen cells

The cells were thawed quickly in a 37°C water bath with gentle agitation. The outside of the vial was disinfected with 70% ethanol, dried with a tissue and cells were immediately transferred to a culture flask containing pre-warmed complete medium. The cells were then incubated at 37°C in a humidified atmosphere with 5% CO₂.

4.2 Isolation of cells

4.2.1 Isolation of primary juvenile cardiac endothelial cells for culture

Before isolating endothelial cells, purified Rat Anti-Mouse CD31 antibodies (BD: 553370) were coupled to magnetic Dynabeads (Invitrogen: 11035) overnight. To do so, 5 µL of Dynabeads per heart were washed with sterile bead buffer (0.1% BSA in PBS) at least five times on a magnetic rack and then resuspended in the same volume of bead buffer. Then, 0.5 µL of 0.5 mg/mL CD31 antibodies were coupled to the Dynabeads. The volumes of the solutions were upscaled according to the number of juvenile mice that would be sacrificed. On the day of the endothelial cell isolation, the unbound antibody was washed at least five times with bead buffer on a magnetic rack and then resuspended in endothelial cell basal medium. The Dynabead-CD31 solution was stored until further use.

The enzyme solution was prepared by dissolving 1 mg/mL Collagenase I (Worthington: LS0004196) and 0.1 mg/mL Deoxyribonuclease I (Worthington: LS002007) in HBSS. Five mL of enzyme solution was prepared per three hearts, which was sterile filtered.

Cardiac endothelial cells were isolated from 8–11-day-old RjOrl:SWISS mice. The mice were euthanized via decapitation with sharp scissors. The hearts were then briefly washed in PBS on ice to remove all the remaining blood. Next, the hearts were cleaned from any remaining vessels and connective tissue, and the atria were removed. Three hearts were pooled on one petri dish on ice and then minced into small pieces with a curved scissor. The minced hearts were resuspended in the enzyme solution and then digested at 37°C at a constant rotation of 18 rpm for 30 to 40 minutes. After digestion, the cells were filtered with a 70 µm cell filter. To flush all single cells, 5 mL of fetal calf serum (FCS) and 15 mL of HBBS buffer were added per three hearts to the cell filter. Then, the Falcon tubes containing the single cells were centrifuged at 400xg for 6 minutes at 4°C. The cell pellet was resuspended in 15 mL HBSS and then again centrifuged at 400xg for 6 minutes at 4°C. The supernatant

was removed and re-suspended with 1 mL of endothelial cell basal medium per three hearts. The solution containing the Dynabead-CD31 was added to the re-suspended cell solution. The solution was incubated at room temperature for 30 minutes with gentle mixing. During the incubation period, 6 cm petri dishes were coated with 0.5% gelatin for 30 minutes at 37°C. The cell suspension-Dynabeads-CD31 solution was then washed with endothelial cell basal medium on a magnetic rack at least five times to purify for cardiac endothelial cells and to get rid of non-endothelial cells. Finally, the cells were resuspended in endothelial cell full medium and seeded on the pre-gelatinized 6 cm petri dishes. The cells were incubated in a cell culture incubator, and the media was regularly renewed until the cells reached confluency.

Once the cells reached confluency, they underwent a purification process using CD102. For each 6 cm petri dish, 30 µL of Dynabeads and 3 µL of purified Rat anti-mouse CD102 (BD: 553326) were utilized. The CD102 antibodies were bound to the Dynabeads as previously done with the CD31 antibodies. To purify endothelial cells, the cells were trypsinized and collected in a falcon tube, followed by centrifugation at 400xg for 6 minutes. Next, the cells were suspended in 1 mL of basal medium and 30 µL of CD102 Dynabeads were added. The cells and beads were incubated at room temperature in a rotating shaker for 30 minutes. Next, the cells were washed five times with basal medium on a magnet, as previously described. Finally, the cells were suspended in full medium and seeded on pre-gelatinized 10 cm petri dishes before being incubated in a cell culture incubator at 37°C. It is possible to split one 6 cm petri dish into a maximum of three 10 cm dishes. The cells may be split up to two additional times until passage n°4.

4.2.2 Isolation of neonatal rat cardiomyocytes for culture

Solutions were prepared, including 1x Ammonium chloride-dextran-sucrose (ADS) kept at room temperature (RT) for Percoll and on ice for withdrawal. 1x red ADS solution was also prepared and kept at RT. Enzyme solution (Collagenase/Pancreatin) was freshly prepared and kept on ice. All solutions were sterilized through filtration.

The hearts were washed in 1x ADS, atria were removed, and the hearts were cut into small pieces. The tissue pieces were transferred to 50ml Falcon tubes (up to 50 hearts per tube) and kept on ice. A pre-digestion step was performed, followed by the main digestion process (approximately 11 times). After each digestion, the supernatant was collected and centrifuged. The cell pellets were resuspended in Foetal Calf Serum (FCS) and stored at 37°C.

A Percoll Gradient was prepared with TOP and BOTTOM Percoll solutions, and the cell suspension was gently layered on top. After centrifugation, the upper layer contained fibroblasts, while the lower layer

contained cardiomyocytes (CM). The cardiomyocytes were collected and transferred to 50ml Falcon tubes.

Cell washing was done by adding 1x ADS (37°C) to the Falcon tubes, followed by centrifugation. The supernatant was removed, and the cell pellet was resuspended in 1x ADS. The process was repeated, and the final pellet was resuspended in Plating Medium (37°C).

Cell counting was performed using Trypan Blue staining, and the cells were seeded on gelatin-coated plates for further experimentation.

4.2.3 Isolation of primary adult murine cardiac endothelial cells and fibroblasts

Before starting the procedure, two plates were prepared: one with phosphate-buffered saline (PBS) and one for the enzyme solution (500 U/ml Collagenase I, 150 U/ml DNase I in RPMI), and were placed on ice to cool. The mice were euthanized via cervical dislocation, and their chest area was disinfected with 70% ethanol. The sternum was cut to expose the heart, and heparin (50 µl per ventricle) was injected into the left and right ventricles while the aorta was pinched with tweezers. The heart was then removed, placed in PBS to remove remaining blood, and cleaned by removing any vessels and connective tissues to obtain a clean heart. The heart was briefly dried on a tissue to remove all remaining blood and weighed accurately.

Next, the heart was placed in a petri dish containing 1 mL of enzyme solution and minced into small pieces with curved scissors. The pieces were transferred into a falcon tube containing an additional 4 mL enzyme solution, and the tubes were placed in a rotator at 16-18 rpm at 37°C for 30 minutes of digestion. During this time, 50 ml falcon tubes were prepared with a 70 µm cell filter on top, and the centrifuge was pre-cooled to 4°C.

After digestion, the cells were filtered with a 70 µm cell filter. To flush all single cells, 5 ml of foetal calf serum (FCS) and 15 ml of magnetic-activated cell sorting (MACS)-buffer were added per heart. The filter was washed carefully to collect as many cells as possible. The tubes were then centrifuged at 400xg for 6-8 minutes at 4°C, and the supernatant was removed. The pellet was re-suspended and washed with 20 ml of MACS-buffer and centrifuged again, and the supernatant was removed. The pellet was then re-suspended in 1mL of MACS-buffer and transferred to an Eppendorf tube. CD146-microbeads (20 µl) were added, and the tubes were left on a tilted table for 30 minutes at room temperature.

The digested hearts were transferred to a falcon containing 20 ml of MACS-buffer for washing. The falcons were centrifuged at 400xg for 6-8 minutes at 4°C, and the supernatant was removed. The pellet was dissolved with 1 ml of MACS-buffer. MS-columns were prepared on a magnetic stand, and collection tubes (15 mL Falcons) were placed on ice to collect everything that passes through the

columns. Prior to applying the cell suspension, the columns were prepared with 500 µl of MACS-buffer. The cell suspension was applied to the prepared MS-column. Once the cell-suspension had passed through the column, the column was washed 3 times with 500 µl of MACS-buffer. After the final wash, 1.5 mL of MACS-buffer was added to the columns. The MACS buffer was then flushed into a newly prepared MS-column on magnet with a piston. The process was repeated with the new MS-column. After the final wash, 1.5 mL of MACS-buffer was added to the columns. The MACS buffer was then flushed into a 15 mL Falcon. Ten mL of MACS buffer was added to the falcon, and the solution was centrifuged at 400xg for 6 minutes at 4°C. The supernatant was carefully removed and then re-centrifuged at 400xg for 1 minute at 4°C to remove the remaining MACS-buffer. Finally, the purified endothelial-cell pellet was resuspended in RNA lysis buffer.

To prepare fibroblasts, the flow-through from the MS-column was filled with MACS-buffer up to a volume of 12 mL and then centrifuged at 400 g for 6 minutes at 4°C. The resulting pellet was resuspended in 1 mL of MACS-buffer and transferred to an Eppendorf tube. Next, 20 µL of Feeder removal beads were added, and the tubes were placed on a tilted table at room temperature for 30 minutes. The subsequent steps were carried out following the same protocol as for the preparation of endothelial cells.

4.2.4 Langendorff isolation of adult murine cardiomyocytes

The isolation of adult cardiomyocytes was performed via the Langendorff perfusion method. The mice were euthanized via cervical dislocation, and the chest area was disinfected with 70% ethanol before opening the sternum to remove the heart. The heart was placed in ice-cold 1x perfusion buffer, and the tissue around the aorta was cleaned under a microscope, leaving some part of the aorta attached to the heart. The aorta was then gently secured onto a cannula with an inner diameter of 1 mm and tied with a silk suture to ensure proper hanging and perfusion through the coronary arteries. The perfusion buffer was then used to flush out all the blood until the colour of the coronary arteries changed.

An enzyme solution containing Liberase DH (5 mg/mL, Roche, 5401089001), trypsin (1%, Gibco, 15090046), and CaCl₂ (100 mM) was then introduced into the system, and perfused at a rate of 3 mL/min for 5 to 6 minutes, while maintaining the temperature at 37°C. The flow-through was collected at the last minute of digestion, in which the digested heart was released from the cannula. Enzymatic digestion was stopped using the Stop I solution, and the heart was homogenized using a 1 mL syringe without a cannula. The cell suspension was passed through a 100 µm cell strainer in a 50 mL Falcon tube to remove clumps.

The cell suspension solution was then centrifuged at 17 xg for two minutes, and the supernatant was removed. The cell pellet was then resuspended in 9 mL of Stop 2 solution. For RNA extraction, the cardiomyocyte cell suspension was transferred to a 15 mL Falcon tube and centrifuged at 17 xg for two minutes. The supernatant was removed, and the cell pellet was lysed in RA1 lysis buffer. For Ion-Optix analysis of isolated cardiomyocytes, the 9 mL of cardiomyocytes containing Stop 2 solution was transferred to a 10 cm petri dish. Calcium concentration was then gradually increased by manually administering it into the digested heart tissue with continuous gentle mixing on a tilted table at room temperature.

Re-introduction of Calcium in a 10 cm petri-dish:

Addition	50 µl 10 mM CaCl ₂	4 min. shake softly
Addition	50 µl 10 mM CaCl ₂	4 min. shake softly
Addition	100 µl 10 mM CaCl ₂	4 min. shake softly
Addition	30 µl 100 mM CaCl ₂	4 min. shake softly
Addition	50 µl 100 mM CaCl ₂	4 min. shake softly
centrifugation	2 min. 17 g (15 ml-tube)	aspirate supernatant

The isolated cardiomyocytes were then ready for further analysis or experimentation. All Langendorff experiments were performed by Nina Weinzierl, Cardiovascular Physiology Department, Medical Faculty of Mannheim – Heidelberg University.

4.3 RNA isolation reverse transcription and quantitative PCR and Agarose gel electrophoresis

4.3.1 RNA extraction from cultured or isolated cells

Cultured or isolated primary cells were lysed using 350 µL of RA1 lysis buffer and the lysate was collected in an Eppendorf tube. To further enhance RNA purity, 350 µL of 75% ethanol were added to the lysate, which was mixed well with the RA1 lysis buffer. This addition helps to remove contaminants such as proteins, salts, and other impurities that may interfere with RNA purification. The lysate was then subjected to NucleoSpin RNA columns, which contain a silica membrane that selectively binds RNA molecules while removing impurities. The RNA extraction process was performed in accordance with the manufacturer's protocol (Machery-Nagel: 740955.250). The RNA was washed with a series of buffers to eliminate residual impurities such as DNA (including DNase digestion) and increase RNA purity. Finally, the purified RNA was eluted from the column using RNase-free water.

4.3.2 RNA extraction from tissue

To extract RNA from whole organs, the organs were first cut into small pieces and snap-frozen in liquid nitrogen. One of these small pieces was then transferred to a 2 ml Eppendorf tube, along with two metal beads. 1 mL of Trizol was added to the tube, and the tissue was disrupted in a tissue lyser 2 at 30 Hz for 2 minutes to break down the tissue and release the RNA.

The resulting lysate was transferred to a new Eppendorf tube, and 200 μ L of chloroform was added. The tube was mixed thoroughly and centrifuged for 15 minutes at 12000g and 4°C to separate the aqueous and organic phases. The upper, aqueous phase containing RNA was carefully pipetted into a new Eppendorf tube. An equal volume of isopropanol was added to the tube and mixed thoroughly to precipitate the RNA.

The lysate was then applied to NucleoSpin RNA columns, which utilize silica membrane technology to bind RNA while excluding contaminants. RNA purification was carried out according to the manufacturer's protocol. Whole organs were cut into small pieces and snap frozen in liquid nitrogen. One of these small organ pieces was transferred to a 2 ml Eppendorf tube and two metal beads were added to it. Next 1 mL of Trizol was added to the tube and the tissue was disrupted in the tissue lyser 2 at 30 Hz for a period for 2 minutes. The liquid was transferred to a new Eppendorf tube and 200 μ L of Chloroform was added to it. Next the tube was well mixed and centrifuged for 15 min at 12000g at 4°C. Next, the supernatant of the phase separation was pipetted into a new Eppendorf tube. An equal volume of Isopronaol was added to the tube and well mixed. Next, the lysate was then subjected to NucleoSpin RNA columns. The RNA extraction process was performed in accordance with the manufacturer's protocol.

4.3.3 cDNA synthesis

For cDNA synthesis, the Maxima cDNA synthesis kit from Thermo Fisher Scientific was used. The RNA concentration was measured and the RNA quality was assessed using a spectrophotometer. cDNA synthesis was performed using the Maxima cDNA synthesis kit according to the manufacturer's instructions. Briefly, a maximum of 1 μ g of total RNA was mixed with oligo(dT)18 primer, random hexamer primer, and dNTP mix, and incubated at 65°C for 5 minutes. After cooling on ice for 2 minutes, the mixture was added to a reaction mixture containing Maxima Enzyme Mix and Maxima RT buffer. The reverse transcription reaction was then carried out at 50°C for 30 minutes followed by heat inactivation at 85°C for 5 minutes. After the synthesis, the cDNA was further diluted and was then used for downstream applications such as qPCR analysis.

4.3.4 Quantitative Real Time PCR

To perform quantitative reverse transcription-polymerase chain reaction (qRT-PCR), primers specific to the target RNA sequence of interest were designed. The primers were designed to span an intron region and selected using the Primer3 program, with criteria including a melting temperature (T_m) between 55-65°C, a GC content of 40-60%, and a length of 18-24 nucleotides. The designed primers were purchased from Sigma in a lyophilized form, reconstituted in nuclease and RNase-free H₂O to a final concentration of 100 μ M.

The qRT-PCR reaction mix was prepared by adding 12.5 μ L of PowerUp SYBR Green Mastermix (2X concentration), 6 μ L of RNase-free H₂O, diluted cDNA template and 0.125 μ L of 100 μ M forward and reverse primers. The Master Mix (without cDNA) was upscaled to match the required number of amplification reactions. The reaction mix was loaded into a transparent qPCR plate and sealed with a transparent foil. The plate was then inserted into an Agilent qPCR machine and the comparative qPCR program was selected.

Firstly, the Uracil DNA Glycosylase (UDG) program was used (2 minutes at 50°C) to eliminate carryover PCR products. A hot start was then performed at 95°C for 2 minutes. The amplification process followed, consisting of a denaturation step at 95°C for 15 seconds, an annealing step at 56°C for 15 seconds and an extension step at 72°C for 1 minute. This cycle was repeated 40 times. The melting temperature of each primer pair was measured at the end of the amplification process to assess primer specificity.

After the reaction, the cycle threshold (C_t) values were determined, representing the cycle number at which the fluorescence signal reaches a predetermined threshold. The qRT-PCR data was analyzed by normalizing the C_t values to a suitable reference gene such as GAPDH, Hprt or 18S, to account for differences in RNA input and reverse transcription efficiency. The relative gene expression levels were calculated using the comparative C_t (ΔC_t) method.

4.3.5 Agarose gel electrophoresis

To determine the size of the nucleic acids amplified in the PCR, an agarose gel electrophoresis was performed. The agarose gel with a 1.5% concentration in TBE buffer was prepared, and Midori Green was added to the gel to enable staining of the DNA. The loading buffer was mixed with the DNA samples and then loaded onto the gel. The gel was then subjected to electrophoresis at 90 volts for a duration of 30 minutes. Following electrophoresis, visualization of the DNA fragments was carried out using UV light in a gel imager. The size of the DNA fragments was estimated by comparing the fragment migration distance to the DNA ladder standards.

4.4 siRNA knockdown and adenoviral overexpression

4.4.1 siRNA mediated knockdown

Small interfering RNAs (siRNAs) that targeted either long non-coding RNA or messenger RNA (mRNA) were purchased from Horizon Discovery. The siRNAs were reconstituted in RNase-free water, resulting in a final concentration of 20 μ M. The transfection mix was prepared by adding 9 μ l of RNAiMax transfection reagent to 150 μ l of Opti-MEM medium in a sterile tube, which was then gently mixed. After incubating for 5 minutes at room temperature, 3 μ l of 20 μ M siRNA was diluted in 150 μ l of Opti-MEM medium.

The diluted siRNA-Opti-MEM solution was added to the RNAiMax-Opti-MEM solution, mixed via vortexing, and incubated for 5 to 10 minutes at room temperature to promote the formation of siRNA-RNAiMax complexes. Prior to treatment, the cells were washed with PBS and the culture medium was refreshed. Next, the siRNA-RNAiMax complex was added dropwise to the cultured cells in the cell culture medium per well in a 6-well plate.

The cells were incubated for 48 hours, as recommended, before lysing or performing functional analysis. To evaluate the specificity of knockdown, the ON-TARGETplus Non-targeting Control Pool was used as a negative control.

4.4.2 Generation of Adenovirus

The AdEasy XL Adenoviral Vector System (AdEasy XL Adenoviral Vector System, #240010) was used for generating recombinant adenoviral vectors for overexpressing Lockd in mammalian cells. In brief, the Lockd cDNA sequence (NR_037955.1) was cloned into the pShuttle-CMV vector, which contained the cytomegalovirus (CMV) promoter. The Lockd cDNA sequence was integrated into the adenoviral genome through homologous recombination in BJ5183-AD-1 cells and between the shuttle vector and the adenoviral backbone vector in XL10-Gold Ultracompetent Cells (pAdEasy-1).

The recombinant adenoviral plasmid was linearized by restriction enzyme digestion, and the linearized DNA was transfected into Hek293 cells, human embryonic kidney cells expressing essential adenoviral genes. The transfection allowed for efficient rescue and packaging of the recombinant adenoviral DNA into infectious adenoviral particles.

The adenoviral vector was amplified by serially passaging the virus through Hek293 cells to increase the viral titer. The recombinant adenoviral particles were then purified from the culture supernatant using centrifugation method to get rid of cellular debris and purify the viral particles.

The titer of the purified adenoviral vector was measured to determine the number of infectious viral particles per unit volume, which was essential for optimizing transduction efficiency in target cells.

The purified adenoviral vector was stored under in -80°C for long-term storage.

4.4.3 Adenoviral infection of mammalian cells

The overexpressing experiments were conducted using a Multiplicity of Infection (MOI) of 50. Prior to infection, 50,000 C166 cells were seeded per well in a 6-well plate. The following day, the cells were washed with phosphate-buffered saline (PBS) and replaced with serum-free Dulbecco's Modified Eagle Medium (DMEM). Adenoviral infection was carried out on the cultured cells for a duration of 4 hours at 37°C and 5% CO₂. Subsequently, the cells were washed twice with PBS and replenished with DMEM supplemented with 10% fetal calf serum (FCS). After 48 hours from the time of infection, the cells were either lysed for analysis or utilized in further experimental procedures.

4.5 Protein lysis, BSA assay, SDS-Page, Western-Blot and Proteomics

4.5.1 Protein lysis

For the extraction of proteins from cultured cells, a lysis buffer comprising of 10 mM Tris, 150 mM NaCl, 4% glycerol, 0.5 mM Natrium disulfit, 1% Triton, 0.1% Nadeoxycholate, 0.05% SDS, 1 mM DTT, and 1x protease inhibitor cocktail was utilized. Prior to lysis, the cells were washed with ice-cold phosphate-buffered saline (PBS) and subsequently lysed with the lysis buffer. The cells were harvested with a cell scraper and collected in an Eppendorf tube. The lysate was then subjected to shock-freezing in liquid nitrogen. Later, the lysate was thawed on ice and centrifuged at a 12000 xg at 4°C for 10 minutes to obtain the soluble fraction that contains the proteins of interest and to remove insoluble debris. The soluble fraction was then transferred to a new tube and frozen until further use.

The protein concentration of the resulting lysate was determined using a protein assay kit such as the Bradford assay or BCA assay. The lysate was then used for downstream applications such as Western blotting, immunoprecipitation, or mass spectrometry.

4.5.2 BSA Assay

BSA assay was conducted on a 96-well plate using the BCA Protein Assay Kit (Thermo Scientific: 23209). Standards of known BSA concentrations were prepared by diluting the BSA standard provided with the kit in 1X assay reagent. In addition, a blank was prepared by adding only 1X assay reagent. 20 µL of each standard and the blank were added in duplicate to the wells of the 96-well plate. Then, 200 µL of working reagent was added to each well, and the plate was incubated at 37°C for 30 minutes to allow for the formation of a purple-colored complex between BSA and the assay reagent. After the

incubation, the plate was cooled to room temperature, and the absorbance was measured at 562 nm using a microplate reader. The concentration of the unknown sample was determined by comparing the absorbance value to the standard curve. The protein concentration was calculated using the equation obtained from the standard curve."

4.5.3 SDS-Page

For SDS-Page, the protein samples were prepared by mixing the cell lysate with 5X Laemmli buffer in a 1:5 ratio. The mixture was boiled for 5 minutes at 95°C to denature the proteins. The samples were then cooled to room temperature and centrifuged briefly to spin down all the liquid.

A 10% polyacrylamide running gel was prepared. After the polymerization of the running gel a prepared stacking-gel was loaded on top. A comb was inserted to form wells for sample loading. The gel was allowed to polymerize for at least 30 minutes (see table under 3.3).

The denatured protein samples were loaded onto the gel wells using a micropipette. A molecular weight marker was also loaded onto the gel to allow for protein size determination.

The gel was placed in an electrophoresis chamber, and the gel wells were filled with running buffer (Tris-glycine-SDS buffer). First an electric current of 80 Volt was applied to the chamber until the loading dye has reached the running gel. Then an electric current of 110 Volt was applied to the chamber and the proteins were separated based on their weight. The gel was run until the tracking dye reached the bottom of the gel.

4.5.4 Western-Blot

The protein sample which has been separated based on size using SDS-PAGE, as described above are transferred from the gel onto a 40 µM polyvinylidene difluoride (PVDF) membrane for subsequent detection by western blotting. To transfer the proteins, the gel was first equilibrated in transfer buffer, consisting of a Tris-glycine buffer containing 20% methanol, to facilitate protein transfer. The membrane was then soaked in methanol for a few minutes to activate it and then briefly washed in transfer buffer. This was followed by layering of the gel onto the membrane, sandwiched between layers of filter paper or sponge.

The sandwich is then placed into a wet transfer system apparatus, and a voltage of 100 Volt for 1 hour and 15 minutes was applied to facilitate the transfer of the proteins from the gel to the membrane.

Once the transfer is complete, the membrane was removed from the apparatus and typically blocked with 3 % bovine serum albumin (BSA) in TBS-T to prevent non-specific binding the primary antibodies. After one hour of blocking, the membrane was probed with a primary antibody diluted in 3% BSA in TBS-T specific to the protein of interest overnight at 4°C with constant shaking. On the next day the

membrane was washed three times for 15 minutes with TBS-T. Then the membrane was incubated for one hour at room temperature with constant shaking with a secondary antibody conjugated to horseradish-peroxidase for detection. After incubation with the secondary antibody, the membrane was washed three times for 15 minutes with TBS-T, to remove any unbound antibodies. To visualize the protein of interest, the membrane was incubated with a chemiluminescent substrate solution, composed of luminol and hydrogen peroxide. The horseradish peroxidase enzyme conjugated to the secondary antibody catalyzes the oxidation of luminol by hydrogen peroxide, leading to the emission of light. This light emission was detected using a chemiluminescence imaging system. The intensity of the signal is proportional to the amount of protein present in the sample, and the signal can be quantified using a software.

4.5.5 Puromycin Labeling

Adenoviral or siRNA treated cells were incubated with Puromycin at the final concentration of 10 µg/mL for a period of 30 minutes at 37°C in a cell culture incubator.

The cells were washed twice with ice-cold PBS to remove puromycin and puromycin-containing medium. Then the cells were lysed in ice-cold cell lysis buffer according to protein lysis protocol described in 4.5.1.

The lysate was then loaded onto a SDS-PAGE and Western Blotting was performed as described before.

4.5.6 Proteomics

Pulled-down protein samples were identified using TMT (Tandem Mass Tag) labelling followed by liquid chromatography-mass spectrometry (LC-MS/MS) at the EMBL Proteomics Core Facility. TMT labelling was performed on the isolated proteins, where each sample was labelled with a unique TMT reagent. The labelled samples were combined and subjected to proteolytic digestion. The resulting peptides were separated by liquid chromatography and analysed by tandem mass spectrometry. The acquired mass spectra were searched against a protein sequence database using appropriate search algorithms, and protein identification was achieved by matching the peptide sequences with high confidence. The relative abundance of the identified proteins across different samples was determined based on the intensities of the TMT reporter ions. The LC-MS/MS analysis provided comprehensive and quantitative information on the pulled-down proteins, enabling the investigation of protein interactions. All proteomic analysis were done at the EMBL Proteomics Core facility, Heidelberg.

4.6 Molecular biology methods

4.6.1 Cytokine stimulation of endothelial cells

Endothelial cells were cultured in 6-well plates until they reached 80% confluency. Serum-free media was prepared containing different cytokines, including 10 ng/mL TGF- β 1, 25 ng/mL FGF2, 25 ng/mL FGF2, 10 ng/mL IL-1 β , 100 ng/mL TNF- α , 20 μ M Phenylephrine, and 20 μ M Isoprenaline. The cells to be treated were washed twice with PBS and exposed to the serum-free media containing the cytokines. Unstimulated cells supplemented with serum-free media served as the control.

Twenty-four hours post-stimulation, the cells were washed with PBS and lysed in RA1 buffer. The stimulation of cells with the cytokines was compared to the control cells.

4.6.2 Actinomycin D RNA stability time-course

To determine the stability of a specific RNA, a time course experiment with Actinomycin C was performed on cultured cells. The cells were seeded in a 6-well plate and allowed to reach 80-90% confluency. Once the cells reached the confluency, they were treated with Actinomycin C (5 μ g/mL) to inhibit transcription initiation. RNA samples were collected from the cells at various time points (0, 2, 4, 6, 8, 16 and 24 hours) after the addition of Actinomycin C. RNA was isolated using the Machery-Nage RNA isolation kit. The RNA concentration and purity were assessed using a NanoDrop spectrophotometer. To determine the stability of the target RNA, cDNA was synthesized from the extracted RNA using the Maxima reverse transcription kit. The cDNA was then used for quantitative PCR analysis with gene-specific primers. The stability of the RNA was determined by plotting the logarithm of the normalized RNA expression level as a function of time after Actinomycin C treatment. The slope of the linear regression of this plot provided the mRNA degradation rate.

4.6.3 Subcellular fractionation

Subcellular fractionation was performed on primary cardiac endothelial cells to obtain chromatin-associated, nuclear-soluble, and cytoplasmic fractions. The method previously described was followed. Briefly, the cells were trypsinized and washed with PBS, and the cell pellet was centrifuged at 500 x g for 5 min. The cells were then lysed in buffer RLN1 and incubated on ice for 5 min. After centrifugation at 300 x g for 2 min at 4°C, the cytoplasmic fraction was collected and stored on ice. The nuclear pellet was further lysed in buffer RLN2 for 5 min on ice, followed by centrifugation at 16,400 x g for 2 min at 4°C. This step separated the nuclear soluble fraction in the supernatant from the chromatin-associated fraction in the pellet. RNA was extracted using the Machery-Nage RNA

extraction kit as per the manufacturer's protocol. Quantification of transcript abundance in each fraction was performed using qRT-PCR.

4.6.4 Sprouting assay

Adenovirus or siRNA treated endothelial cells were used for this assay. The cells were subjected to two washes with phosphate-buffered saline (PBS) and were detached from the culture plate through the use of trypsin-EDTA. The reaction was then halted through the addition of PBS containing 10% FBS, and the cells were subjected to centrifugation at $200 \times g$ for 5 min. The supernatant was subsequently discarded, and the cells were resuspended in cell culture medium before being counted through the use of a hemacytometer. A total of 50,000 cells were required to prepare hanging drops for C166 cells, while 60,000 cells were needed for HUVECs.

The cells were then mixed with 4 ml of cell culture medium in a fresh 15 ml conical tube. A 1 ml methocel stock solution was subsequently added to the mixture to improve spheroid formation. The solution was mixed carefully and then transferred to a sterile multichannel pipette reservoir. Using a 12-channel pipette, 25 μ l drops of the solution were pipetted onto a 10 cm square Petri dish. The drops were then incubated upside-down in a humidified cell culture incubator set at 37°C and 5% CO₂ for 24 h.

To prepare the methocel solution that would be needed the next day, the methocel stock solution was mixed with 20% FBS. The solution was then mixed carefully to prevent the formation of air bubbles.

The hanging drops that contained the spheroids were washed off through the pipetting up and down with 10 ml of PBS using a 10 ml serological pipette. The solution was subsequently transferred to a 15 ml conical tube. The spheroids were then subjected to centrifugation at $200 \times g$ for 5 min, and the supernatant was aspirated. Two milliliters of methocel containing 20% FBS were subsequently added, and the spheroids were mixed.

For the preparation of the collagen matrix, the collagen stock solution, 10x Medium 199, 0.2 M NaOH, and methocel containing 20% FBS were placed on ice. A collagen medium was then created by mixing 4 ml of 3 mg/mL collagen stock solution with 0.5 ml of 10x Medium 199 on ice. The pH value was adjusted through the addition of sterile, ice-cold 0.2 M NaOH dropwise until the collagen medium changed color from yellow to orange (approximately 250 μ l). The tube was then inverted carefully to ensure thorough mixing. The preparation of the collagen medium on ice was essential to prevent collagen polymerization.

The spheroids were resuspended in methocel containing 20% FBS, and 2 ml of collagen was added to the mixture. The solution was then mixed carefully to prevent the formation of air bubbles, and 1 ml

of the spheroid-collagen solution was added per well of a 24-well plate. The plate was subsequently incubated in a humidified cell culture incubator set at 37 °C and 5% CO₂ for 30 min to allow for the polymerization of the collagen matrix. Finally, the spheroids were stimulated with 100 µl of basal medium and 25 ng/ml FGF2 (in basal medium) by adding it dropwise on the collagen matrix.

4.6.5 BrdU proliferation assay

The BrdU proliferation assay from Roche was employed to assess DNA synthesis and cell proliferation. The necessary materials used in the assay included BrdU Labeling Solution (Roche, Cat. No. 11647229001), FixDenat Solution (Roche, Cat. No. 10901604001), Anti-BrdU-POD Antibody (Roche, Cat. No. 11633716001), Substrate Solution (Roche, Cat. No. 11644753001), and Wash Buffer (Roche, Cat. No. 10899276001).

2000 C166 cells were seeded in 96-well plates and incubated overnight to ensure proper adherence. The next day a sufficient amount of cell culture medium containing BrdU Labeling Solution was prepared to cover the cells in each well, with the appropriate concentration.

Subsequently, the cells were labeled with BrdU by removing the medium and adding BrdU Labeling Solution to each well, fully covering the cells. The cells were then incubated for the recommended labeling duration, of typically 2 hours.

Next, the cells were subjected to fixation and denaturation. The BrdU Labeling Solution was removed, and FixDenat Solution was added to each well or slide. The cells were fixed and their DNA denatured by incubating at room temperature for 30 minutes.

Following fixation and denaturation, the cells were subjected to thorough washing with the Wash Buffer, which was performed three times.

For the detection of BrdU-labeled DNA, Anti-BrdU-POD Antibody was added to each well or slide, appropriately diluted in the Wash Buffer as directed in the product manual. The cells were incubated at room temperature for 90 minutes to allow the primary antibody to bind to the BrdU-labeled DNA. After incubation with the primary antibody, the cells underwent an additional three washes with the Wash Buffer to eliminate any unbound primary antibody.

Subsequently, the substrate solution was introduced to each well or slide, ensuring complete coverage of the cells. The cells were incubated in the dark for a specific duration, for at least 5 minutes.

Finally, the absorbance or fluorescence signal was measured using an appropriate detection instrument. For example, absorbance at 450 nm was measured using a microplate reader, while fluorescence was measured using a fluorescence microscope or plate reader.

4.6.6 Propidium iodide staining and cell cycle determination

Cell cycle analysis is a widely used technique to study the distribution of cells in different phases of the cell cycle. Propidium iodide (PI) staining is a common method to analyse the cell cycle distribution of fixed cells. In this study ab139418, a PI staining kit was used to analyse the cell cycle distribution of fixed cells.

Firstly, the cells were harvested and washed with cold PBS twice to remove any debris or serum components. Then, the cells were fixed with 70% ethanol in PBS overnight at -20°C. The fixed cells were washed with cold PBS to remove the ethanol and resuspended in 1 ml of PI staining solution containing 50 µg/ml PI and 100 µg/ml RNase A. The cells were incubated for 30 minutes at 37°C in the dark to allow PI to intercalate into the DNA and RNase A to digest RNA.

After the incubation, the stained cells were analysed using a flow cytometer. The excitation wavelength was set to 488 nm, and the emission was detected at 630 nm. The cell cycle distribution was analysed using a flow cytometry software by plotting the DNA content (x-axis) against the cell count (y-axis). The software was used to gate out debris and aggregates, and only the single-cell population was considered for analysis.

4.6.7 smFISH probe labelling

smFISH probe labelling was performed following the established protocol developed by Imre Gaspar et. al [254] To generate smFISH probes for labelling, first a reaction of Amino-11-ddUTP with dye-NHS-ester was set up. Specifically, 4 µL of 10 mM Amino-11-ddUTP was mixed with 2 µL of 20 mM dye-NHS-ester and 0.6 µL of 1 M NaHCO₃, resulting in a final reaction containing a two-fold molar excess of dye-NHS-ester and 0.1 M pH 8.3 NaHCO₃. The reaction was incubated at room temperature for 2-3 hours. Next, 0.66 µL of 100 mM Tris pH 7.4 was added to quench unreacted NHS ester, resulting in a final Tris-HCl pH 7.4 concentration of 10 mM. Then, 0.75 µL of water was added to achieve a final volume of 8 µL. The final concentration of labelled ddUTP was 5 mM.

Next, 4 µL of 150 µM designed pool oligonucleotides was mixed with 1 µL of labelled ddUTP, 0.6 µL of TdT enzyme, 3 µL of TdT buffer, and 6.4 µL of H₂O. The final volume of the reaction was adjusted to 15 µL. Specifically, 500 pmol of the designed pooled oligonucleotides was combined with the labelled ddUTPs in 1x TdT buffer and 0.006 U/pmol ssDNA TdT enzyme was added. The reaction was incubated overnight at 37°C.

On the following day, 60 µL of 1 M NaAc pH 5.5 and 1 µL of 5 mg/mL linear acrylamide were added to the reaction. The final concentration of NaAc was 300 mM. The whole solution was then transferred to a 1.5 mL Eppendorff tube and 800 µL of -20°C pre-cooled 100% EtOH was added and mixed well. The reaction was incubated at -80°C for 1 hour. The oligonucleotides were then pelleted at 16000 xg

at 4°C for 20 minutes and the pellet was washed in 1 mL of 4°C 80% EtOH with vortexing. The pellet was pelleted again at 16000 xg at 4°C for 20 minutes and washed two more times by transferring to a fresh tube each time. The residual EtOH was removed and the pellet was air dried.

Finally, the pellet was reconstituted in 25 µL of nuclease-free water and absorbance at 260 OD and OD_{260/280} was measured to estimate the degree of labelling and percentage of recovery, respectively.

4.6.8 RNA immuno-precipitation

For the RNA-immunoprecipitation (RIP), two confluent 15 cm culture dishes were used. The media was removed, and the cells were washed with PBS. Each dish was then scraped into 300 µL of 1x polysome lysis buffer. The lysates from both dishes were combined in a fresh Eppendorf tube (600 µL per tube). The lysates were vigorously vortexed and passed 5 times through a 27-gauge needle to homogenize and break the chromatin. The resulting mixture was stored at -80°C until further use.

Before performing the immunoprecipitation 50 µL of lysate was taken as an input control sample and stored at 4°C.

For the immunoprecipitation, the antibody and the respective IgG control were coupled with the cell lysate overnight at 4°C. On the next day, 50 µL of beads per IP were removed and washed twice with 500 µL of NT-2 on a magnet. The supernatant was removed, and the beads were resuspended in 600 µL of NET-2 buffer and kept on ice. An IgG pre-clear step with streptavidin beads was conducted to remove background. Then, 600 µL of the beads were added to the lysate, and the lysate was incubated with the beads for 2 hours at 4°C. The supernatant was removed, and 1 mL of ice-cold NT2 was added to the beads. The beads were washed five more times with ice-cold NT2.

For RNA purification, the beads were resuspended in 150 µL of proteinase K buffer, and 20 µL of proteinase K was added (P8107S, NEB). The mixture was incubated at 55°C for 45 minutes with constant shaking at 900 rpm. After digestion, the solution was placed on a magnet, and the supernatant was transferred to a fresh tube. Then, 200 µL of phenol chloroform was added and vortexed thoroughly. The solution was transferred to a heavy lock tube and centrifuged at 15,000 xg for 15 minutes at 4°C. Next, the aqueous phase was transferred to a fresh tube.

For the Input RNA, 7.5 µL of 20% SDS, 50 µL of NT2 buffer, and 18 µL of Proteinase K were added. The mixture was incubated at 55°C for 45 minutes with constant shaking. Subsequently, the Input and RIP RNA were purified using the Zymo Clean and Concentrator Kit according to the manufacturer's instructions, and eluted in 20 µL of RNase-free H₂O.

4.6.9 Chromatin Immuno-RNA Precipitation

Chromatin RNA Immunoprecipitation (ChIRP) is a technique that allows for the specific isolation and characterization of chromatin-associated long non-coding RNAs (lncRNAs). By utilizing complementary antisense oligonucleotides targeting the lncRNA of interest, ChIRP enables the enrichment and subsequent analysis of chromatin-bound RNA-protein complexes, providing valuable insights into the roles of lncRNAs in chromatin regulation and gene expression

The chromatin RNA immunoprecipitation (ChIRP) assay was performed following the established protocol developed by Howard Chang and Chia-Chen Chu, with minor adaptations [255].

To design anti-sense oligo probes to pull-down Lockd, the free online Biosearch Technologies' Stellaris FISH Probe Designer was utilized. The probe design parameters were set to tile the entire ncRNA from 5' to 3', with one 20-mer probe every 100 bp of RNA, and a spacing of 80 between probes. Additionally, the target GC% was set to 45. Furthermore, during the design process, repeats and regions homologous to non-target genomic sequences were avoided.

Custom oligos were then ordered with a 3'-Biotin-TEG modification and HPLC purification. The probes were numbered based on their position along the RNA from 5' to 3', and each was reconstituted at 100 mM with water. Two pools of probes were prepared, with one pool consisting of even numbered probe (2, 4) and the other consisting of numbered probe (1, 3). The concentration of each probe was verified using Nanodrop, and both pools were stored at -20 °C when not in use.

Duplicate experiments were performed, with one using the odd numbered probes and the other using the even numbered probes. This was done in order to control for non-specific pull-down, with valid ncRNA-dependent signals expected to be detected with both pools of probes and stand out from non-specific noise. This "two probe" pool strategy is important for ChIRP-Seq experiments.

C166 cells were grown in dishes until confluency was achieved. A total of 40 million cells were required, with 20 million cells used for each ChIRP with both odd and even probe pools. The cells were rinsed once with PBS and trypsinized, followed by the trypsinization the cells were re-suspended with media. The resulting single cell suspension was counted, and 20 million cells were transferred into 15 mL tubes. The cells were pelleted at 300 xg for 4 min and the media was aspirated. Subsequently, each tube containing 20 million cells was resuspended in 20 mL of PBS and pelleted again at 300 xg for 3 min. The PBS was then decanted, and the remaining liquid was carefully aspirated.

Fresh 1% glutaraldehyde in PBS was prepared for use just before use. For every 40 million cells, 30 mL of 1% glutaraldehyde solution was prepared by adding 1.2 mL of 25% glutaraldehyde stock to 28.8 mL of PBS. To begin the cross-linking process, cells were initially resuspended in a small volume of 1% glutaraldehyde until no chunks were visible, and then the volume was topped up to 15 mL. The cells were mixed by inverting and cross-linked for 10 minutes on an end-to-end rotator or shaker at room

temperature. The cross-linking reaction was then quenched with 1.5 mL of 1.25 M glycine, and rotation was continued for another 5 minutes. Cells were then pelleted at 800 xg for 5 minutes at 4°C, and the supernatant was discarded. The pellet was washed once with 10 mL of chilled PBS, pelleted again at 800 xg for 5 minutes at 4°C, and the supernatant was discarded. The washed, cross-linked cell pellet was then resuspended with 1 mL of chilled PBS per 20 million cells and transferred to a tube. The cells were pelleted again at 1000 xg for 3 minutes at 4°C, and the supernatant was decanted, and as much PBS as possible was removed with a pipette tip. The cell pellets were then flash-frozen in liquid nitrogen and stored at -80°C until further use.

The frozen cell pellets were thawed at room temperature. The cells were pelleted at 1000 xg for 3 minutes at 4°C and the supernatant was discarded. The last traces of supernatant were removed using a fine pipette tip. To determine the cell pellet weight, an empty tube identical to the one used to pellet the cells was weighed on an analytical balance, and each cell pellet was weighed and recorded.

For the preparation of complete lysis buffer, fresh protease inhibitor cocktail PMSF and RNase inhibitor were supplemented just before use. The buffer was prepared in a 10X volume based on the weight of the cell pellet, for example, 1 mL for 100 mg. The complete lysis buffer was mixed well, and the cell pellets were resuspended in the buffer in a 10X volume.

In the experiment, the cell lysate was aliquoted into 15 mL polystyrene tubes. The lysate was then sonicated in a cold room using a Sonicator to obtain a sonicated DNA size range of 200 bp, which was achieved in 4 hours. 5 µL of lysate was transferred to a fresh Eppendorf tube to evaluate shearing. The sample was mixed with 90 µL Proteinase K buffer and 5 µL Proteinase K, and incubated at 50 °C for 45 min.

The sonicated DNA from step was purified using the ChIP DNA Clean and Concentrator kit (Zymo Research D5205). The purified DNA was eluted in 30 µL Zymo Elution buffer and the DNA size was checked on a 1% agarose gel. If the bulk of the sonicated DNA smear was between 100-500 bp, the sonication was considered complete.

The remaining sonicated chromatin was centrifuged at 16100 xg for 10 min at 4 °C and the samples were flash-froze the in liquid nitrogen, and stored it at -80 °C until further use.

The chromatin samples were thawed at room temperature. For RNA and DNA INPUT, 10 µL aliquots of chromatin were extracted and preserved on ice until ChIRP processing was completed.

For each ChIRP reaction (odd & even), 4 mL of hybridization buffer was prepared and mixed thoroughly. Thereafter, 1 mL of chromatin was transferred into two separate 15 mL tubes, one for ChIRP with odd probes and the other with even probes, and 2 mL of hybridization buffer was added to both.

The ChIRP probes, odd or even, were thawed and 1 μ L of 100 μ M probe pool was added to separate per 1 mL of chromatin. The mixtures were thoroughly combined and incubated at 37 °C for 4 hours with rotation in an incubator.

Twenty minutes prior to hybridization completion, the Streptavidin magnetic C1 beads were prepared. For every 100 pmol of probes, 100 μ L beads were utilized. The beads were washed twice with unsupplemented lysis buffer, using a magnetic stand for separation.

The beads were resuspended in their original volume of lysis buffer, which was freshly supplemented with 0.1M PMSF, PIC, and RNase inhibitor. After the hybridization reaction was complete, 100 μ L of washed beads were added to each ChIRP reaction, mixed thoroughly, and incubated at 37 °C for 30 min with rotation in the hybridization oven.

Wash buffer (5 mL per ChIRP reaction) was prepared and mixed thoroughly. The wash buffer was pre-warmed to 37 °C and 0.01x volume of 0.1M PMSF was added before use.

The beads were washed 5 times with 1 mL of wash buffer at 37 °C. For the first wash, the lysate was removed on a magnetic stand, the beads were re-suspended in 1 mL of wash buffer, and transferred to an Eppendorf tube. The mixture was incubated at 37 °C with rotation for 5 min.

For subsequent washes, the tubes were briefly spun, and the beads were separated on a magnetic stand. The wash buffer was removed, the beads were resuspended in a fresh 1 mL of wash buffer, and the mixture was incubated at 37 °C with rotation for 5 min.

In the final wash, the beads were extensively resuspended in 1 mL Wash buffer, and 100 μ L was removed for RNA isolation. The remaining 900 μ L was for DNA purification. After removing the wash buffer from both, the tubes were briefly spun and returned to the magnetic stand, and the last traces of wash buffer were removed with a pipette tip.

The bead sample reserved for RNA was resuspended in 95 μ L of Proteinase K buffer. The RNA INPUT aliquot reserved previously was mixed with 85 μ L of Proteinase K buffer. Proteinase K was added to both the beads and the RNA INPUT, and the samples were incubated at 50 °C for 45 min with constant shaking. The tubes were then briefly spun and incubated at 95 °C for 10 min. The samples were chilled on ice for 5 min, followed by the addition of 500 μ L TRI Reagent. The mixture was vortexed vigorously for 10 sec and incubated at room temperature for 10 min. The samples were either stored at -80 °C or processed for Trizol RNA extraction.

For each pair of ChIRPs and DNA INPUT, 1 mL of DNA Elution buffer was prepared. 10 μ L of RNase A (10 mg/mL, R4642) were added per 1 mL of DNA Elution buffer just before use, and the mixture was vortexed to mix.

Beads reserved for DNA were resuspended in 150 μL of DNA Elution buffer supplemented with RNases, and 10 μL of DNA INPUT was diluted in 140 μL of the same buffer. The samples were incubated at 37 °C for 30 min with shaking.

The tubes containing beads were placed on a magnetic stand for 1 min, and the supernatants were transferred to fresh Eppendorf tubes.

The DNA Elution was repeated a second time with another 150 μL of DNA Elution buffer plus RNases, and the resulting samples were transferred to the same tube as the first elution (total ~300 μL).

15 μL of Proteinase K was added to each sample, and they were incubated at 50 °C for 45 min with shaking.

The DNA samples were purified using the ChIP DNA Clean and concentrator kit (Zymo Research D5205) following the manufacturer's protocol. The DNA was eluted in 30 μL of Zymo Research DNA Elution buffer and sent for sequencing.

4.6.10 ChIRP - Mass Spectrometry

Chromatin RNA Immunoprecipitation followed by Mass Spectrometry (ChIRP-MS) is a technique that combines the specificity of RNA-protein interactions captured by ChIRP with the high-resolution protein identification capabilities of mass spectrometry. By integrating these two methods, ChIRP-MS enables the comprehensive analysis of protein interactions with long non-coding RNAs (lncRNAs), providing insights into the functional roles of lncRNAs and their associated protein partners in cellular processes.

The ChIRP-Mass Spectrometry assay was performed following the established protocol developed by Howard Chang and Chia-Chen Chu, with minor adaptations [256].

Eleven 15 cm plates were used per ChIRP-MS experiment, corresponding to approximately 70 million cells. The cells were trypsinized and harvested as described before in the ChIRP protocol.

The cells were resuspended in 3% formaldehyde (diluted in PBS), and cross-linked for 30 minutes at room temperature with continuous mixing. Subsequently, the formaldehyde cross-linking reaction was quenched by treating the cells with 0.125 M glycine for 5 minutes. The cells were pelleted at 800 xg, washed with PBS, pelleted again, and all liquid was carefully aspirated. The weight of the cell pellets was measured and recorded. Finally, the cell pellets were snap-frozen in liquid nitrogen and stored at -80 °C until further use.

Next day, the cells were thawed on ice and dissolved in cell lysis buffer at a ratio of 100 mg cell pellet per 1 mL buffer, followed by thorough resuspension. The cell lysate was sonicated using a sonicator in the 4°C room for a period of one hour. A 5 μL aliquot of the lysate was taken and diluted with 90 μL of pK buffer (without pK enzyme), and centrifuged at 16000 xg for 10 minutes. If a visible pellet was

present, the supernatant was transferred to a fresh tube, and the pellet was resuspended in 95 μL of pK buffer (without pK enzyme). To both the "soluble" and "insoluble" fractions, 5 μL of pK enzyme (5% v/v) was added. The mixture was incubated at 50 $^{\circ}\text{C}$ for 45 minutes. DNA extraction was performed using a PCR purification kit following the manufacturer's instructions. The extracted DNA was run on a 1% agarose gel, and it was ensured that the majority of the DNA was present in the "soluble" fraction. If not, sonication was continued until the lysate clarified. Importantly, unlike ChIRP-seq, there was no requirement to sonicate the DNA into smaller fragments, and the average DNA lengths typically remained above 1-2 kb when most of the cell lysate was solubilized. The lysate was then centrifuged at 16k xg for 10 minutes at 4 $^{\circ}\text{C}$, and the resulting supernatant was transferred to fresh tubes and stored at -80 $^{\circ}\text{C}$ until further use.

A portion of the lysate was reserved for "input" samples, with a typical volume of 10 μL for RNA and protein each, which were maintained at 37 $^{\circ}\text{C}$ during the hybridization and wash steps. The remaining lysate was aliquoted equally for experimental samples and RNase-controls, approximately 70 million cells each. Each sample was subjected to preclearing by incubating with 30 μL of washed C-1 beads at 37 $^{\circ}\text{C}$ for 30 minutes with mixing in a 37 $^{\circ}\text{C}$ cell incubator. The C-1 beads were washed by placing aliquots on a magnet until clear, carefully removing the bead storage solution, resuspending the beads pellet with 100 μL of cell lysis buffer, and placing the beads back on the magnet for a total of two washes. Simultaneously with the preclearing step, RNase A was added to the control lysate at a final concentration of 10 $\mu\text{g}/\text{mL}$ to serve as the RNase treatment control. Following preclearing, the samples were placed on the magnet to transfer the supernatants to fresh tubes, and the cleared samples were subjected to another supernatant transfer to ensure thorough removal of any remaining C-1 beads. To all the samples, hybridization buffer and ChIRP probes (1 μL of 100 μM probes per 1 mL of lysate) were added at 2 $\times$ volume, and the samples were incubated at 37 $^{\circ}\text{C}$ with end-to-end rotation for 4 hours in a 37 $^{\circ}\text{C}$ cell incubator. The even and odd probes from the ChIRP experiment were used together in this experiment. Before the completion of hybridization, the C-1 beads were washed three times on a magnet stand with cell lysis buffer (using 100 μL of beads per 1 μL of 100 μM probes), and all buffer was removed after the last wash. After hybridization, the tubes were briefly spun down, and 1 mL of sample was used to resuspend and transfer the beads into the reaction, followed by continued mixing at 37 $^{\circ}\text{C}$ for 30 minutes. The contents were then briefly spun down, and the tubes were placed on a Dynamag for 1-2 minutes or until the solution became clear. The liquid was decanted, and residual liquid was removed using a pipette tip. Subsequently, the beads were washed with wash buffer for a total of five washes, each performed at 37 $^{\circ}\text{C}$ with constant mixing for 5 minutes. During the last wash, 5 % of the beads were transferred to a fresh tube for RNA analysis to confirm the pull-down, while the bulk material was utilized for protein extraction and analysis. The residual buffer on

the magnet was removed after the last wash, and the samples were then used for RNA or protein extraction.

The RNA was extracted as described before in the ChIRP experiment.

For the protein extraction, the samples were resuspended (on beads) in 100 μ L biotin elution buffer. The proteins were eluted from beads at room temperature with constant mixing for 20 min, and then at 65 °C for 10 min. Supernatant was then transferred to a fresh tube and the beads were re-eluted in 100 μ L biotin elution buffer. The same procedure was then repeated a second time. The two eluents were pooled for protein precipitation. 50 μ L TCA (25% v/v) was added to the clean eluent, mixed thoroughly by vortexing, and precipitated overnight at 4 °C. The next day, proteins were pelleted at 16,000 rcf at 4 °C for 30 min. Supernatant was carefully removed from the side of tubes, and protein pellet was washed once with cold acetone before being immediately pelleted again at 16,000 rcf at 4 °C for 5 min. Then the acetone was removed. Protein pellets were then briefly re-centrifuged, residual acetone removed and pellets were air-dried for 1 min. Protein pellets samples were solubilised in 100 μ L of protein storage buffer. Final protein samples were then taken to the proteomics core facility for mass-spectrometry.

4.6.11 RNA antisense purification – Mass spectrometry

RNA antisense purification coupled with mass spectrometry (RAP-MS) is an additional technique used for the identification and characterization of RNA-protein interactions. By selectively capturing RNA molecules and associated proteins through hybridization with complementary antisense oligonucleotides, followed by stringent purification and mass spectrometry analysis.

RAP-MS was performed following the established protocol developed by Colleen A McHugh and Mitchell Guttmann, with minor adaptations [257].

Five antisense capture probes, each consisting of 50 nucleotides, were designed to target the Lockd RNA sequence. The probes were strategically tiled along the entire transcript, ensuring comprehensive coverage. To prevent unintended hybridization, probes with the potential to bind to off-target sequences were excluded, and probes containing more than 8 consecutive bases of repetitive sequences were eliminated from consideration. The designed DNA oligonucleotides were then ordered with 5'-biotin modification.

For each RAP-MS sample, three 15 cm tissue culture plate containing confluent C166 cells were utilized. The medium was removed from the plate, and washed with 10 mL of ice-cold 1x PBS. The PBS wash was subsequently discarded. The plates were then exposed to UV cross-linking at 254 nm, with a total energy of 0.8 J/cm². Following cross-linking, the plates were removed from the cross-linker and kept on ice throughout the remainder of the procedure. (The control samples were non-cross-linked)

1ml of lysis buffer was added to the cells. The cells were then scraped from the plate and transferred to a 2 mL tube. The lysate was then left on ice for 10 minutes.

The cell suspension was passed through a 26-G needle for 5 cycles. Following that, the cell lysate was incubated at 37 °C for 12 min after adding 1x DNase salt solution and 330 U TurboDNase. Subsequently, the lysate was mixed with an equal volume of 2x Hybridization Buffer and centrifuged at 16,000 x g for 10 min at 4 °C. The supernatant was then transferred to a fresh tube and flash-frozen in liquid nitrogen. The lysate was stored at -80 °C until it was ready for RAP captures.

The next day, the cell lysate was thawed on ice. For each sample, 100 µL of streptavidin-coated magnetic beads were used. The beads were separated on a magnetic rack, and the storage buffer was removed. Subsequently, the beads were washed in 1 mL of 10 mM Tris-HCl pH 7.5, followed by magnetic separation and removal of the supernatant. This process of bead washing was repeated four more times using 10 mM Tris pH 7.5 and two times using 1x Hybridization Buffer. After the final wash, the supernatant was removed from the beads using magnetic separation, and the cell lysate was transferred to the beads and resuspended. The mixture was incubated at 37 °C for 30 min with constant mixing at 900 rpm on a thermomixer. After the incubation, the beads were then magnetically separated, and the supernatant was transferred to fresh tubes. This step was repeated to transfer the lysate to fresh tubes a second time to ensure complete removal of beads. Next, a sample containing 100,000 cells worth of lysate was taken and transferred to a PCR strip tube, which served as the "RNA Input" sample.

Denature appropriate 3 µL of 100 µM probe by heating at 85 °C for 3 min, then place on ice.

The lysate and probe were mixed, followed by incubation for 2 hours at 67 °C with intermittent mixing at 900 rpm on a thermomixer. Simultaneously, streptavidin beads were prepared as previously described. The beads were magnetically separated and the final wash was removed. After the 2-hour incubation, a sample of lysate corresponding to 100,000 cells was taken and transferred to a PCR strip tube, representing the "RNA Input + Probe" sample. The beads were resuspended in lysate and incubated for 30 minutes at 67 °C with intermittent mixing at 900 rpm on a thermomixer. Subsequently, the beads were magnetically separated and the supernatant was removed. A sample of supernatant corresponding to 100,000 cells was taken and transferred to a PCR strip tube, representing the "RNA Flow-Through" sample. The beads were washed 6 times, with 1 mL of 1x Hybridization Buffer. Each wash was incubated for 5 minutes at 67 °C. After the last wash, a 1% portion of the beads from the total volume was removed and transferred to a PCR strip tube, constituting the "RNA elution" sample.

The remaining beads were magnetically separated and the supernatant was removed. The beads were resuspended in 1 mL of Benzonase Elution Buffer. Then, 125 U of benzonase nuclease were added to

the sample. The sample was incubated for 2 hours at 37 °C with intermittent mixing at 900 rpm on a thermomixer to digest nucleic acids and release proteins from the beads. The beads were magnetically separated again and the supernatant was transferred to a fresh microcentrifuge tube. This step was repeated three times in order to remove all traces of streptavidin beads. The last supernatant obtained represents the "protein elution" sample. The protein elution samples were taken to the EMBL proteomics core facility for mass-spectrometry.

4.7 Stainings

4.7.1 Staining of cultured cells

Cells were cultured on coverslips or in culture dishes until reaching the desired confluency. The culture medium was then removed, and the cells were gently washed with phosphate-buffered saline (PBS) to eliminate any residual media components. Fixation of the cells was achieved by incubating them with a 4% formaldehyde in PBS solution at room temperature for 5 minutes. After fixation, the formaldehyde solution was removed, and the cells were washed with PBS to remove any excess fixative. For permeabilization, a 0.3% Tween 20 in PBS solution was added to the cells and incubated at room temperature for 10 minutes, allowing better staining penetration. Subsequently, the Tween solution was removed, and the cells were washed again with PBS.

To conduct staining and improve specificity, the cells were incubated overnight at 4°C with a staining solution (3% BSA in PBS) containing the desired antibodies. After staining, the cells were washed with PBS to remove any unbound staining reagents. Then, a staining solution (3% BSA in PBS) containing the desired fluorescent dyes or secondary antibodies was added to the cells and allowed to incubate for one to two hours at room temperature. Nuclear counterstaining, along with mounting medium, was applied to the cells before addition of a cover slip for microscopy.

Finally, the stained cells were observed and analysed using a fluorescence microscope (Leica DMi8).

4.7.2 Fluorescence *in situ* hybridization

C166 cells were cultured on PCA slides until the cells reached the desired confluency of 70 %. The PCA wells were washed three times with 1xPBS. Subsequently, cells were fixed at room temperature for 10 min with 4% formaldehyde. A further wash step with 1xPBS was carried out. The cells were then permeabilized for 10 min at room temperature using 0.1% TritonX-100 in 1xPBS.

For pre-hybridization, cells were incubated with wash buffer at room temperature for 30 minutes. Simultaneously, the hybridization buffer for the samples was prepared. The buffer was thoroughly

vortexed and warmed in a 37°C shaker to ensure complete dissolution of dextran sulfate. The smFISH probes, along with Phalloidin 488 (diluted at 1:50), were added to the hybridization buffer, reaching a final concentration of 250nM for the probes.

To each PCA slide, 100 µl of pre-warmed (37°C) hybridization buffer containing smFISH probes and Phalloidin was added. The slides were then incubated at 37°C for 16-18 h in a humid chamber containing wet tissues.

On the following day, the slides were rinsed once and washed twice for 30 min each with pre-warmed wash buffer at 37°C. During the last wash 1:2000 Dapi was added to visualise the nuclei during microscopy. A single rinse with 0.1% Triton in PBS was performed. The coverslips were mounted using ProLong Diamond mounting medium (P10144, ThermoFisher). After allowing approximately 1 to 2 hr for drying at room temperature in the dark, the PCA slides were ready for imaging. For long-term storage, the slides could be stored at -20°C.

4.7.3 RNAScope of Lockd in the heart tissue

The RNAScope Multiplex Fluorescent Reagent Kit v2 assay was performed on fixed-frozen heart tissue samples to visualise Lockd and Cdh5 RNA on Sham and 1wTAC heart sections. The protocol was carried out according to the manufacturer's protocol.

In brief, the frozen heart tissue samples were cut into 10 µm thick sections using a cryostat. The sections were then mounted on positively charged microscope slides and air-dried at room temperature for 1 hour.

The tissue sections were fixed with 4% paraformaldehyde (PFA) at cold temperatures for at least 30 minutes. After fixation, the sections were washed with 1x phosphate-buffered saline (PBS) to remove excess fixative.

The RNAScope Target Retrieval Reagents were preheated, and the tissue sections were incubated with Protease IV at room temperature for 30 minutes to facilitate probe penetration. The sections were then hybridized with the Multiplex Fluorescent probe mix, which consisted of the target-specific Cdh5 and Lockd probes and Opal dyes.

After each step, the tissue sections were gently washed with wash buffer to remove any unbound reagents.

The signal amplification step was performed at 40°C for 30 minutes. Following the amplification, the tissue sections were counterstained with DAPI and mounted with fluorescent mounting medium. After the mounting medium dried, the fluorescent signals were visualized using a fluorescence microscope.

4.7.4 Sirius-Red staining

Picro-sirius red staining was employed as a commonly used technique to assess the accumulation of total collagen in the extracellular matrix (ECM) as an indicator of heart fibrosis. Tissue slides were fixed by immersing them in 100% ice-cold acetone at room temperature (RT) for 2 minutes. Subsequently, the slides were immersed in a solution of picric acid and 37% formaldehyde for 30 minutes. This picric acid solution consisted of 150 mL saturated picric acid and 50 mL of the aforementioned formaldehyde solution. Following fixation, the slides were rinsed in 70% ethanol and transferred into a solution containing picric acid and Direct Red 80 dye (Sigma Aldrich 365548) for 60 minutes.

Afterward, the slides underwent a 10-minute incubation in 10 mM hydrochloric acid (HCl) and were subsequently washed with 70% and 100% ethanol for 5 minutes each. Finally, the tissue slides were washed in Roticlear solution (Roth Chemicals, A538.6) and mounted using RotiMount (Roth Chemicals, HP68.1).

The stained tissue sections were captured using a Zeiss Axio Scan.Z1 microscope equipped with a bright-field mode at a 20X magnification. Image analysis was conducted utilizing ZEN 2.6 Blue Edition software from Carl Zeiss, and quantification was performed using ImageJ software.

It is noteworthy that the picric acid solution employed in this protocol functioned as both a fixative for the tissue slides and a clearing agent for subsequent staining with Direct Red 80 dye. Furthermore, the hydrochloric acid treatment was utilized to enhance the contrast of the collagen fibers within the tissue sections.

4.7.5 Heart tissue staining

The slides were allowed to dry at room temperature (RT) for 30 minutes. The tissue was fixed with 4% formaldehyde for 20 minutes at RT. Subsequently, the slides were washed with 1xPBS for 3 times, 10 minutes each, at RT. Permeabilization of the tissue was carried out using 0.3% Triton in PBS for 20 minutes at RT, followed by washing the slides with 1xPBS for 3 times, 10 minutes each, at RT. Blocking was performed by incubating the slides in 3% BSA in PBS (filtered with 0.2 μ m) for 20 minutes. The slides were then incubated with primary antibody (1:250) overnight at 4°C. The next day, the slides were washed three times with 1x PBS at room temperature then incubated with WGA (1:100), IB4 (1:50) and secondary antibody (1:1000) in BSA for 1 hour at RT in the dark. Following this, the slides were washed once with 1xPBS + 0.01% Triton in a dark glass cuvette, followed by 2 more washes with 1xPBS in a dark glass cuvette. Finally, the slides were mounted using Vectashield® HardSet™ Antifade Mounting Medium with DAPI and left to dry for at least 1 hour at RT in the dark.

4.8 *In vivo* methods

4.8.1 Animal testing and animal welfare

All studies, including the use and care of mice and rats, were conducted in accordance with ethical principles and approved by the Regierungspräsidium Karlsruhe, Germany, under protocols 35-9185.81/G-144/18 and I-22/03. All experiments involving surgical procedures and subsequent physiological assessments were conducted in compliance with the German Animal Welfare Act.

Wild-type ICR/CD1 mice were bred in-house, while C57BL/6N mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France). The animals were housed in a temperature and humidity-controlled room ($22 \pm 2^\circ\text{C}$ and 35–60 % humidity) with a 12-hour light/dark cycle and provided with unlimited access to water and standard food.

4.8.2 Transverse aortic constriction

The mice used for transverse aortic constriction and Sham surgeries were all 9 weeks $\pm$ 6 days old. The mice were anesthetized using a whole-body chamber with 2.5 % isoflurane and an airflow of 1 L/minute. To prevent cooling of the mouse, a heated operating table at 37°C was used during the whole procedure. Anaesthesia was continued using a mask inhalation of 2.5 % isoflurane. Analgesia was administered 20 minutes prior to surgery through the intra peritoneal injection of Buprenorphine at a dose of 0.1 mg/kg body. Bepanthen eye ointment was applied to prevent corneal dehydration prior to the start of the operation. The ventral thorax up to the neck was shaved and subsequently disinfected with 70% ethanol. After ensuring deep anaesthesia through the lack of the toe pinch reflex, a median skin incision was made at the level of the fourth rib to the sternal notch. The mouse was then intubated tracheally, and controlled ventilation was provided using a Harvard Respirator (Model 683) with 2.5% isoflurane inhalation. The mouse was securely fixed to the operating table, and an upper median sternotomy was performed up to the level of the second rib (approximately 4 mm). After carefully separating the thymus, the aortic arch was dissected free. Subsequently, the aortic arch was ligated between the two carotid arteries using surgical suture material (Ethicon, Ethilon 7-0) around a 26-gauge needle, which was immediately removed after ligation. To support the compensation of the acute increase in afterload and prevent bradycardic cardiac arrhythmias, a subcutaneous injection of 0.1 mg/kg body weight Atropine sulfate was administered once. After the operation, the sternum was reassembled, and the muscles were adapted using single button stitches. The skin was then closed using tissue adhesive Histoacryl. Isoflurane inhalation was discontinued, and the mouse was continuously ventilated with pure oxygen. Spontaneous breathing was observed by monitoring the activity of the respiratory muscles or by disconnecting the tube from the ventilator.

Once spontaneous breathing was established, the mouse was finally disconnected from the ventilator and extubated. The mouse was kept in an incubator at 35°C until it fully recovered. The entire operation lasted approximately 15 minutes. The procedure for sham-operated mice was identical, except that no aortic ligation was performed. Postoperative analgesia was provided by administering Metamizole in drinking water for five days postoperatively at a dose of 200 mg/kg body weight.

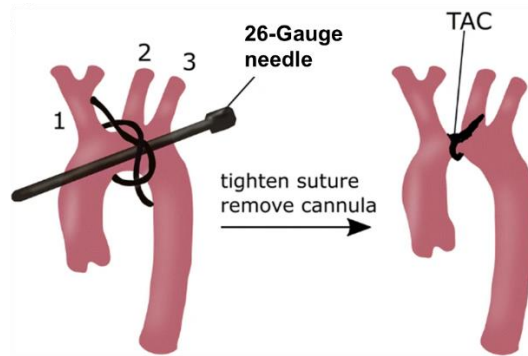


Figure 4: Schematic representation of the transverse aortic constriction (TAC) procedure

The scheme shows the location of constriction in the aortic arch, precisely positioned between the innominate artery and the left common carotid artery, using a 26-gauge needle. Once the needle is withdrawn from the constriction site, the left ventricle experiences elevated pressure, resulting in the development of cardiac hypertrophy. Illustration adapted from Bacmeister, Lucas et al. "Inflammation and fibrosis in murine models of heart failure." Basic research in cardiology (2019)

4.8.3 Echocardiography

Echocardiography was performed on the mice one and two-weeks after transverse aortic constriction or Sham surgery in order to determine the left ventricular heart function and dimension.

The mice were anesthetized in a whole-body chamber with 2.5% isoflurane and an airflow of 1 L/minute. Anesthesia was maintained using a mask (isoflurane 2.5%, airflow 0.8 l/min). The ventral thorax was depilated with an animal clipper and covered with preheated ultrasound gel. During the operation, the mouse was kept at body temperature by using a heated operating table (37°C). The left ventricular function, heart size and heart mass were determined using a 7.5 MHz transducer (Visualsonics).

In addition, 2 days after the TAC operation/sham, the blood flow of both carotids were compared with one another (to estimate the gradients generated by TAC across the ligature of the aortic arch).

After image acquisition, the obtained files were transferred to the Vevo software for analysis. Left ventricular (LV) dimensions, were calculated. All echocardiography measurements were performed by Merve Keles, Cardiovascular Physiology Department, Medical Faculty of Mannheim – Heidelberg University.

4.8.4 GapmeR induced *in vivo* Lockd knockdown

To achieve efficient knockdown of Lockd *in vivo*, commercially available LNA antisense GapmeRs from QuiaGen were utilized. The first potential antisense GapmeRs were configured at Geneglobe. Standard LNA *in vitro* GapmeRs were initially purchased to test the efficiency of Lockd knockdown in C166 endothelial cells. LNA Standard GapmeRs were resuspended in PBS to obtain a concentration of 20 μ M. The knockdown of Lockd in C166 cells was performed in the same way as siRNA knockdown. After determining an efficient LNA antisense GapmeR that achieved > 99% knockdown of Lockd in C166-ECs, 20 mg of antisense LNA GapmeR *in vivo* ready was purchased.

In vivo ready GapmeRs were resuspended in PBS to a concentration of 4 mg/mL and aliquoted into 400 μ L solutions to avoid freeze-thaw cycles. The GapmeRs were further diluted in PBS to obtain the appropriate concentration for injections. The control and 2-week TAC-operated mice received 4 doses of 100 μ L of 5 mg/kg mouse GapmeRs injections. The GapmeRs were subcutaneously injected into a skin fold on the back of the mice. The mice received their first injection two days after the echocardiogram for the control mice and two days after TAC for the operated mice. Then, the mice received the second, third, and fourth doses every third day over a period of 14 days. The one-week TAC cohort mice received 100 μ L of 5 mg/kg GapmeRs one day after TAC-operation. Three and five days after the operation, the mice received a dose of 100 μ L of 7.5 mg/kg of GapmeRs to match the amount of GapmeR received by all cohorts. The mice were then euthanized for organ extraction or primary cell isolation.

4.8.5 Organ removal

All adult mice were sacrificed via cervical dislocation. The mice were fixed on a table and their chest and peritoneal area was disinfected with 70% ethanol. Heart, Lung, Liver and tibias were taken for analysis. To ensure proper relaxation of the ventricles, the excised hearts were first washed in cold 1xPBS and subsequently in 0.5 M KCl/PBS. All other excised organs were first washed in ice cold PBS, briefly dried on a tissue, weighed, cut into small pieces and then shock-frozen in liquid nitrogen. For histology, the heart base was oriented with the cut surface facing downwards in embedding medium (O.C.T.) and frozen in n-pentane which was floating in liquid nitrogen. The embedded tissues were stored at -80°C.

4.9 RNA Sequencing

4.9.1 Bulk RNA sequencing

For RNA sequencing, isolated RNA from both isolated endothelial cells and whole heart tissue was utilized. The quality of the RNA samples was evaluated using the Agilent 2100 Fragment Analyser. Subsequently, library preparation was conducted employing the DNBSEQ Eukaryotic Strand-specific mRNA library, with BGI in Hong Kong. RNA sequencing was performed using single-ended reads, with a sequence read length of 50 bases on the Illumina HiSeq 2500 platform, also carried out by BGI.

To analyze the RNAseq data, first adapter sequences from the fastq files were removed using the R package FastqCleaner. Following the trimming process, the reads were aligned to the reference genome (mm10) using the R package bowtie2. For gene annotation, the bioMaRt R package was utilized. To ensure comparability, the library size was normalized using counts per million (cpm) reads and subsequently transformed the data into log2 values. To identify genes with significant changes in expression between compared groups, the edgeR package of R was employed and the significance threshold was set at $p\text{-Value} < 0.05$.

In addition, to gain further insights into gene function, Gene Ontology (GO) enrichment analysis was conducted using the Metascape online tools [258]. This analysis allowed to explore the biological functions and processes associated with the differentially expressed genes, providing valuable information for the interpretation of the results.

4.9.2 Single cell sequencing from cardiac endothelial cells

Single-cell RNA sequencing was performed by isolating cardiac endothelial cells (ECs) from freshly pelleted samples as mentioned before. After fluorescence-activated cell sorting (FACS), the single ECs were suspended in a resuspension buffer and loaded onto a 10x Genomics Chromium Controller chip following the manufacturer's protocol. Murine EC scRNA-seq indexed libraries were prepared using the Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1 according to the manufacturer's instructions. Library quantification and quality assessment were conducted using the Bioanalyzer High Sensitivity DNA Analysis Kit and High Sensitivity D5000 ScreenTape System. The indexed libraries were sequenced using the NextSeq 500/550 High Output Kit v2.5.

The single-cell RNA sequencing data were processed using CellRanger version 3.1.0 from 10x Genomics. The reads were mapped to the mm10 reference genome (version 3.0.0). Analysis was performed using the Seurat package (version 4.0.2) in R (version 4.0.5). Cells expressing at least 200 genes and with less than 25% mitochondrial counts were filtered. The matrix was then log-normalized

and scaled. Unsupervised clustering was performed with a resolution of 0.5 using 10 and 5 dimensions respectively, following the Satja Lab Tutorial.

Differentially expressed genes were identified using the FindAllMarkers function with the bimod statistical test in the Seurat package for focused analyses. Genes with adjusted P-values < 0.05 were considered differentially expressed [259](Manuscript submitted for publication). The single cell sequencing analysis was done by Aya Abouissa, Cardiovascular Physiology Department, Medical Faculty of Mannheim – Heidelberg University.

4.10 Software analysis

4.10.1 Combined RNA sequencing overlap

The software KNIME was utilized to combine and filter various RNA sequencing data. Firstly, the individual datasets were imported into KNIME as separate input files. Then, a combination node was employed to merge the datasets into a single dataset, ensuring compatibility in terms of format and column structure.

Subsequently, filtering was performed using the filtering nodes available in KNIME, such as $p\text{-Value} < 0.05$ and $\text{Log}_2\text{FC} > 0.6$. These nodes allowed the application of specific criteria, such as gene expression thresholds or statistical significance values, to exclude unwanted or irrelevant data points from the combined dataset. The filtering process resulted in a refined dataset containing only the desired RNA sequencing data.

Finally, the processed and filtered RNA sequencing data were exported from KNIME as output files, ready for further downstream analysis. The resulting dataset provided a consolidated and refined collection of data, facilitating subsequent interpretation.

4.10.2 ImageJ analysis

Cell sizes and numbers as well as fluorescence intensity were analysed using ImageJ software. First, images of cells were acquired by microscopy and saved as digital image files. The images were then opened in ImageJ for analysis.

To measure cell sizes, the appropriate scale was set using a known reference length on the image. This ensured accurate conversion of pixel measurements to physical units.

The cell boundaries were traced by manually outlining each cell using the freehand selection tool. Once the cells were selected, the measurement function in ImageJ was used to calculate the area or diameter of each cell. The software provided the measurements in pixel units. To convert the pixel

measurements to physical units, the scale previously set using the known reference length was applied. This allowed the determination of the actual sizes of the cells in micrometers. The cell size data, including the measurements of individual cells, were recorded and analysed statistically.

To analyze fluorescence of microscopy pictures, a known reference intensity was used to establish the scale in ImageJ, ensuring accurate conversion of pixel values to physical units. Regions of interest (ROIs) were selected encompassing the fluorescent areas of interest within the images.

For each ROI, the software measured the integrated density, providing quantitative measurements of fluorescence. Background fluorescence was taken into account by selecting an adjacent region outside the fluorescent area as the background ROI. The software calculated the average fluorescence intensity of the background region, which was subsequently subtracted from the fluorescence measurements of the ROIs.

The obtained fluorescence data were recorded and subjected to statistical analysis using appropriate software or spreadsheet programs.

4.11 Statistics

The presented data in this study are expressed as the mean $\pm$ standard error of the mean (SEM), unless otherwise specified. To compare the averages of two independent groups, a Two-Tailed Unpaired t-Test was employed, allowing us to assess whether there existed a significant difference between the two groups.

For scenarios involving three or more independent groups, a One-Way ANOVA was applied. This analysis facilitated the identification of significant differences between at least one group and the others.

To explore the effects of two independent categorical variables on a continuous dependent variable, a Two-Way ANOVA was conducted. This statistical approach enabled us to understand the individual and combined impacts of the variables on the outcome.

All statistical analyses were performed using GraphPad Prism Version 8.4.3. A significance level (α) of 0.05 was adopted for all tests to determine statistical significance.

Prior to conducting the analyses, the assumptions of each test were assessed, including normality and equal variance. In instances where the data did not meet these assumptions, appropriate transformations or non-parametric tests were utilized as alternative methods, ensuring the validity and reliability of the statistical analyses.

5. Results

5.1 Lockd emerges as a lncRNA candidate for functional investigation in the early phase of cardiac remodelling in cardiac endothelial cells

The objective of this study is to examine the participation of long non-coding RNAs (lncRNAs) derived from endothelial cells in the cardiac response to hemodynamic overload, with a specific focus on their impact on cardiovascular function. In order to identify the endothelial lncRNAs likely to play a role in cardiac stress response, a comprehensive analysis of multiple RNA sequencing datasets was performed. The datasets used in this study comprised bulk RNA sequencing data obtained from cardiac endothelial cells and cardiomyocytes isolated from adult mice. These samples were collected one-week after the transverse aortic constriction (TAC), a procedure that leads to cardiac pressure overload and triggers cardiac remodelling and pathological cardiac hypertrophy. The specific time point of one-week post-TAC was chosen to capture the early molecular changes and dynamic responses occurring in these cell types during the initial stages of cardiac remodelling. The RNA sequencing data were subjected to a filtering process to identify specific lncRNAs that displayed upregulation in cardiac endothelial cells one-week following transverse aortic constriction. Additionally, my analysis aimed to identify lncRNAs that exhibited higher overall expression levels in cardiac endothelial cells compared to cardiomyocytes one-week after the induction of pressure overload (Figure 5).

To ensure robustness and reliability, stringent filtering criteria were applied to the obtained results. Specifically, lncRNAs were considered for further analysis if they demonstrated a more than 50 % increase in expression between Sham and TAC and a statistically significant p-value below 5 %. As part of the second filtering process, lncRNAs were subjected to further analysis if they exhibited a more than 50% increase in expression in the cardiac endothelial cells after TAC compared to the cardiomyocytes after TAC. Additionally, these lncRNAs were required to have a statistically significant p-value below 5%, indicating their differential expression in the two cell types. This filtering step allowed us to focus on lncRNAs that showed significant upregulation specifically in the endothelial cells in response to TAC and that were higher expressed in endothelial cells, potentially highlighting their role in endothelial-specific responses to cardiac pressure overload.

The data obtained from both filtering processes were overlapped to generate a list of long non-coding RNAs that were utilized for subsequent analyses. This selection process allowed us to focus on the most promising candidates for further investigation.

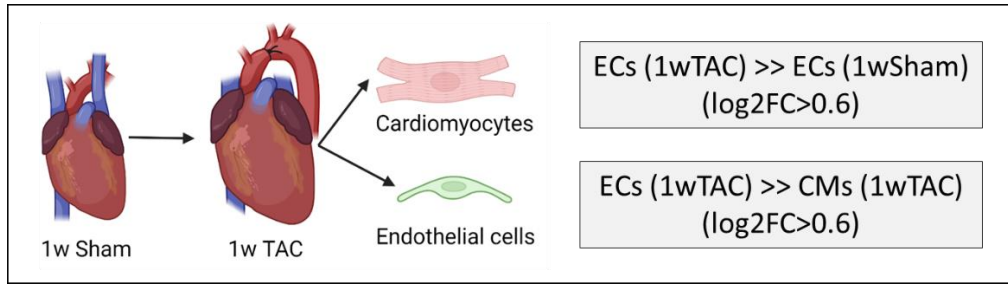
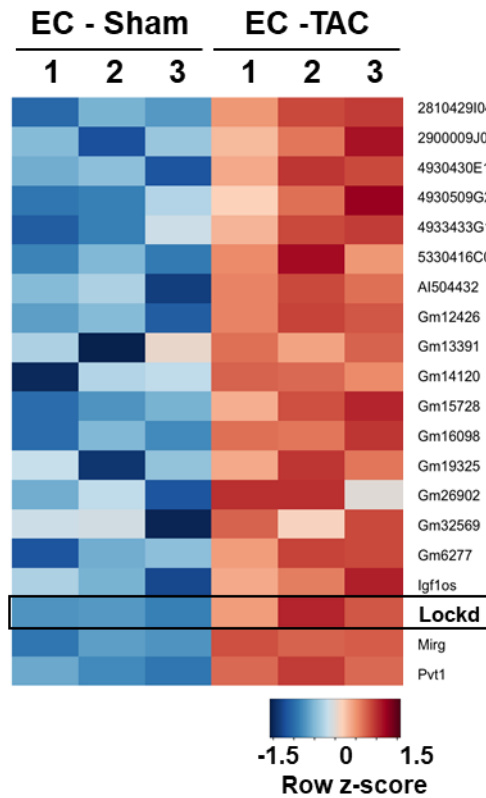


Figure 5: Pathway for the identification of differentially expressed genes in cardiac endothelial cells following 1 week of transverse aortic constriction (TAC).

As anticipated, the analysis of gene expression patterns during cardiac overload uncovered substantial differences in the expression levels of multiple genes, including 20 long non-coding RNAs (lncRNAs). Through screening and filtering, the lncRNA Pvt1 emerged as a notable candidate. Previous studies have already explored Pvt1 in the context of cardiac pressure overload-induced cardiac hypertrophy, lending credibility to our screening process [260, 261]. Of notable significance, a remarkable upregulation of a long non-coding RNA (lncRNA) called Lockd specifically in the cardiac endothelial cell population one-week after transverse aortic constriction (TAC), in comparison to the Sham-operated cardiac endothelial cells (Figure 6A) was observed. Lockd exhibited a fold change of 4.2 in the cardiac endothelial cells post-TAC compared to the Sham cardiac endothelial cells ($p\text{-Value} = 8E^{-12}$). Additionally, Lockd exhibited higher expression levels in endothelial cells relative to cardiomyocytes one-week following TAC induction (Fold change = 3.7; $p\text{-Value} = 7.9E^{-9}$) (Figure 6B).

The upregulation of Lockd in cardiac endothelial cells during the early phase of TAC-induced cardiac overload suggests that Lockd may have a potential involvement in the molecular response to increased hemodynamic stress and subsequent cardiac remodelling. The specific enrichment of Lockd in the cardiac endothelium following TAC indicates it may play a potential role within this cellular subset, for example contributing to endothelial cell-mediated processes associated with cardiovascular adaptation and remodelling.

A



B

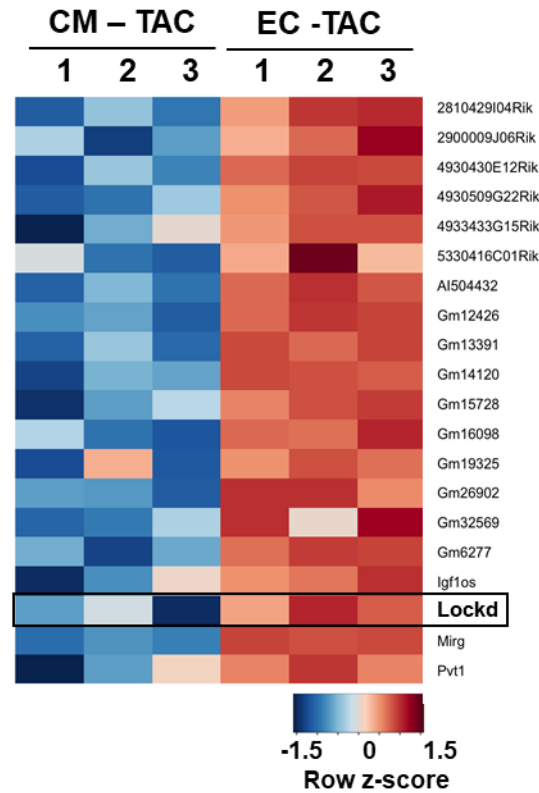


Figure 6: Identification of differentially expressed lncRNAs in cardiac endothelial cells following 1 week of transverse aortic constriction

(A) Heatmap depicting the expression levels of lncRNAs in cardiac endothelial cells one-week after TAC compared to Sham-operated mice. The filtering criteria applied included a log2 fold change (log2FC) greater than 0.6 and a p-value lower than 0.05 (B) Heatmap depicting the expression levels of lncRNAs in cardiac endothelial cells and cardiomyocytes one-week after TAC. The filtering criteria applied included a log2 fold change (log2FC) greater than 0.6 and a p-value lower than 0.05.

Lockd, a murine intergenic long non-coding RNA (lncRNA), was found to be positioned directly downstream of the protein-encoding gene *Cdkn1b* and upstream of the protein-encoding gene *Apold1* (Figure 7).

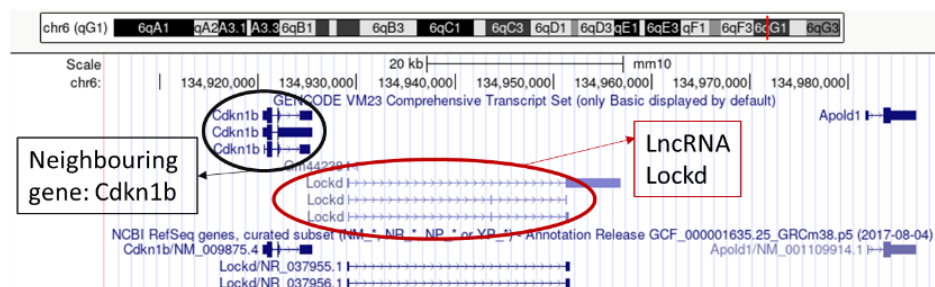


Figure 7: Localisation of Lockd on the murine genome

5.2 Lockd has a very low coding probability

The coding potential of Lockd was assessed using the online available open reading frame finder. The analysis revealed that Lockd possesses the potential to encode micro-peptides (Figure 8A). However, blasting these potential micropeptides using the Basic Local Alignment Search Tool from the National Library of Medicine failed to identify any of these putative micro-peptides associated with Lockd.

Furthermore, Lockd exhibited an *in-situ* coding probability of 3 %, similar to the previously characterized non-coding RNA (ncRNA) Malat1. In contrast, the neighbouring protein-coding gene Cdkn1b displayed a coding probability of 98%. These coding probability estimates provide insights into the likelihood of Lockd being a non-protein-coding transcript (Figure 8B).

The absence of identified micro-peptides associated with Lockd and its relatively low *in situ* coding probability align with the notion that Lockd may function as a non-coding RNA as it has an even lower coding probability than other well described lncRNAs. In contrast, Cdkn1b exhibits a high coding probability and it is known to code for a peptide.

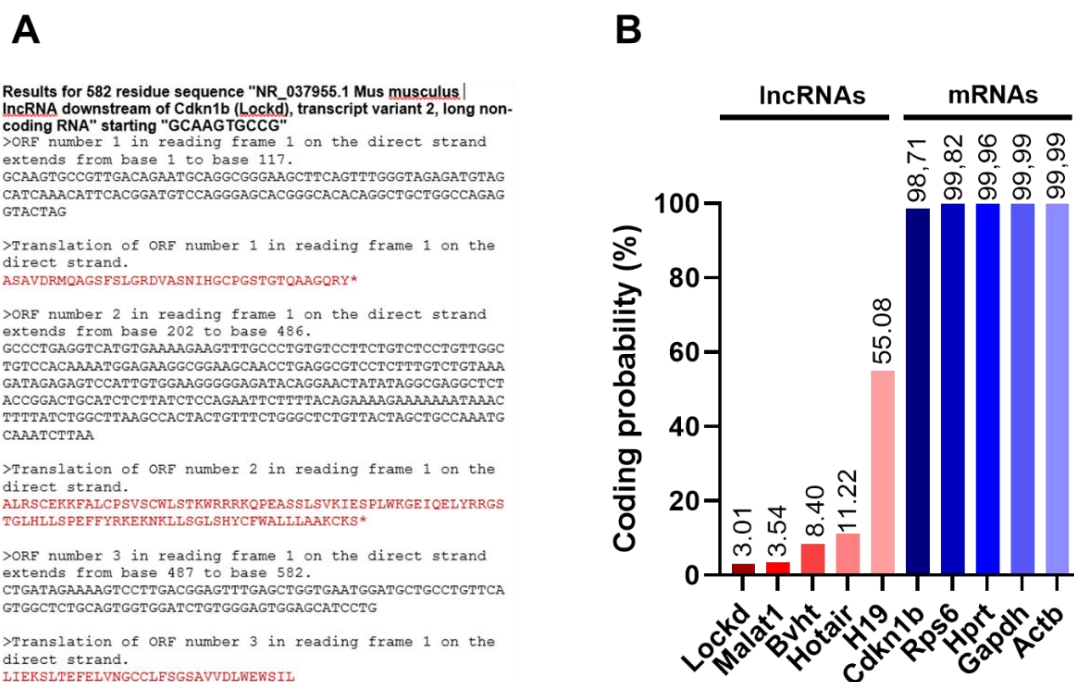


Figure 8: Assessment of the coding potential of Lockd and other lncRNAs and protein-coding mRNAs

(A) Assessment of open reading frames on the Lockd lncRNA using online available tool *ORF Finder* (genecorner.ugent.be/orf_find). (B) Percentage of probability of RNAs to code for peptides determined via the Coding-Potential Assessment Tool, CPAT (bio.tools/cpat).

5.3 Lockd is up-regulated in the early phase of cardiac remodelling

In the RNA sequencing analysis, an up-regulation of Lockd was observed in both cardiac endothelial cells and cardiomyocytes one-week following pressure overload, induced by transverse aortic constriction (TAC). To validate this observation, adult 9 weeks old RjOrl:SWISS (CD-1)mice were subjected to TAC surgery, a model for pressure overload-induced cardiac hypertrophy and heart failure. The heart weight to body weight ratio was measured as an indicator of cardiac hypertrophy (Figure 9A), and comparative quantitative polymerase chain reaction (qPCR) was performed on isolated RNA from heart tissue one-week post-TAC and Sham surgeries. The results revealed a significant approximately six-fold up-regulation of Lockd in TAC hearts compared to Sham hearts (Figure 9B).

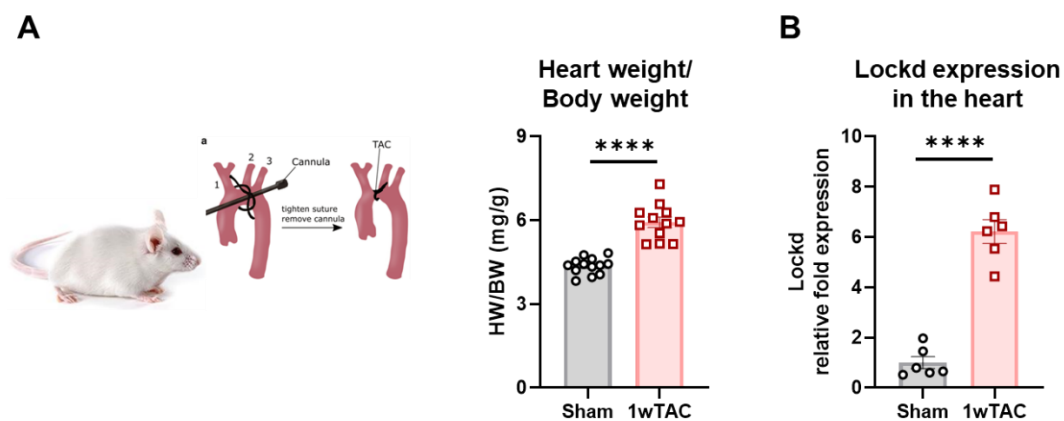


Figure 9: Validation of Lockd upregulation in pressure overload-induced cardiac hypertrophy

(A) Measurement of heart weight to body weight ratio (mg/g) in Swiss mice subjected to transverse aortic constriction (TAC) surgery or Sham surgery. The heart weight to body weight ratio serves as an indicator of cardiac hypertrophy. (B) qPCR analysis of Lockd expression in heart tissue isolated from TAC and Sham-operated mice one-week post-surgery. qPCR data was normalised to the house keeping gene Hprt. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with ****, $P < 0.0001$.

To further investigate the cellular specificity of Lockd up-regulation, the main cardiac cell types, cardiomyocytes, endothelial cells, and fibroblasts one-week after TAC and Sham surgeries were isolated. Cardiomyocytes were isolated using the Langendorff perfusion method, while cardiac endothelial cells and cardiac fibroblasts were isolated via the MACS cell separation method. Interestingly, the highest up-regulation of Lockd was observed in cardiomyocytes (14-fold increase), while significant up-regulation was detected in endothelial cells (6-fold increase), with endothelial cells having the highest cell specific Lockd expression one-week after TAC. However, no significant change in Lockd expression was observed in cardiac fibroblasts (Figure 10).

Lockd expression in cardiac cell types

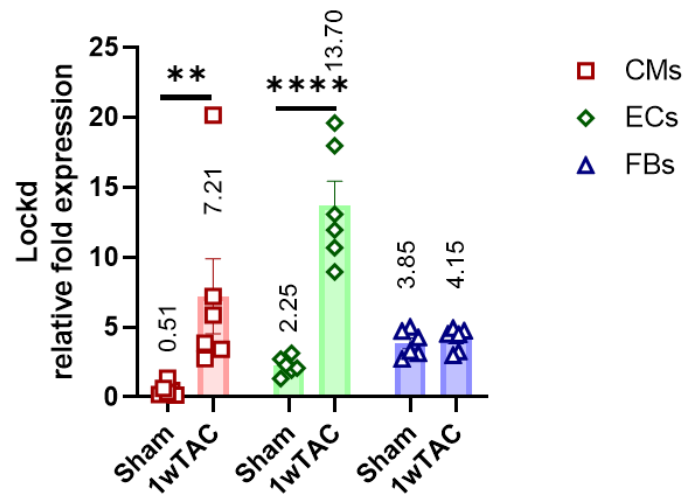


Figure 10: Cellular specificity of Lockd up-regulation in pressure overload-induced cardiac hypertrophy

qPCR analysis of Lockd expression in cardiomyocytes, cardiac endothelial cells and cardiac fibroblasts from TAC and Sham-operated mice one-week post-surgery. qPCR data was normalised to the house keeping gene Hprt. Data represents mean and error bars represent SEM. Significance was calculated by 2way ANOVA with **, $P < 0.01$ and ****, $P < 0.0001$.

In order to visualise the localisation patterns of Lockd in the cardiac tissue, RNAscope staining was performed on heart tissues one-week after sham or transverse aortic constriction surgery. RNAscope is an *in-situ* hybridization method specifically designed to visualize RNA molecules in fixed-frozen tissue samples. The procedure involves target retrieval, tissue fixation, probe hybridization, signal amplification, and fluorescent detection, enabling multiplexing and providing insights into gene expression patterns within tissues.

RNAscope staining was conducted for the endothelial specific Cdh5 to as an endothelial cell marker and Lockd to examine its presence in the heart. It was observed that Lockd was indeed present in both the Sham and 1w TAC hearts, as was Cdh5 RNA. Interestingly, in line with previous results, higher Lockd fluorescence was observed one-week after TAC compared to Sham hearts, supporting the hypothesis of Lockd upregulation, which had been quantified previously.

Furthermore, evidence of both co-localization and non-co-localization of Lockd and Cdh5 was found in both Sham and TAC hearts, indicating that Lockd was expressed in both endothelial and non-endothelial cells within the heart (Figure 11).

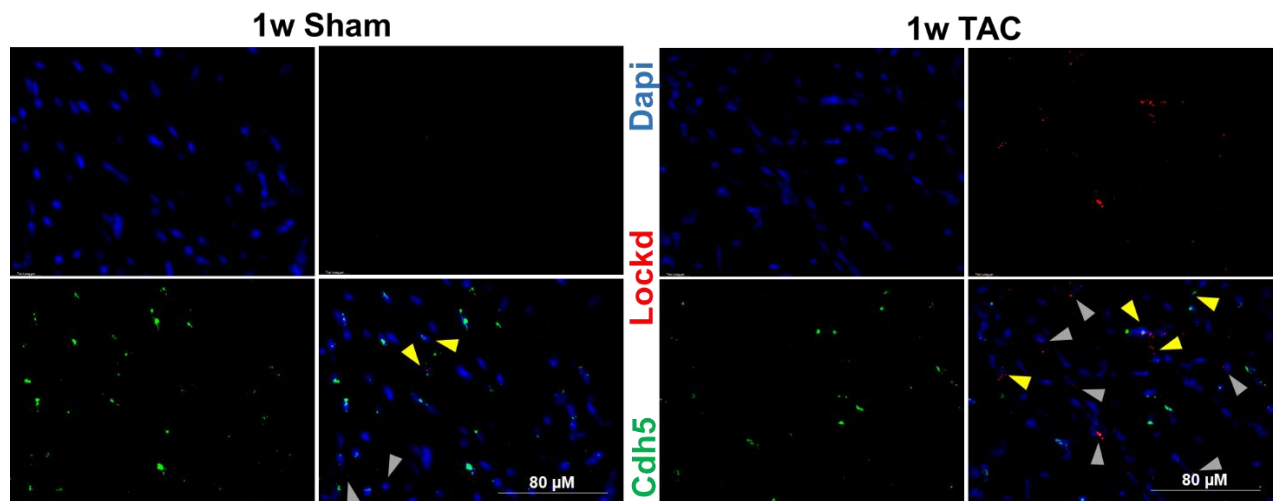


Figure 11: Visualisation of Lockd in the cardiac tissue

RNAscope staining was conducted for Cdh5 to localize endothelial cells and for Lockd to examine its presence in the heart in both Sham and 1wTAC hearts. Yellow arrows show Lockd and Cdh5 positive cells. Grey arrows show Lockd only positive cells. Scale bar = 80 μ M

The expression of Lockd's neighboring gene, Cdkn1b, in the context of induced cardiac pressure overload was investigated, as closely located genes can often share common regulatory elements and promoters and co-expression may be observed among such neighbouring genes [262]. Interestingly, while Lockd showed upregulation one-week post-pressure overload, a contrasting pattern for Cdkn1b expression was observed. Both in whole heart tissue (Figure 12A) and isolated cardiac endothelial cells (Figure 12B), Cdkn1b exhibited a mild downregulation. These findings indicate a lack of co-expression between Lockd and Cdkn1b, as their expression levels did not correlate. Thus, it appears that the regulation of Lockd and Cdkn1b expression is independent of each other, suggesting distinct functional roles and regulatory mechanisms for these genes in the context of cardiac pressure overload.

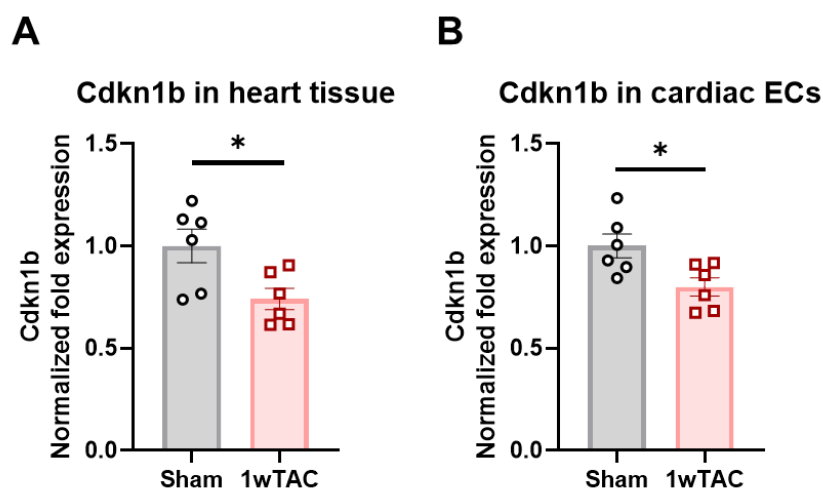


Figure 12: Expression analysis of Cdkn1b, the neighbouring gene of Lockd

(A) qPCR quantification of Cdkn1b mRNA expression in the heart tissue 1 week post Sham and TAC surgeries. (B) qPCR quantification of Cdkn1b mRNA expression in cardiac endothelial cells 1 week post Sham and TAC surgeries. qPCR data was normalised to the house keeping gene Hprt. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$.

To examine the temporal expression pattern of Lockd during pressure overload-induced cardiac remodelling, a TAC time course on C57Bl6/N mice was conducted. Non-operated mice were used as controls and compared to 1 week, 2 weeks and long-term TAC (> 8 weeks TAC) operated mice (Figure 13A) Comparative qPCR analysis was performed on whole heart tissue, cardiac endothelial cells, and cardiac fibroblasts. The results revealed that Lockd expression peaked one-week after TAC, followed by a decrease but sustained up-regulation in whole heart tissue (Figure 13B). Similarly, Lockd expression exhibited a peak at one-week post-TAC in cardiac endothelial cells, and at later time points, Lockd expression remained approximately twice as high as in non-operated control hearts (Figure 12C). No differential regulation of Lockd could be observed in cardiac fibroblasts after TAC time course (Figure 13D). These findings suggest that Lockd might play a role in the early response to pressure overload-induced cardiac remodelling in both cardiomyocytes and endothelial cells. Furthermore, the sustained up-regulation of Lockd expression implies its potential involvement in persisting cardiac stress conditions. The expression profile of Cdkn1b was also investigated (Figure 13E). No correlation in expression between Lockd expression and its neighbouring gene was observed, suggesting an independent mechanism of transcriptional regulation.

These results demonstrate the up-regulation of Lockd in cardiac endothelial cells and cardiomyocytes following pressure overload-induced cardiac remodelling. The cellular specificity of Lockd up-regulation suggests its potential involvement in the early response to cardiac remodelling, particularly in cardiomyocytes and endothelial cells.

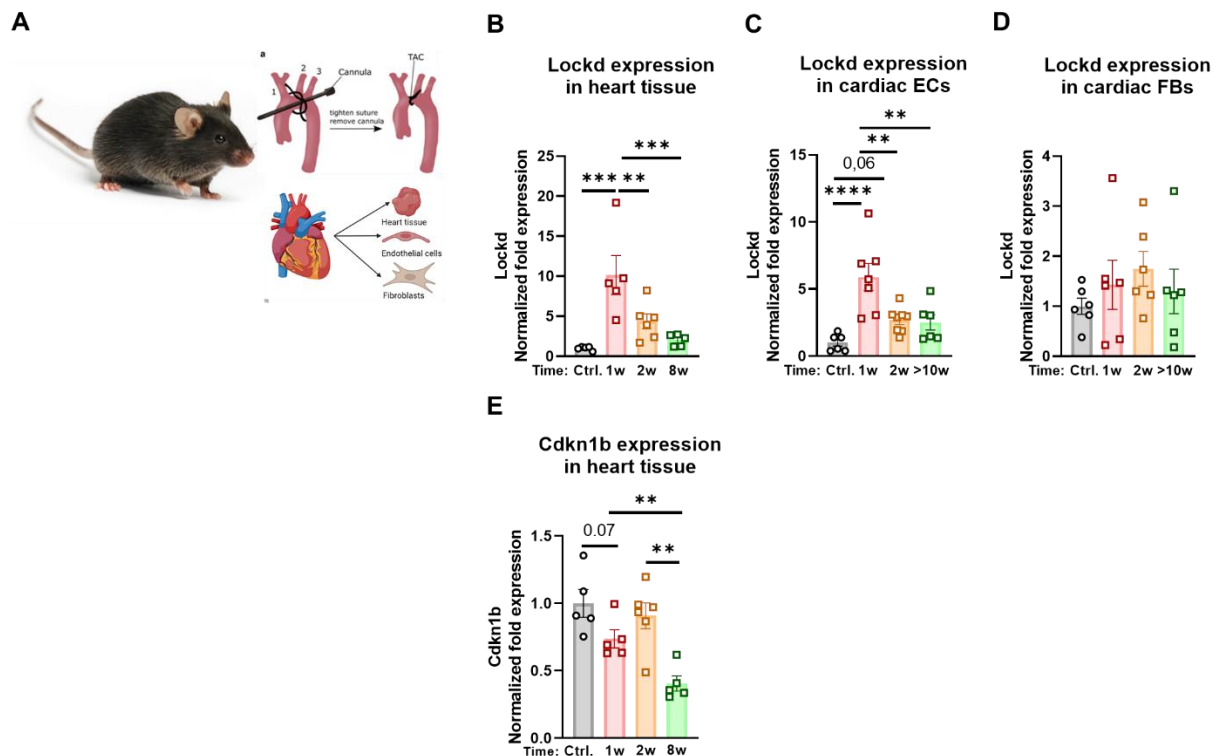


Figure 13: Temporal expression pattern of Lockd during pressure overload-induced cardiac remodelling

(A) Experimental design: Unoperated mice (Control) were compared to mice at 1 week, 2 weeks, and long-term post-TAC. (B) Expression of Lockd in whole heart tissue. (C) Expression of Lockd in cardiac endothelial cells. (D) Expression of Lockd cardiac fibroblasts. (E) Expression profile of Cdkn1b in whole heart tissue. qPCR data was normalised to the house keeping gene Hprt. Data represents mean and error bars represent SEM. Significance was calculated by One-Way ANOVA, with **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

5.4 Lockd loci is conserved in the human genome

In order to investigate if an analogue of the murine long non-coding RNA Lockd is also present in humans, and thereby confirm that Lockd might also be relevant in human biological processes, evolutionary conservation analysis was performed.

The potential functional relevance of the identified long non-coding RNA, Lockd, in the context of human biology was investigated by examining its conservation in humans.

While the human counterpart of Lockd is not annotated in the human genome, genomic analyses have demonstrated a high degree of conservation at the Lockd locus in humans. By performing a murine Lockd genome blast against the human genome, two conserved regions corresponding to the first and second exons of mouse Lockd were identified (Figure 14A). These conserved regions were further

validated on the human genome using the UCSC genome browser, confirming their alignment with the mouse Lockd loci, specifically downstream of Cdkn1b and upstream of the Apold1 gene. Additionally, the potential human Lockd sequence was blasted against the mouse genome, revealing overlap between the human and mouse Lockd, indicating high conservation during evolution (Figure 14B).

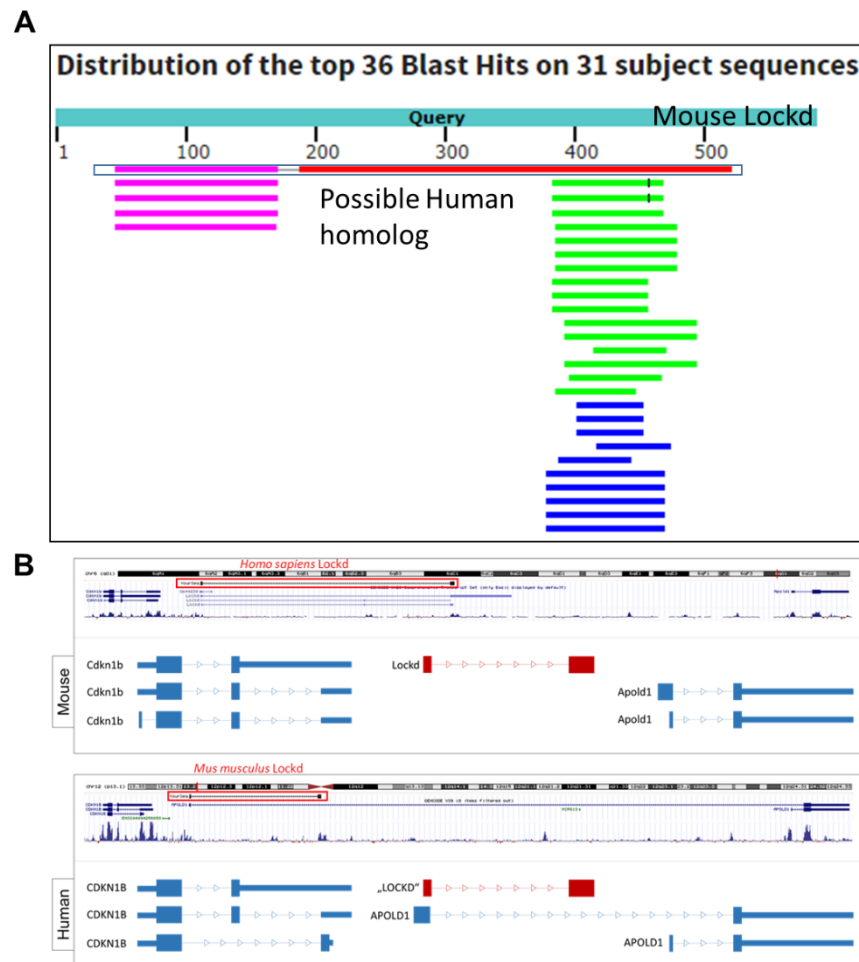


Figure 14: Genomic conservation analysis of Lockd locus between mouse and human

(A) Murine Lockd genome blast against the human genome. (B) Validation of conserved regions of mouse Lockd on the human genome and validation of conserved regions of human Lockd on the murine genome.

Alignment analysis demonstrated a significant degree of conservation between the murine Lockd transcript and the potential human Lockd transcript. A local alignment tool, which finds and aligns related regions of sequences revealed up to 73% identity and 80.9% similarity between the mouse and human loci (Figure 15A). Notably, both species exhibit a similar genomic context for the Lockd locus. In humans, it is situated immediately downstream of the Cdkn1b gene, with the first exon overlapping with the first exon of an Apold1 transcriptional variant and the second exon overlapping

with the first intron of that specific Apold1 variant. (Figure 14B). The potential human Lockd transcript variant, 482 bases, is also smaller than the murine counterpart, 582 bases.

Intriguingly, intron-spanning qPCR performed on various human cell lines (Human Umbilical Vein Endothelial Cells (HUVECs) and Human Cardiac Microvascular Endothelial Cells (HCMEC)) did not yield any PCR products. However, specific qPCR targeting only the second exon of the potential human Lockd generated an amplicon, 77 base pairs in size, which was visualized on an Agarose gel. Importantly, a no-Retrotranscriptase control qPCR did not produce any amplicon of exon 2 of human Lockd, suggesting that at least exon 2 of the human Lockd undergoes active transcription (Figure 15B).

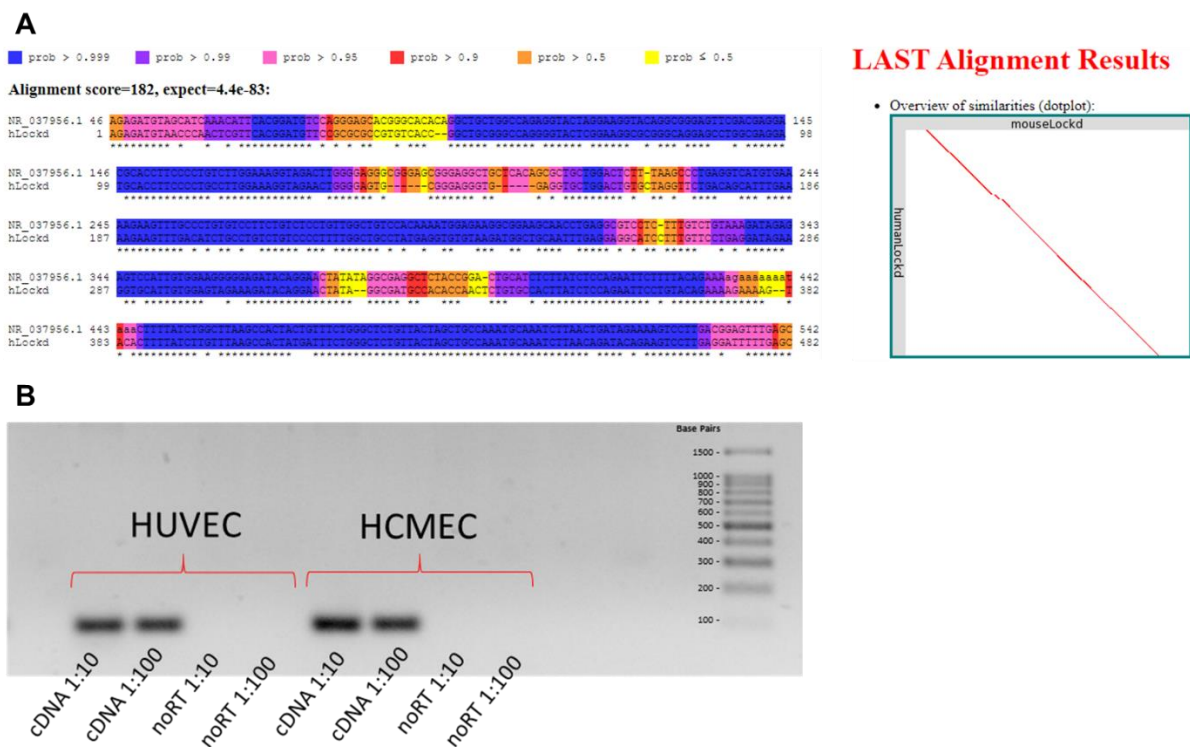


Figure 15: Comparative analysis of Lockd conservation and transcription between mouse and human

(A) Large-Scale Alignments with Spaced Seeds was used as a tool for sequence alignment. Mouse Lockd and human Lockd alignment and identification of regions of similarity between the sequences, indicating potential homology or evolutionary conservation (fasta.bioch.virginia.edu). (B) Agarose gel showing PCR amplicon and transcriptional analysis of potential human Lockd Exon 2 in human endothelial cells.

5.5 Lockd is up-regulated in human failing hearts

Considering the high conservation of Lockd in humans, it was interesting to investigate whether Lockd undergoes differential regulation in the context of human heart failure compared to healthy

individuals. For this purpose, tissue samples from individuals diagnosed with heart failure who had received a left ventricular assist device (LVAD) were obtained. Additionally, heart tissue samples from otherwise healthy individuals, who experienced unfortunate fatalities, such as in car accidents, were included for comparison. All samples that were analysed were from patients having an average age of 60 (Figure 16A)

Utilizing a comparative qPCR, Lockd expression levels in these human tissue samples were evaluated. The findings revealed that Lockd was upregulated in the hearts of individuals with heart failure, establishing a significant association between Lockd expression and human cardiac dysfunction (Figure 16B).

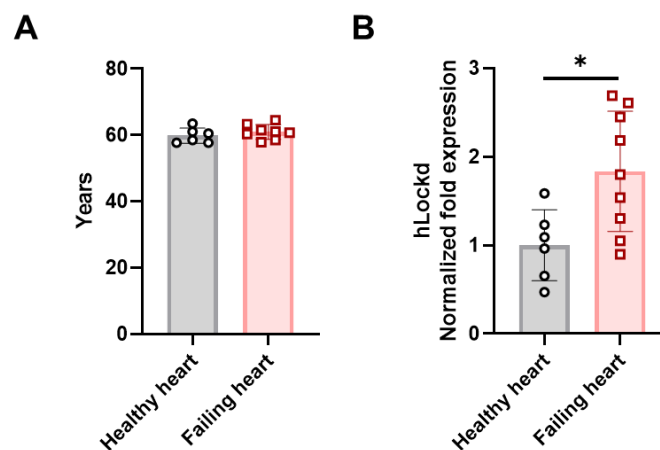


Figure 16: Human Lockd expression in human heart failure

(A) The average age of the patients. (B) qPCR analysis of the expression levels of human Lockd in heart tissue samples. Data normalised to the house keeping gene Gapdh. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$.

5.6 Lockd expression is high during the developmental phase

Next, the expression profile of Lockd during cardiac development was investigated. Comparative quantitative PCR analysis was performed using heart tissue isolated at different stages of development. Specifically, heart samples were collected from mice at postnatal day 1 (P1), P5, P21, and 8 weeks of age (adult stage).

The results revealed that Lockd exhibited the highest expression in newborn mice (P1) and subsequently demonstrated a gradual decrease in expression as the mice transitioned into adulthood.

This age-dependent expression pattern of Lockd during cardiac development mirrored observations made in other organs, such as the liver and brain, where similar trends were observed (Figure 17).

The highest expression of Lockd during the growth phase of development is of particular interest, as cardiac hypertrophy is known to be associated with the re-expression of fetal gene patterns.

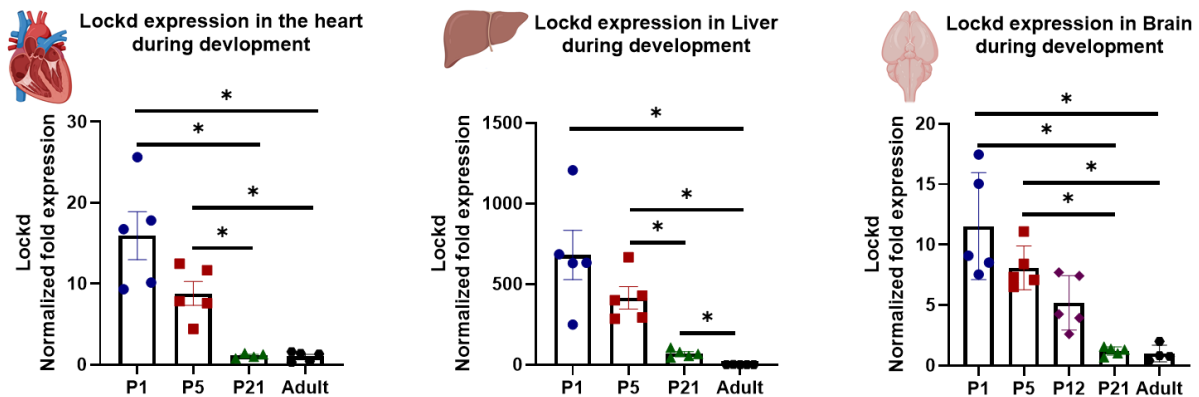


Figure 17: Lockd expression during development

qPCR analysis of the expression profile of Lockd during development. Hearts, liver and brain were isolated from mice at different stages of development. qPCR data was normalised to the house keeping gene U6. Data represents mean and error bars represent SEM. Significance was calculated by One-Way Anova with *, $P < 0.05$.

5.7 Lockd is a stable RNA molecule

Subsequently, the stability of Lockd RNA was evaluated through a timecourse experiment using Actinomycin D. Actinomycin D is a potent transcription inhibitor that hinders the process of DNA-dependent RNA synthesis. It functions by binding to DNA and obstructing the formation of the transcription initiation complex, thereby impeding the generation of new RNA molecules. By subjecting cells or tissues to Actinomycin D treatment, the decay of Lockd RNA over time was monitored, enabling to assess its RNA stability [263]. This allows to study the stability of existing RNA molecules over time by collecting samples at different time points after Actinomycin D treatment and assessing the abundance of the target RNA using q-PCR. The decrease in RNA levels over time provides insights into the degradation kinetics and stability of the RNA of interest.

The results revealed that Lockd RNA exhibited high stability, comparable to that of the house-keeping gene Gapdh. Lockd displayed a half-life of approximately 24 hours, indicating a slow decay rate and a

robust resistance to degradation. In contrast, the RNA half-life of Myc, which is known to have a short RNA stability, was less than 1 hour, signifying rapid degradation kinetics (Figure 18) [264].

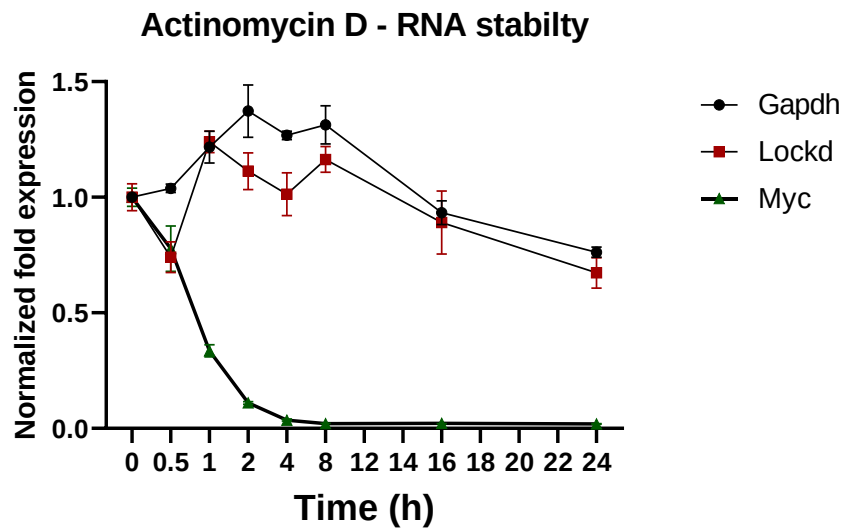


Figure 18: Stability of Lockd RNA assessed by Actinomycin D timecourse experiment
qPCR analysis of RNA degradation kinetic upon Actinomycin D treatment. qPCR data was normalised to RNA input concentration. Data represents mean and error bars represent SEM.

5.7 Lockd expression is regulated by angiogenic cytokines in endothelial cells

To investigate the regulatory factors influencing Lockd expression in endothelial cells, 80% confluent C166 endothelial cells (Figure 19A) and P3 primary juvenile cardiac endothelial cells (Figure 19B) were stimulated with various cytokines in serum-free media for a duration of 24 hours.

Through this experimental approach, it was observed that specific pro-angiogenic factors, such as Vegf, Fgf2, and Angpt2, led to a significant upregulation of Lockd expression *in vitro*. In addition, cells which were supplemented with serum-containing (10% FCS) also showed an up-regulation of Lockd, suggesting that proliferating cells up-regulate Lockd. Interestingly, the anti-angiogenic cytokine TGF- β exhibited a downregulatory effect on Lockd expression *in vitro*.

These findings provide evidence for the regulatory influence of different cytokines on Lockd expression in endothelial cells, with pro-angiogenic factors promoting upregulation and the anti-angiogenic cytokine Tgf- β inducing downregulation, highlighting the intricate interplay between cytokine signalling and Lockd expression in endothelial cell physiology.

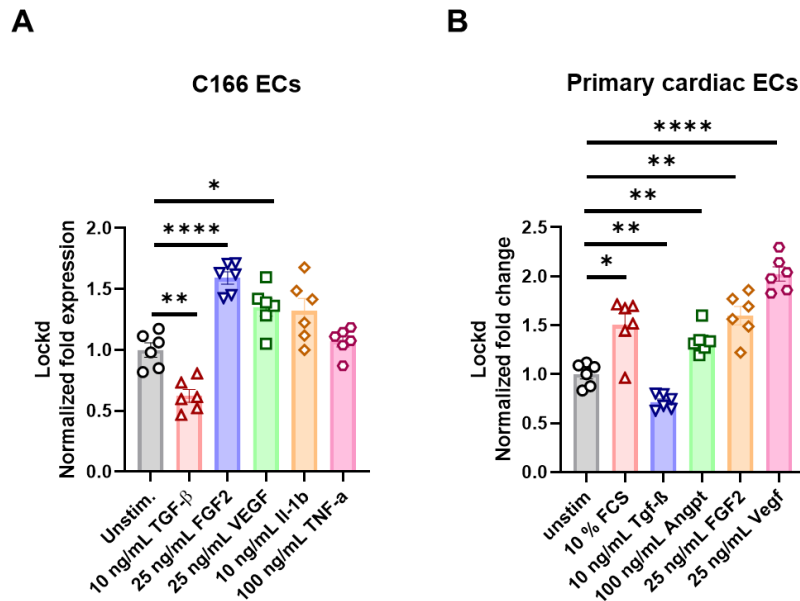


Figure 19: Regulation of Lockd expression in endothelial cells in response to cytokine stimulation

(A) qPCR analysis of Lockd expression after stimulation of C166; a yolk-sac endothelial cell-line with various cytokines. (B) qPCR analysis of Lockd expression after stimulation of isolated primary juvenile cardiac endothelial cells with various cytokines. qPCR data was normalised to RNA input concentration. Significance was calculated by One-Way ANOVA with *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$

5.8 Lockd inhibits angiogenesis

In the early phase following induced cardiac overload, specifically approximately one-week after transverse aortic constriction, Lockd exhibited its peak expression in cardiac endothelial cells. This time point coincides with the activation of endothelial cells for neovascularization in response to cardiac remodelling during the compensatory phase [265]. Considering the differential regulation of Lockd by angiogenic cytokines in endothelial cells, it is reasonable to hypothesize that Lockd might play a specific role in angiogenesis during this compensatory phase.

To investigate this hypothesis, experiments involving both Lockd overexpression and knockdown were conducted using cultured C166 endothelial cells. The down-regulation of Lockd was achieved by employing small interfering RNA (siRNA), resulting in a significant 90% reduction in Lockd levels within the cells (Figure 20A). The siRNA-treated cells were then utilized to form endothelial spheroids, which were embedded in a collagen-methylcellulose matrigel supplemented with basal medium or basal medium supplemented with 25 ng/mL Fgf2—a potent inducer of angiogenesis from endothelial spheroids [266] (Figure 20B). The sprouting capacity of the endothelial spheroids was evaluated by quantifying the number and length of sprouts. The number of sprouts per spheroid was considerably

increased in the Lockd knockdown cells, under both basal conditions and in the presence of Fgf2, showing a substantial increase in the number of sprouts per spheroid compared to siCtrl-treated spheroids. Furthermore, siLockd-treated spheroids supplemented with Fgf2 displayed a significantly greater number of sprouts per spheroid compared to siCtrl-treated spheroids supplemented with Fgf2. Notably, siCtrl-treated spheroids exhibited a trend towards increased sprouting only when stimulated with Fgf2 (Figure 20C). Regarding the length of sprouts, siLockd-treated spheroids exhibited significantly greater sprout length compared to siCtrl-treated spheroids, specifically in the context of Fgf2 stimulation. However, under basal conditions, no difference in sprout length was observed between siCtrl and siLockd-treated spheroids (Figure 20D). Collectively, these results demonstrate that the downregulation of Lockd expression significantly increases the sprouting capacity of endothelial cell spheroids, particularly when stimulated with Fgf2. These findings suggest a role for Lockd in regulating endothelial cell sprouting and angiogenic potential (Figure 20E).

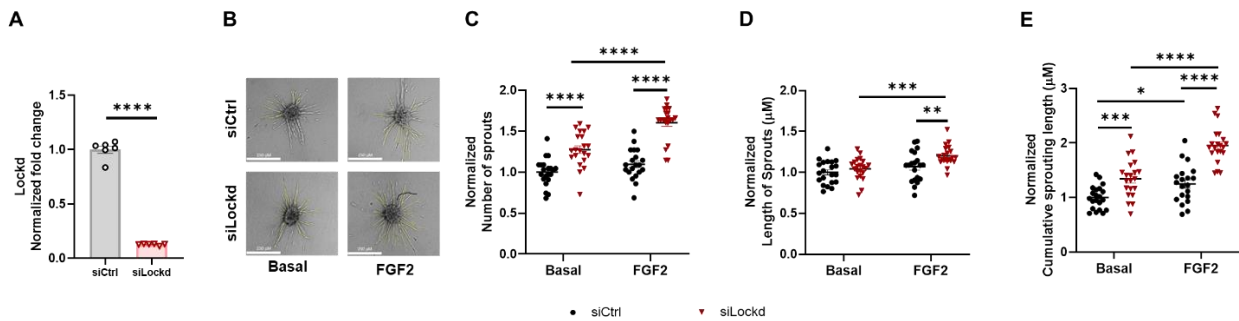


Figure 20: Knockdown of Lockd enhances sprouting capacity in endothelial cell spheroids

(A) Lockd levels were down-regulated by employing small interfering RNA (siRNA), resulting in a significant 90% reduction in Lockd expression within the cells. qPCR data was normalised to the house keeping gene Gapdh. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with ****, $P < 0.0001$. (B) Endothelial spheroids formed from siRNA-treated cells were embedded in a collagen-methylcellulose matrigel supplemented with basal medium or basal medium supplemented with 25 ng/mL FGF2. Scale bar = 250 μ M. (C) Quantification of the number of sprouts per spheroid. (D) Evaluation of sprout length per spheroid. (E) Cumulative sprouting length per endothelial spheroid. Data represents mean and error bars represent SEM. Significance was calculated by 2way ANOVA with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

In order to investigate the impact of Lockd overexpression on endothelial cell sprouting, experiments using adenoviral infection in cultured C166 endothelial cells were conducted. Ad.Lockd was administered at a multiplicity of infection (MOI) of 50, resulting in an average six fold overexpression of Lockd within the cells, comparable to the observed upregulation in cardiac endothelial cells one-week after TAC (Figure 21A).

The adenovirus-infected cells were then employed to generate endothelial spheroids, which were embedded in a collagen-methylcellulose matrigel supplemented with basal medium or basal medium supplemented with 25 ng/mL Fgf2 (Figure 21B).

Our findings revealed that spheroids infected with Ad.Lockd exhibited a significantly reduced number of sprouts compared to those infected with Ad.βGal, when treated with Fgf2. Furthermore, the increase in the number of sprouts per spheroid was lower in the Fgf2-treated Ad.Lockd-infected spheroids compared to the control group (Figure 21C).

Moreover, the sprout length in Ad.Lockd-infected spheroids was significantly shorter than that observed in Ad.βGal-infected spheroids under both basal and Fgf2-treated conditions. While the sprout length increased in Ad.βGal-infected spheroids upon Fgf2 stimulation, this response was not observed in the spheroids infected with Ad.Lockd (Figure 21D).

Taken together, these results demonstrate a diminished overall sprouting capacity in spheroids infected with the Lockd overexpression adenovirus compared to those infected with the βGal overexpression adenovirus. The differential effect on sprouting was particularly pronounced in Ad.Lockd-infected spheroids when stimulated with the angiogenic cytokine Fgf2 (Figure 21E).

These findings suggest an inhibitory role of Lockd in angiogenesis, as evidenced by reduced sprouting capacity in response to Lockd overexpression and enhanced sprouting upon Lockd knockdown. Moreover, this data emphasizes the potential significance of Lockd in regulating angiogenic processes during the compensatory phase of cardiac remodelling.

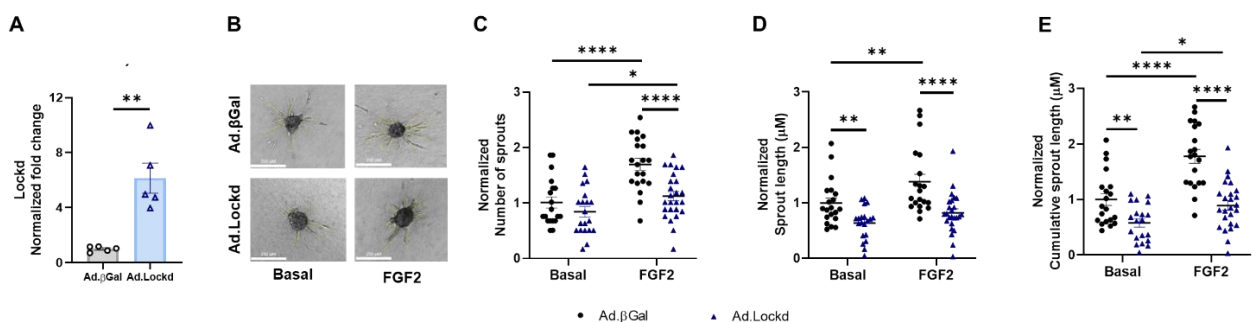


Figure 21: Lockd overexpression inhibits endothelial cell sprouting

(A) qPCR quantification of Lockd overexpression of C166 cells after adenoviral infection with Ad.Lockd or Ad.βGal at an MOI of 50. qPCR data was normalised to the house keeping gene Gapdh. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with ****, $P < 0.0001$. (B) Ad.βGal and Ad.Lockd-infected endothelial cells were used to generate endothelial spheroids, which were embedded in a collagen-methylcellulose matrigel supplemented with basal medium or basal medium supplemented with 25 ng/mL FGF2. Scale bar = 250 μm. (C) Quantification of the number of sprouts per spheroid. (D) Evaluation of sprout length per spheroid. (E) Cumulative sprouting length per endothelial spheroid. Data represents mean and

error bars represent SEM. Significance was calculated by 2way ANOVA with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

5.9 Lockd inhibits endothelial proliferation

After observing a difference in sprouting angiogenesis, the focus of my investigation shifted towards examining endothelial proliferation. This was done in order to investigate whether endothelial cell proliferation could be implicated in the observed differences in sprouting angiogenesis. To achieve this, the BrdU proliferation assay was employed, a method for quantifying the incorporation of BrdU (Bromodeoxyuridine) into newly synthesized DNA, thus providing an assessment of the relative abundance of actively proliferating cells.

In order to assess the impact of Lockd on endothelial cell proliferation, siRNA-mediated down-regulation of Lockd was utilized. The siLockd-treated cells exhibited higher levels of BrdU incorporation compared to cells treated with siCtrl, indicating increased proliferation in the absence of Lockd expression (Figure 22A). Conversely, cells infected with Ad.Lockd, which resulted in overexpression of Lockd, demonstrated reduced BrdU incorporation when compared to cells infected with Ad. β Gal (Figure 22B).

These findings suggest that Lockd may exert a regulatory influence on the proliferation of endothelial cells. Knockdown of Lockd led to enhanced proliferation, while overexpression of Lockd resulted in attenuated proliferation.

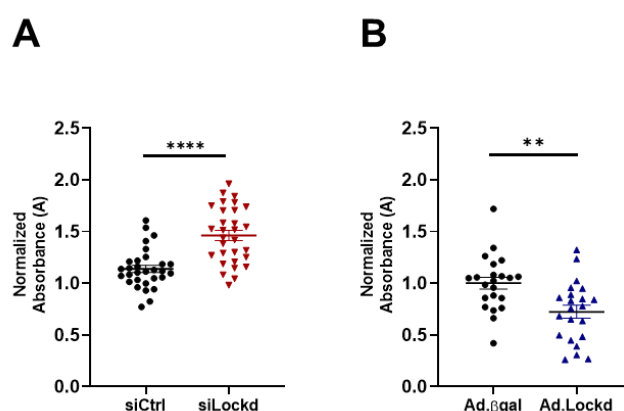


Figure 22: Lockd regulates endothelial proliferation

(A) Normalized (to control) absorbance of BrdU incorporation in siRNA treated cells. (B) Normalized absorbance of BrdU incorporation in Adenovirus-infected cells. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with **, $P < 0.01$; ****, $P < 0.0001$.

As a further method to assess the potential modulation of endothelial cell proliferation by Lockd, an immunostaining assay was performed using antibodies against Aurora B, a known marker of proliferating cells, as well as Phosphohistone 3 (PH3). Phalloidin and DAPI staining were used as markers of the cellular cytoskeleton and nuclei, respectively. Subsequently, the populations of Aurora B-positive and Aurora B-negative cells were quantified.

Lockd knockdown cells exhibited an increased percentage of Aurora B-positive cells compared to siCtrl cells (Figure 23A). Conversely, the overexpression of Lockd with an MOI of 50 resulted in a reduced number of Aurora B-positive cells compared to Ad.βGal-infected cells (Figure 23B).

Similarly, Lockd knockdown cells demonstrated an elevated percentage of PH3-positive cells compared to siCtrl cells (Figure 23C). Conversely, the overexpression of Lockd with an MOI of 50 led to a decreased number of PH3-positive cells compared to Ad.βGal-infected cells (Figure 23D).

These findings further support the notion that Lockd inhibits endothelial cell proliferation.

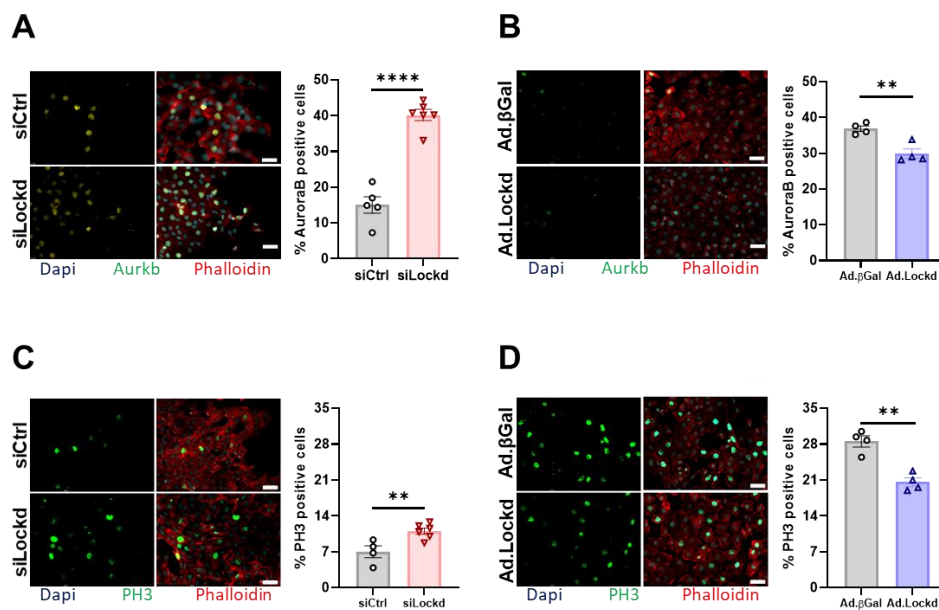


Figure 23: Assessment of endothelial cell proliferation by immunostaining of Aurora B and Phosphohistone 3

(A) Immunostaining using antibodies against Aurora B, a marker of proliferating cells, was performed to evaluate the potential modulation of endothelial cell proliferation by Lockd knockdown. Phalloidin and DAPI staining were employed to visualize the cellular cytoskeleton and nuclei, respectively. (B) Aurora B immunostaining to quantify the modulation of endothelial cell proliferation by adenoviral Lockd overexpression. (C) Immunostaining using antibodies against Phosphohistone 3, a marker of proliferating cells, was performed to evaluate the potential modulation of endothelial cell proliferation by Lockd knockdown. Phalloidin and DAPI staining were employed to visualize the cellular cytoskeleton and nuclei, respectively. (D) Phosphohistone 3 immunostaining to quantify the modulation of endothelial cell proliferation by adenoviral Lockd overexpression. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with **, $P < 0.01$; ****, $P < 0.0001$. Scale bar = 50 μ m

5.10 Lockd does not influence migration of endothelial cells

The observed difference in sprouting angiogenesis led to the investigation of whether endothelial cell migration could have played a role in the observed effects. Endothelial cell migration is crucial during angiogenesis, and vessel repair processes [267]. Endothelial cell proliferation leads to an increase in cell numbers, involving mitosis and cell division, while migration involves the movement of individual cells to establish new blood vessels and shape the vascular network. Both processes are fundamental for physiological and pathological angiogenesis

To evaluate the effect of Lockd on endothelial cell migration, the scratch assay in conjunction with mitomycin C treatment was employed. Mitomycin C, an antibiotic that forms a covalent bond with DNA, hinders DNA synthesis and subsequently inhibits cell proliferation [268]. Mitomycin C was used to exclude effects of cell proliferation on scratch wound closure. In this assay, a scratch was made in confluent C166 cells treated with either siLockd or siCtrl, or infected with either Ad.Lockd or Ad.βGal. The cells were pre-incubated with 10 µg/ml mitomycin C for two hours before the scratch was made using the tip of a 200 µl pipette. The closure of the scratches was quantified after a 24-hour period.

Interestingly, the analysis revealed no discernible differences in scratch closure between cells treated with siLockd or siCtrl (Figure 24A), as well as cells infected with Ad.Lockd or Ad.βGal (Figure 24B). These results indicate that Lockd does not significantly influence endothelial cell migration under the conditions tested.

Taken together, these findings suggest that Lockd primarily exerts an inhibitory effect on angiogenesis and endothelial cell proliferation, while not significantly affecting endothelial cell migration.

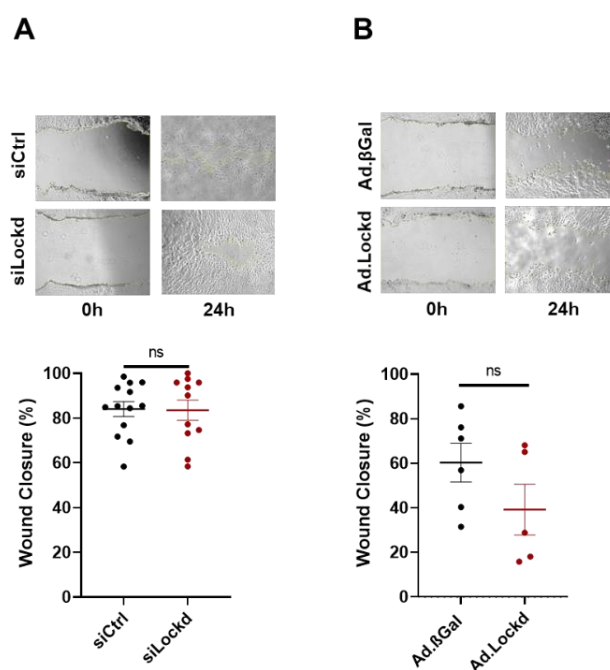


Figure 24: Assessment of endothelial cell migration using scratch closure assay

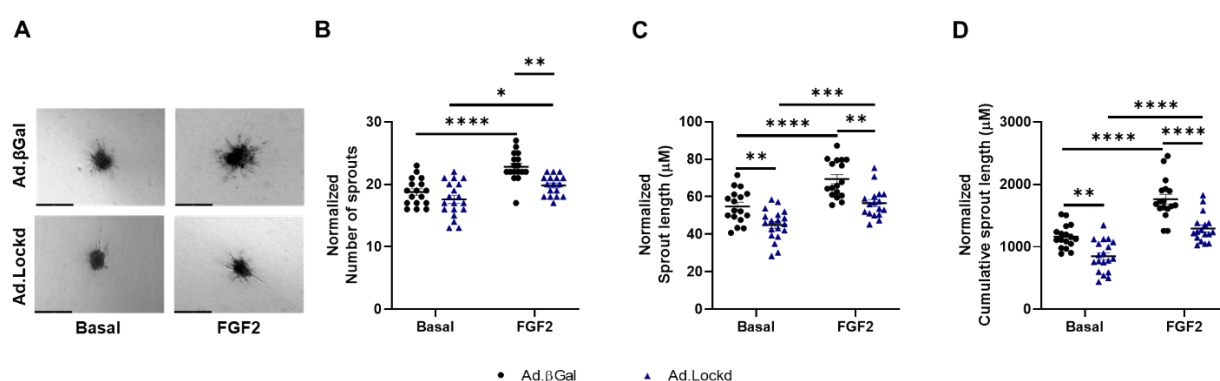
(A) Measurement of percentage of scratch closure to investigate the impact of Lockd knockdown on endothelial cell migration. (B) Quantification of scratch closure to assess the effect of Lockd overexpression on endothelial cell migration. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with ns, $P > 0.05$.

5.11 Lockd inhibits angiogenesis of HUVECs

In order to investigate if the effects of Lockd on angiogenesis are conserved in primary human endothelial cells, a sprouting assay was conducted using HUVECs (human umbilical vein endothelial cells). HUVECs were infected with an MOI (multiplicity of infection) of 50 with murine Ad.Lockd or Ad.βGal, and the resulting endothelial spheroids were embedded in a methylcellulose-collagen matrix (Figure 25A).

Our observations revealed a tendency towards a lower number of sprouts in the Lockd overexpression cells compared to cells infected with Ad.βGal (Figure 25B). Similarly to the findings in C166 mouse endothelial cells, a reduction in the length of the sprouts was observed (Figure 25C), and the overall sprouting capacity of the HUVEC spheroids was diminished in the Lockd overexpressing cells (Figure 25D).

Furthermore, consistent with the results obtained from mouse endothelial cells, the difference in total sprouting capacity was even more pronounced when the HUVEC endothelial spheroids were treated with 25 ng/μL Fgf2, a known inducer of angiogenesis. These findings suggest that Lockd may also have an inhibitory effect on angiogenesis in human endothelial cells.

**Figure 25: Effect of Lockd overexpression on endothelial cell sprouting**

(A) Ad.βGal and Ad.Lockd-infected HUVECs were used to generate endothelial spheroids, which were embedded in a collagen-methylcellulose matrigel supplemented with basal medium or basal medium supplemented with 25 ng/mL FGF2. Scale bar = 250 μM. (B) Quantification of the number of sprouts per spheroid. (C) Evaluation of sprout length per spheroid. (D) Cumulative sprouting length per endothelial spheroid. Data represents mean and error bars represent SEM. Significance was calculated by 2way ANOVA with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

5.12 Lockd inhibits proliferation of HUVECs

The potential upstream role of Lockd in angiogenesis in human endothelial cells was examined by investigating its impact on the proliferation of HUVECs (human umbilical vein endothelial cells). Specifically, the human Lockd transcript was targeted for knockdown using two newly designed siRNAs: sihLockd1 achieved approximately 30% knockdown, while sihLockd3 achieved a knockdown of 50% (Figure 26A).

Upon the knockdown of Lockd in HUVECs, a significant increase in BrdU incorporation into newly synthesized DNA was observed, suggesting that the downregulation of Lockd led to a pronounced enhancement of HUVEC proliferation (Figure 26B).

Conversely, overexpressing murine Lockd in HUVECs resulted in a notable decrease in BrdU incorporation, indicating that Lockd overexpression led to a substantial reduction in HUVEC proliferation (Figure 26C).

These results demonstrate that Lockd can modulate HUVEC proliferation, potentially playing a regulatory role in angiogenic processes. This suggests that the effect of Lockd in proliferation are conserved in humans

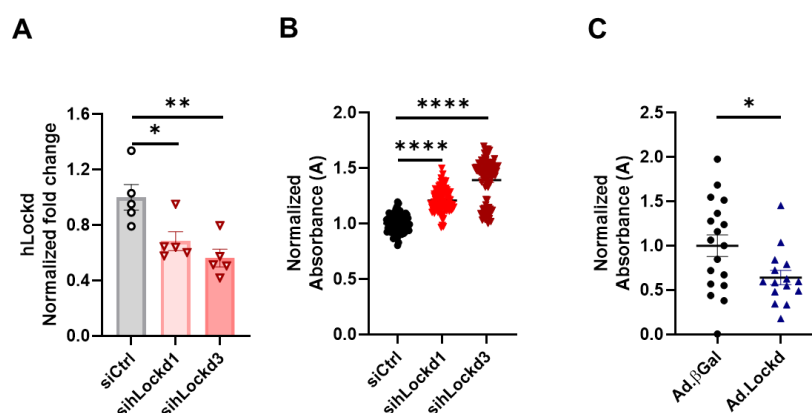


Figure 26: Impact of Lockd modulation on the proliferation of human umbilical vein endothelial cells (HUVECs)

(A) Knockdown efficiency of human Lockd was assessed using two newly designed siRNAs, sihLockd1 and sihLockd3. (B) Following the knockdown of Lockd in HUVECs using siRNAs, BrdU incorporation, indicative of DNA synthesis and cell proliferation, was observed and compared to cells treated with control siRNA. Data represents mean and error bars represent SEM. Significance was calculated by One-way ANOVA with *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. (C) Overexpression of murine Lockd in HUVECs followed by BrdU proliferation assay. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$.

5.13 Lockd is induced during mitosis

The localization of Lockd within subcellular fractions was of particular interest to understand in which cellular localization Lockd might act. Subcellular fractionation of juvenile primary cardiac endothelial cells was done in order to fractionate for cytoplasmic, nucleoplasmic and chromatin fractions. The subcellular fractionation analysis of primary cardiac juvenile mouse ECs revealed the presence of Lockd in both the nucleus and cytoplasm, similar to the protein-coding RNA Gapdh and a higher cytoplasmic fraction than the lncRNAs Malat1, Xist and U2 (Figure 27A).

Given Lockd's relatively high expression for a long non-coding RNA (lncRNA), Fluorescence *in situ* hybridization (FISH) was conducted on C166 endothelial cells to precisely determine its cellular localization. Dapi staining was employed for DNA visualization, while phalloidin staining was used to visualize actin filaments. The findings revealed that Lockd exhibited predominantly perinuclear localization, with additional presence in the nucleus and cytoplasm, thus supporting the subcellular fractionation results (Figure 27B).

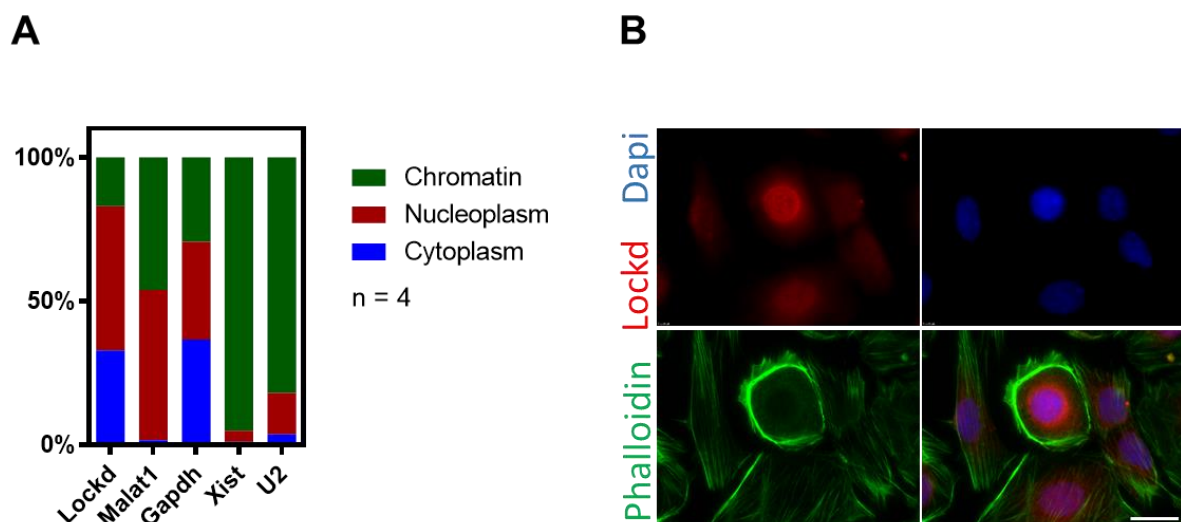


Figure 27: Subcellular localization of Lockd in endothelial cells

(A) qPCR quantification of RNAs after subcellular fractionation of primary cardiac juvenile mouse ECs. (B) Fluorescence *in situ* hybridization (FISH) in combination with Dapi and phalloidin staining. Scale bar = 25 μ M

During the FISH experiments, an intriguing observation was made that mitotic cells displayed notably higher Lockd fluorescence signals. To further investigate this phenomenon, the Lockd fluorescence intensity was quantified in non-mitotic and mitotic cells. Dapi staining was used to identify cells undergoing chromatin condensation or those in the metaphase or anaphase stages of mitosis. During mitosis, chromatin condenses and becomes highly condensed and tightly packed. In interphase cells,

the chromatin appears as a more diffuse and dispersed pattern, while in cells undergoing mitosis, the chromatin appears as distinct, condensed masses. During metaphase and anaphase, the chromosomes are maximally condensed and aligned at the equatorial plate (metaphase) or being pulled apart towards opposite poles of the cell (anaphase). DAPI staining highlights the condensed chromosomes in these stages, allowing to identify cells in metaphase or anaphase of mitosis.

The analysis revealed a significant increase in Lockd fluorescence intensity in dividing cells compared to non-mitotic cells (Figure 28A). This observation suggests a potential involvement of Lockd during cellular division, emphasizing its dynamic regulation in the context of cell cycle progression.

To investigate the dynamics of Lockd during cell cycle progression, Lockd expression was examined in cells treated with Mitomycin C, a DNA replication inhibitor that induces cell cycle arrest at the G1 phase, thereby rendering the cells non-proliferating and quiescent. The aim was to assess the impact of cell cycle arrest on Lockd expression.

The Mitomycin C treated cells exhibited a significant down-regulation of Lockd expression compared to untreated cells, indicating a suppression of Lockd during non-proliferative conditions (Figure 28B). This observation suggests that Lockd expression is tightly linked to the active cell cycle and may play a role in promoting cell proliferation.

Conversely, cells were also treated with Nocodazole for a short duration (4 hours) prior to cell lysis. Nocodazole disrupts the dynamics of microtubule assembly/disassembly by binding to beta-tubulin, thereby impairing the formation of metaphase spindles during cell division [269, 270]. As a result, cells treated with Nocodazole are unable to progress through the cell cycle phases.

Upon short-term stimulation with Nocodazole, a significant up-regulation of Lockd expression was observed compared to untreated cells (Figure 28C). This finding provides further evidence for the dynamic regulation of Lockd during cell cycle phase transitions and suggests its involvement in cellular processes associated with cell cycle progression.

Treatment with Mitomycin C, which induces cell cycle arrest at the G1 phase, resulted in down-regulation of Lockd expression, indicating its link to active cell proliferation. Conversely, Nocodazole treatment, which halts cell cycle progression at the G2/M phase, led to an up-regulation of Lockd expression. These findings suggest that Lockd plays a role in promoting cell proliferation and is dynamically regulated during cell cycle phase transitions.

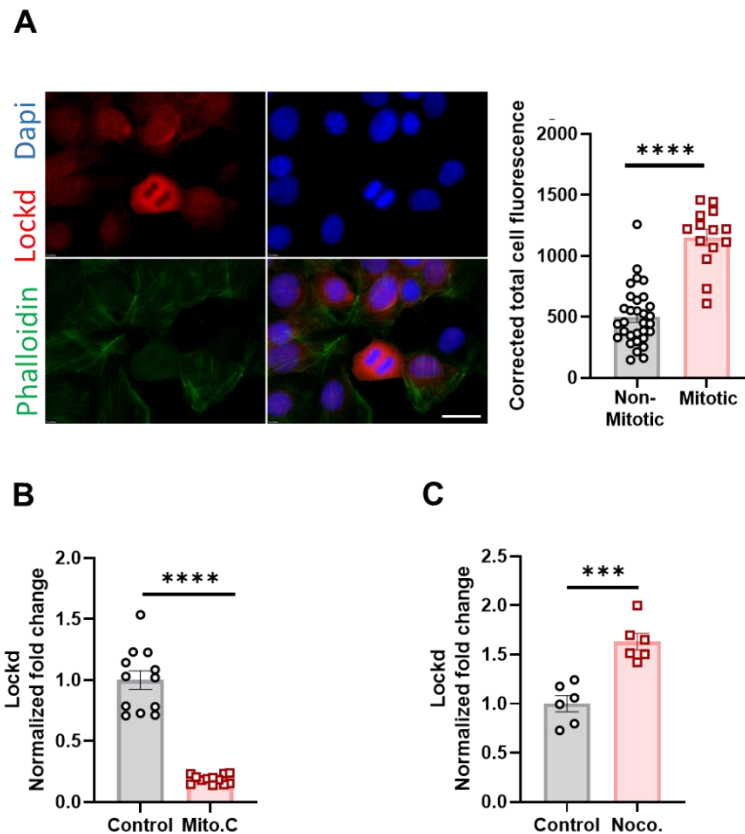


Figure 28: Dynamic regulation of Lockd during cell cycle progression

(A) Fluorescence *in situ* hybridization (FISH) in combination with Dapi and phalloidin staining for the quantification of Lockd fluorescence intensity in non-mitotic and mitotic cells. Scale bar = 25 μ M (B) Quantification of Lockd expression after Mitomycin C induced G1 cell cycle arrest. (C) Quantification of Lockd expression after 4h stimulation of cells with Nocodazole. qPCR data was normalised to the house keeping gene Gapdh. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

To ascertain if Lockd is also upregulated in mitotic endothelial cells in an *in vivo* setting, single-cell sequencing data obtained from adult cardiac endothelial cells were examined. Intriguingly, Lockd was predominantly expressed in one representative cluster, cluster N° 9, which is associated with cell cycle and cell division (Figure 29A). Within this cluster, Lockd was co-expressed with endothelial markers such as Cdh5 and mitosis markers including Mki67 and Aurkb (Figure 29B).

Moreover, within this cluster, Lockd ranked as the 15th most upregulated gene and the most highly upregulated lncRNA (Figure 29C). These findings highlight the significant upregulation of Lockd specifically in mitotic endothelial cells, both *in vitro* and *in vivo*, underscoring its potential involvement in cell cycle regulation and division.

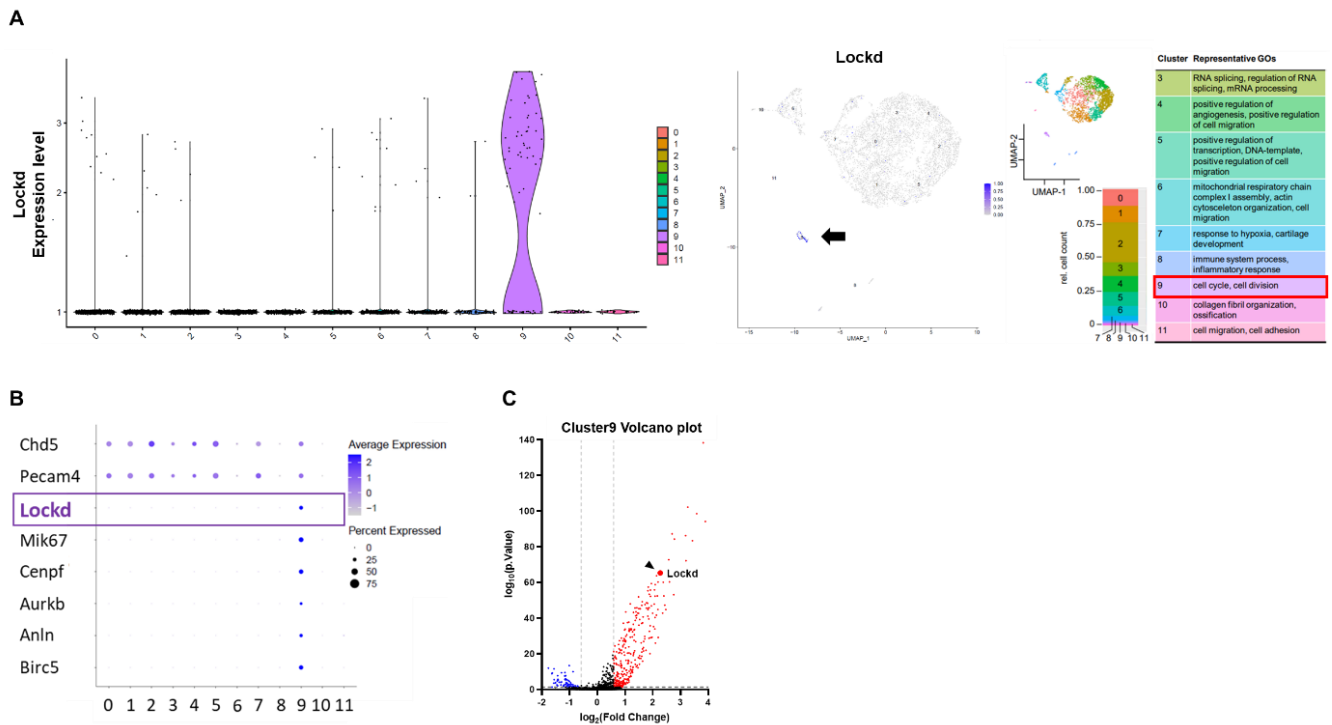


Figure 29: Lockd upregulation in mitotic endothelial cells *in vivo*

(A) Analysis of single-cell sequencing data obtained from adult cardiac endothelial cells revealed that Lockd was predominantly expressed in cluster N° 9, which is associated with cell cycle and cell division. The violin plot demonstrates the higher expression levels of Lockd in cluster N° 9 compared to other clusters. (B) Within cluster N° 9, Lockd exhibited co-expression with endothelial markers such as Cdh5 and mitosis markers including Mki67 and Aurkb. The bubble plot highlights the overlapping expression patterns of Lockd with these markers. (C) In the same cluster, Lockd ranked as the 15th most upregulated gene and the most highly upregulated long non-coding RNA (lncRNA), as indicated by the volcano plot.

5.14 Lockd knockdown influences the expression of cell cycle processing genes

In order to gain comprehensive understanding of the potential biological processes influenced by Lockd, bulk RNA sequencing analysis was conducted on endothelial cells (ECs) treated with siLockd and siCtrl. The transcriptomic data revealed significant alterations in gene expression, both upregulation (806 genes; p.Value < 0.05) and downregulation (1115 genes; p.Value < 0.05), providing valuable insights into Lockd's functional implications (Figure 30A).

Subsequently, a Gene Ontology (GO) enrichment analysis was performed on the differentially expressed genes (False discovery rate < 0.05) to elucidate the enriched biological processes. Notably, the most prominently upregulated genes (286 genes; FDR < 0.05) were found to be associated with the cell cycle pathway, suggesting a potential role of Lockd in cell cycle regulation. Conversely, downregulated GO terms (392 genes; FDR < 0.05) encompassed crucial biological processes including

apoptosis, regulation of blood vessel development, ECM organization, and heart development (Figure 30B).

To validate the effectiveness of Lockd knockdown, quantitative PCR assays were employed. The results demonstrated a highly efficient knockdown of Lockd expression of 90 %. Through qPCR validation, several genes associated with endothelial to mesenchymal transition (EndMT), such as Sm22 and Fsp, exhibited downregulation upon Lockd knockdown, further corroborating the involvement of Lockd in EndMT-associated processes. Similarly, genes related to ECM organization, including Collagen 4 and Foxc2, displayed downregulation, while Prg4 exhibited upregulation.

Furthermore, genes associated with cell cycle progression, such as Uhmk1, Ccnd3 and Cdc25b, were significantly upregulated upon Lockd knockdown, suggesting a potential regulatory role of Lockd in cell cycle progression. Additionally, differential regulation of angiogenesis-related genes, including Pdgfb and Mtor, was observed (Figure 30C).

These findings provide compelling evidence that may Lockd play a crucial role in diverse biological processes, such as cell cycle regulation, ECM organization, and angiogenesis.

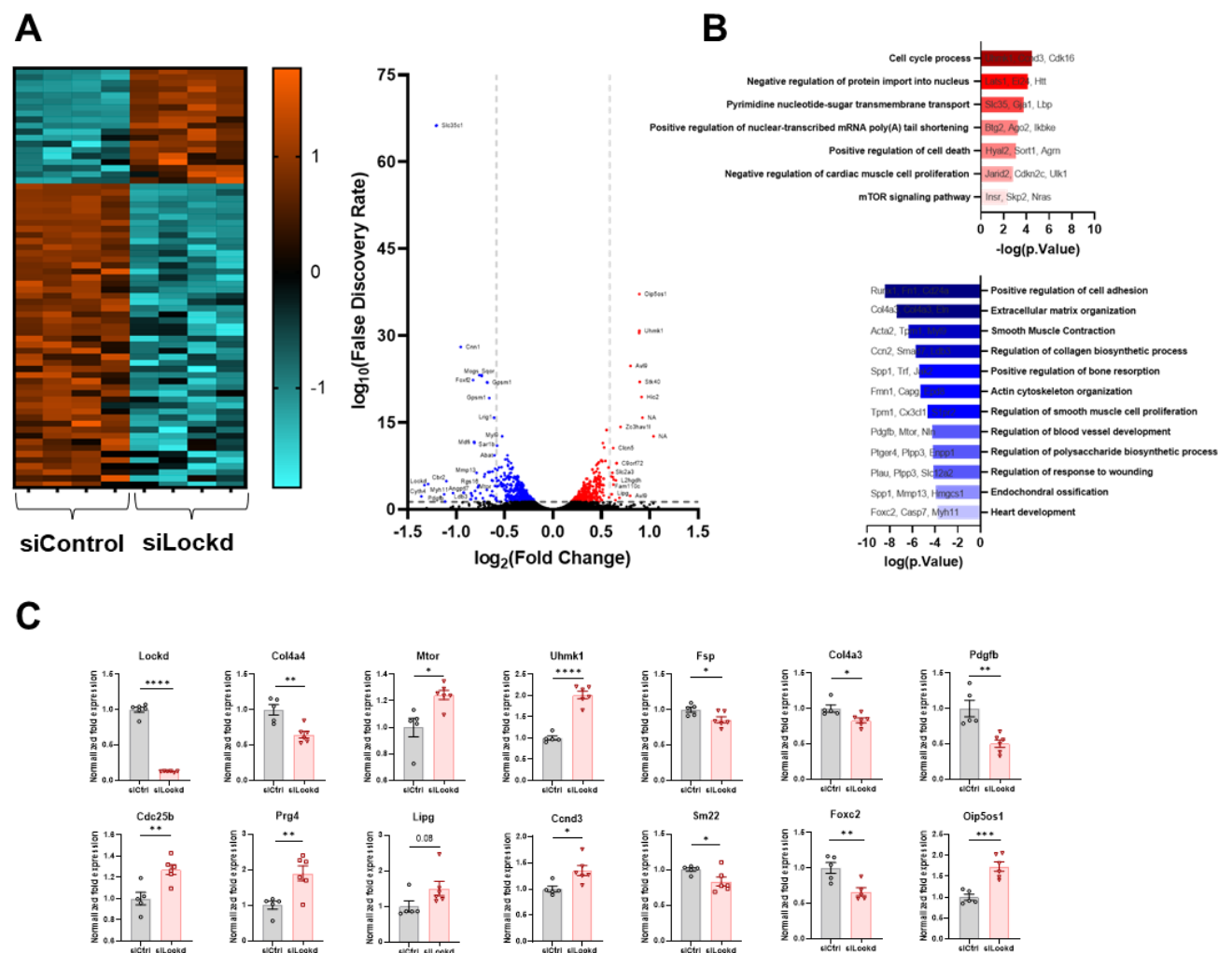


Figure 30: Lockd-mediated regulation of gene expression and biological processes in endothelial cells

(A) Heat map representation of gene expression profiles in cells subjected to siRNA knockdown. The heat map displays the expression levels of a selected set of genes across different conditions. The colour scale indicates the relative expression levels, with orange indicating higher expression and blue indicating lower expression. The siRNA knockdown samples exhibit distinct gene expression patterns compared to the control samples, indicating the impact of gene silencing on the cellular transcriptome. And Volcano plot illustrating the differential gene expression analysis between siRNA knockdown and control samples. The x-axis represents the log₂ fold change, indicating the magnitude of change in gene expression, while the y-axis represents the -log₁₀ adjusted false discovery rate, reflecting the statistical significance of the observed differential expression. Each dot represents a gene, and genes exhibiting significant differential expression are highlighted in red and blue. (B) Gene Ontology (GO) analysis reveals enrichment of biological processes associated with differentially expressed genes. Bar plot illustrating the enriched Gene Ontology (GO) terms associated with upregulated genes (red) and down-regulated genes (blue) following Lockd modulation. The y-axis represents the GO terms, while the x-axis represents the enrichment score, indicating the degree of enrichment in log₁₀ p.value. (C) qPCR analysis of genes associated endothelial-to-mesenchymal transition, extracellular matrix organization, cell cycle progression and of angiogenesis-related genes. qPCR data was normalised to the house keeping gene Gapdh. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, P < 0.05; ***, P < 0.001; ****, P < 0.0001.

5.15 Lockd exerts an indirect influence on its neighboring gene through trans-regulation

The most significantly upregulated protein-coding gene identified upon Lockd knockdown in C166 endothelial cells was the cell cycle regulator Uhmk1. Despite limited knowledge about Uhmk1, it is recognized as a serine/threonine protein kinase that facilitates cell cycle progression from G1 to S phase. This is achieved through the phosphorylation of the cyclin-dependent kinase inhibitor Cdkn1b, also known as p27, at position Ser10, leading to its nuclear export and subsequent degradation. Cdkn1b is lying directly upstream of Lockd in the mouse as well as the human genome (see above). Cdkn1b serves as a critical cell cycle regulator for maintaining cells in the G1 phase [271-274].

To investigate the potential relationship between Lockd and Cdkn1b phosphorylation, Western-Blot analyses targeting Cdkn1b and phospho- Cdkn1b were conducted. To investigate the potential mechanisms underlying the potential changes in Cdkn1b phosphorylation, a 4-hour treatment with Mg132, a proteasome inhibitor, was employed prior to cell lysis. This treatment aimed to inhibit the proteasomal degradation of proteins, including phosphorylated and ubiquitinated Cdkn1b, and facilitate their detection. Remarkably, upon Lockd knockdown, an elevated level of Cdkn1b phosphorylation were observed. There was the hypothesis that there may be an increase in ubiquitinated Cdkn1b as well, as the band above corresponds to the addition of one ubiquitin molecule (~8.5 kDa) (Figure 31A). Additionally, a significant reduction in total Cdkn1b levels was

noted, suggesting an augmented entry of cells into the cell cycle following Lockd knockdown (Figure 31B).

Conversely, in C166 endothelial cells overexpressing Lockd, an accumulation of Cdkn1b was observed, which promotes the maintenance of cells in the quiescent G1 phase (Figure 31C).

To gain deeper insights into the functional interplay between Lockd and Uhmk1, an attempt was made to rescue the observed increased cell proliferation phenotype resulting from Lockd knockdown. For this purpose, siRNA targeting Uhmk1 was employed. Efficient knockdown of Uhmk1 mRNA was achieved in C166 endothelial cells, with approximately 70% reduction observed (Figure 31D). As previously demonstrated, Lockd knockdown resulted in enhanced endothelial cell proliferation. However, when both Lockd and Uhmk1 RNA were simultaneously knocked down in C166 endothelial cells, the proliferation rate returned to basal conditions. Therefore, the combined knockdown of Uhmk1 and Lockd proved sufficient to rescue the phenotype of increased cell proliferation *in vitro* (Figure 31E).

These findings suggest a regulatory relationship between Lockd and the cell cycle modulator Uhmk1 *in vitro*. The upregulation of Uhmk1 upon Lockd knockdown, accompanied by alterations in Cdkn1b levels and phosphorylation, provides valuable insights into the intricate mechanisms underlying endothelial cell proliferation (Figure 31F).

This shows that Lockd knockdown in C166 endothelial cells leads to an upregulation of Uhmk1 expression, which, in turn, results in increased phosphorylation and degradation of Cdkn1b, a cyclin-dependent kinase inhibitor responsible for maintaining cells in the G1 phase. Overexpression of Lockd in C166 endothelial cells leads to the accumulation of Cdkn1b, promoting the maintenance of cells in the quiescent G1 phase. By modulating Uhmk1 expression and subsequently affecting Cdkn1b levels and phosphorylation, this might be one of the mechanisms in which Lockd may serve as a player in the delicate balance of cell cycle regulation in endothelial cells during proliferation

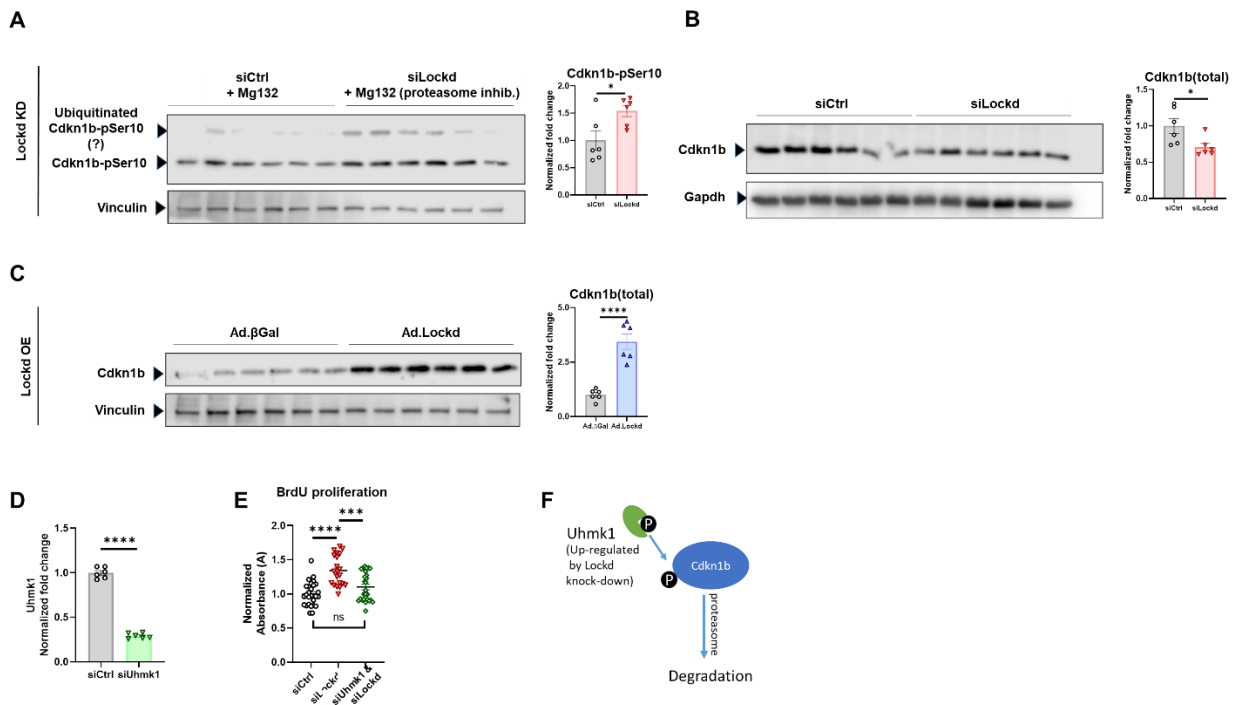


Figure 31: Lockd-mediated regulation of Uhmk1 and Cdkn1b in endothelial cell proliferation

(A) Western blot analysis of phosphoSer10-Cdkn1b and Vinculin (as a normalizer) upon Lockd siRNA knockdown and 4-hour treatment with Mg132 to inhibit proteasomal degradation. (B) Western blot analysis of Cdkn1b and Gapdh (as a normalizer) upon Lockd siRNA knockdown. (C) Western blot analysis of Cdkn1b and Vinculin (as a normalizer) upon Lockd adenovirus overexpression in C166 endothelial cells. (D) The efficiency of siRNA mediated Uhmk1-mRNA knockdown in C166 endothelial cells was assessed using quantitative polymerase chain reaction. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$; ****, $P < 0.0001$. (E) BrdU proliferation assay assessing the impact of combined Lockd and Uhmk1 knockdown on endothelial cell proliferation. Data represents mean and error bars represent SEM. Significance was calculated by One-way ANOVA with ns, $P > 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$. (F) Image representing the regulatory relationship between Lockd, Uhmk1, and Cdkn1b *in vitro*.

5.16 Dynamic expression of Lockd influences the progression of the cell cycle

To investigate the influence of Lockd dynamics on endothelial cell cycle progression, the effect of Lockd knockdown and overexpression on Propidium iodide staining in C166 endothelial cells was examined. Lockd knockdown was achieved through siRNA-mediated silencing, while Lockd overexpression was induced by adenoviral delivery in C166 endothelial cells.

Propidium iodide staining, a widely used technique for assessing the cell cycle stage of proliferating cells, was employed. This staining method exploits the ability of propidium iodide to intercalate with DNA, enabling precise measurement of DNA content and allowing the determination of the relative distribution of cells in different phases of the cell cycle. Subsequently, the resulting fluorescence

intensity was quantified using flow cytometry analysis, providing valuable insights into the cell cycle profile of the investigated proliferating cell population [275].

Upon Lockd knockdown in endothelial cells, a notable increase in the percentage of cells entering the S phase of the cell cycle (Figure 32A) was observed. This effect was observed in both unstimulated cells and those stimulated with FGF2 (Figure 31B), a potent growth factor for endothelial cells [276]. Furthermore, a comparison was conducted between unstimulated siCtrl-transfected cells and FGF2-stimulated siCtrl-transfected cells. Here the effect of FGF2 resulted in more cells in the S-phase of cell cycle (Figure 32C). Conversely, Lockd overexpression led to an elevated number of cells in the G1 phase and a reduced number of cells in the G2/M phase of the cell cycle (Figure 32D). This alteration in cell cycle distribution was consistent in both unstimulated cells and cells stimulated with 25 ng/uL FGF2 for a 24-hour duration (Figure 32E). Additionally, a comparison was made between unstimulated Ad.βGal-infected cells and FGF2-stimulated Ad.βGal-infected cells. The results revealed a notable impact of FGF2 stimulation, leading to an increased proportion of cells in the S-phase of the cell cycle and a reduced number of cells in the G0/G1 phase (Figure 32F).

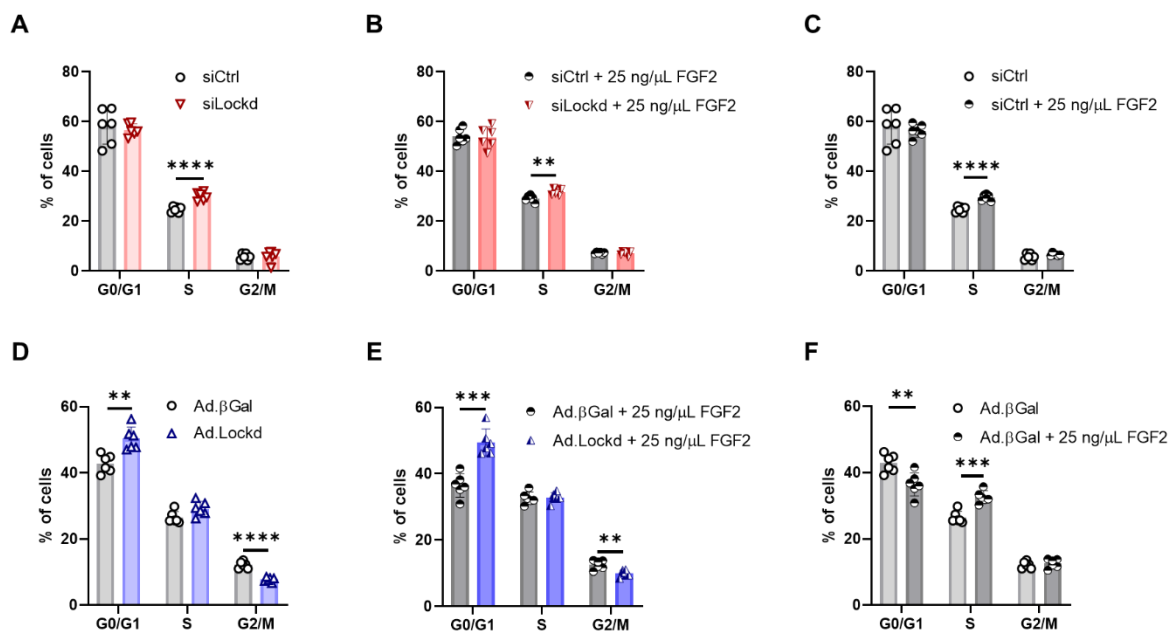


Figure 32: Lockd modulation of endothelial cell cycle progression analyzed by propidium iodide staining and flow cytometry

(A) Propidium iodide staining was performed on siCtrl and siLockd-treated cells to assess the cellular distribution in the cell cycle. The resulting fluorescence intensity was quantified, and the cells were categorized into distinct cell cycle phases based on their DNA content. The distribution of cells in the G0/G1, S, and G2/M phases was determined, and the data were plotted accordingly. (B) Propidium iodide staining was performed on siCtrl and siLockd-treated cells following 24 hours of FGF2 stimulation to assess the cellular distribution in the cell cycle. (C) The distribution of cells in the G0/G1, S, and G2/M phases on untreated siCtrl and siCtrl-treated cells following 24 hours of FGF2 stimulation to assess the cellular distribution in the cell cycle. (D) Propidium iodide staining was performed on Ad.βGal infected and Ad.Lockd-infected cells to assess the cellular distribution in the

cell cycle. The resulting fluorescence intensity was quantified, and the cells were categorized into distinct cell cycle phases based on their DNA content. The distribution of cells in the G0/G1, S, and G2/M phases was determined, and the data were plotted accordingly. (E) Propidium iodide staining was performed on Ad.βGal and Ad.Lockd-infected cells following 24 hours of FGF2 stimulation to assess the cellular distribution in the cell cycle. (F) The distribution of cells in the G0/G1, S, and G2/M phases on untreated Ad.βGal and Ad.βGal-infected cells following 24 hours of FGF2 stimulation to assess the cellular distribution in the cell cycle. Data represents mean and error bars represent SEM. Significance was calculated by multiple t-test and statistical significance determined using the Holm-Sidak method with **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

During the propidium iodide sorting procedure, events that were smaller than the G1 phase were also quantified. These events are commonly associated with apoptotic processes and generated by apoptotic bodies. No discernible differences in the percentage of apoptotic events were detected in siRNA-treated cells (Figure 33A). Strikingly, Lockd overexpressing cells exhibited a significantly reduced percentage of apoptotic events compared to cells infected with Ad.βGal (Figure 33B).

Taken together, these findings suggest that Lockd serves as a negative regulator of the cell cycle, favouring cellular quiescence in the G1 phase while potentially inhibiting apoptosis.

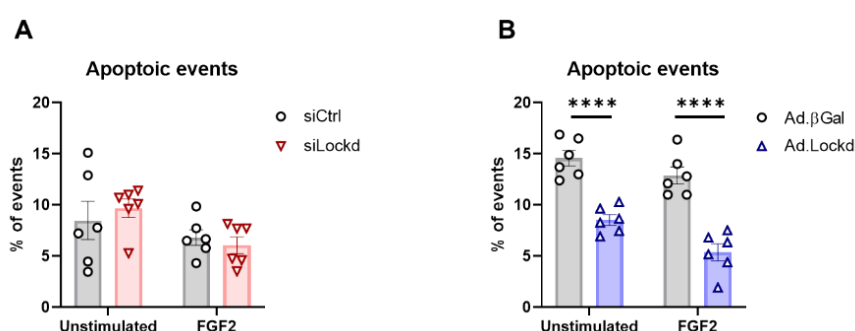


Figure 33: Quantification of apoptotic events cells using propidium iodide staining

(A) The percentage of apoptotic events (events smaller than the G1 phase) in siRNA-treated cells. (B) The percentage of apoptotic events (events smaller than the G1 phase) in Adenovirus treated cells. Data represents mean and error bars represent SEM. Significance was calculated by multiple t-test and statistical significance determined using the Holm-Sidak method with ****, $P < 0.0001$.

5.17 Lockd confers protection against apoptosis

To investigate the potential impact of Lockd on apoptosis, the Caspase3/7 assay was employed, a well-established technique for assessing the activation of caspase-3 and caspase-7, key executioner caspases involved in the apoptotic process [277]. By utilizing a fluorogenic substrate specifically

cleaved by active caspase-3 and caspase-7, the presence of apoptotic cells was detected and quantified based on the fluorescence intensity emitted by the cleaved substrate. This assay provided valuable insights into the extent of apoptosis occurring within the investigated cell population.

In the experimental setup, siRNA Lockd knock-down cells and adenoviral-infected Lockd overexpression C166-cells were treated with 800 μ M H_2O_2 for a duration of 4 hours in serum-free media, known to induce apoptosis [278, 279]. A significant increase in Caspase3/7 activity in the Lockd knockdown cells was observed, suggesting a higher propensity for apoptosis in the absence of Lockd (Figure 34A). Conversely, a trend towards reduced Caspase3/7 activity in the Lockd overexpressing cells was observed, indicating a potential role for Lockd in the inhibition of apoptosis (Figure 34B).

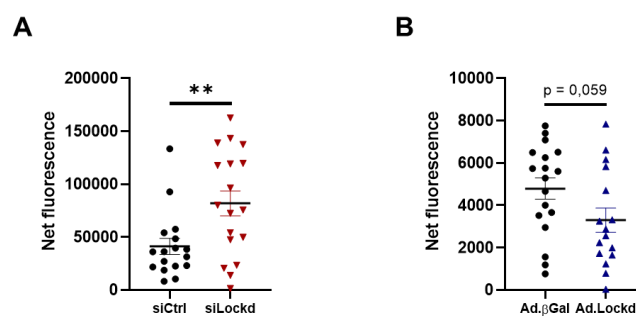


Figure 34: Caspase3/7 activity in Lockd knockdown and Lockd overexpression cells treated with H_2O_2 -induced apoptosis

(A) Net fluorescence measurement of Caspase 3/7 activity in the Lockd knock-down cells compared to control cells. (B) Caspase3/7 activity quantification in Lockd overexpressing cells compared to cells infected with Ad.βGal. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with **, $P < 0.01$.

5.18 Lockd knockdown has no impact on heart function under normal cardiac conditions

The subsequent objective of the study was to investigate the impact of Lockd knock-down on heart function in an *in vivo* setting. To accomplish this, Antisense LNA GapmeRs were employed as a methodological approach. Antisense LNA GapmeRs are single-stranded oligonucleotides specifically designed for *in vivo* silencing of both lncRNA and mRNA targets. These oligonucleotides possess modified 3' and 5' ends, conferring protection against nucleases. The binding of GapmeRs to their respective RNA targets leads to RNaseH-mediated cleavage and subsequent degradation of the targeted RNA molecules [280, 281].

In the experimental design, Lockd GapmeRs and Control GapmeRs were administered to 9-week-old BL6 mice via subcutaneous injection. A total of four injections were administered over a period of two

weeks, with a dosage of 5 mg per kg of body weight per injection. Subsequently, a comprehensive assessment of heart function was conducted (Figure 35A).

Two days after the final injection, the mice were euthanized, and organ isolation was performed. Notably, there were no significant differences in the body weight of the mice between the Lockd and Control GapmeR-injected groups (Figure 35B). Moreover, under normal heart conditions without induced cardiac stress, no significant variations were observed in the heart-to-body weight ratio or lung-to-body weight ratio between the Lockd GapmeR-injected mice and the Control GapmeR-injected mice (Figure 35C).

To evaluate the efficacy of Lockd knock-down in the heart tissue (Figure 35D), including cardiac endothelial cells (Figure 35E) and cardiomyocytes (Figure 35F), quantitative PCR was employed to quantify Lockd expression levels. Efficient knock-down of Lockd was achieved in these specific cardiac cell populations. In addition to the heart, Lockd knockdown was also assessed in Lung and Liver endothelial cells. Interestingly, no knockdown of Lockd could be detected in lung endothelial cells (Figure 35G). On the other hand, a very efficient knockdown was quantifiable in liver endothelial cells (Figure 35H). However, in whole liver tissue, no knockdown of Lockd was achieved (Figure 35I), which could be explained by the already very low Lockd expression in the Liver (Ct Values > 35).

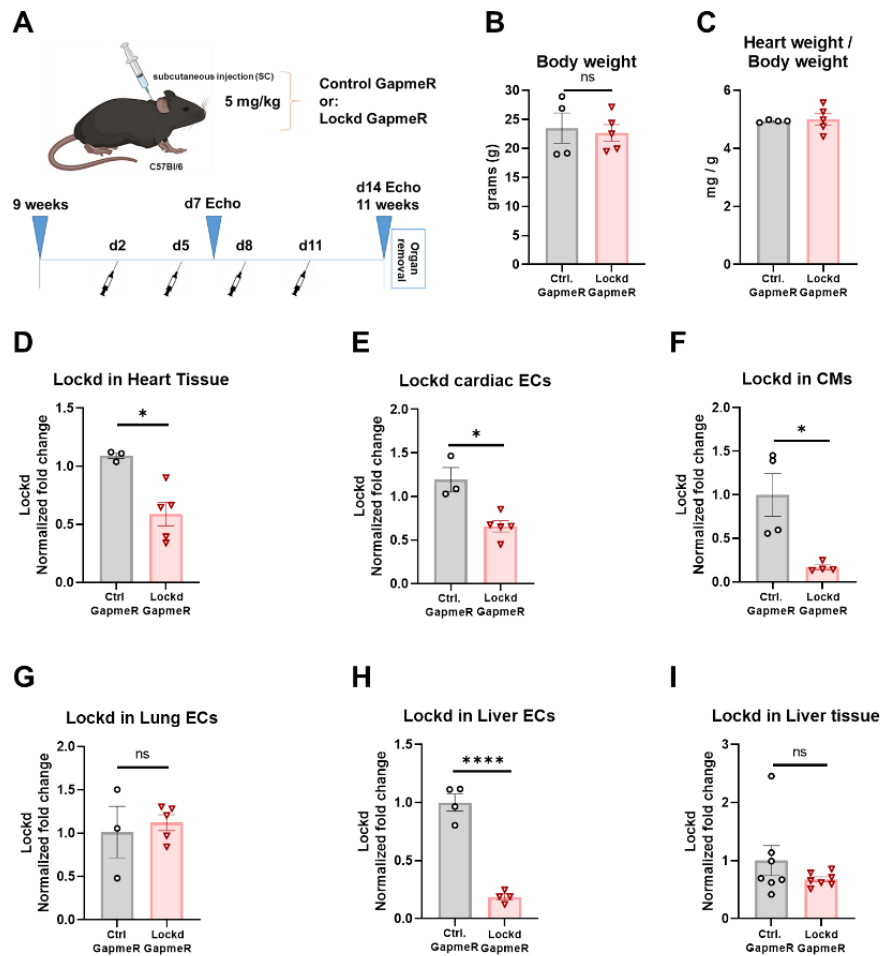


Figure 35: *In vivo* assessment of Lockd knockdown and its impact on heart function

(A) Experimental design for Lockd knock-down in mice using GapmeRs. (B) Body weight analysis of mice injected with Lockd GapmeRs or Control GapmeRs. (C) Analysis of heart-to-body weight ratio in Lockd GapmeR-injected mice and Control GapmeR-injected mice under normal heart conditions without induced cardiac stress. (D) Quantitative PCR analysis of Lockd expression levels in heart tissue. (E) Quantitative PCR analysis of Lockd expression levels in cardiac endothelial cells. (F) Quantitative PCR analysis of Lockd expression levels in cardiomyocytes. (G) Quantitative PCR analysis of Lockd expression levels in Lung endothelial cells. (H) Quantitative PCR analysis of Lockd expression levels in liver endothelial cells. Quantitative PCR analysis of Lockd expression levels in whole liver tissue. qPCR data was normalised to the house keeping gene Hprt. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with ns, $P > 0.05$; *, $P < 0.05$; ****, $P < 0.0001$.

Furthermore, heart function was assessed at one-week and two weeks following GapmeR injections. (Figure 36A). During measurements no significant changes were detected regarding heart rate (beats per minutes) (Figure 36B). No significant changes were detected in ejection fractions (Figure 36C), left ventricular wall thickness (Figure 36D), and end-diastolic volume (Figure 36E). No significant differences in heart function were observed between the Lockd GapmeR-injected mice and the Control GapmeR-injected mice at both time points. These results indicate that Lockd knock-down,

administered under normal physiological conditions, did not result in notable alterations in heart function.

Collectively, the *in vivo* experimental approach utilizing Antisense LNA GapmeRs facilitated efficient knock-down of Lockd expression in the heart tissue, encompassing cardiac endothelial cells and cardiomyocytes. No significant changes in body weight, heart-to-body weight ratio, lung-to-body weight ratio, or heart function were observed following Lockd knock-down, thus indicating that Lockd does not exert a substantial influence on heart function under these specific conditions.

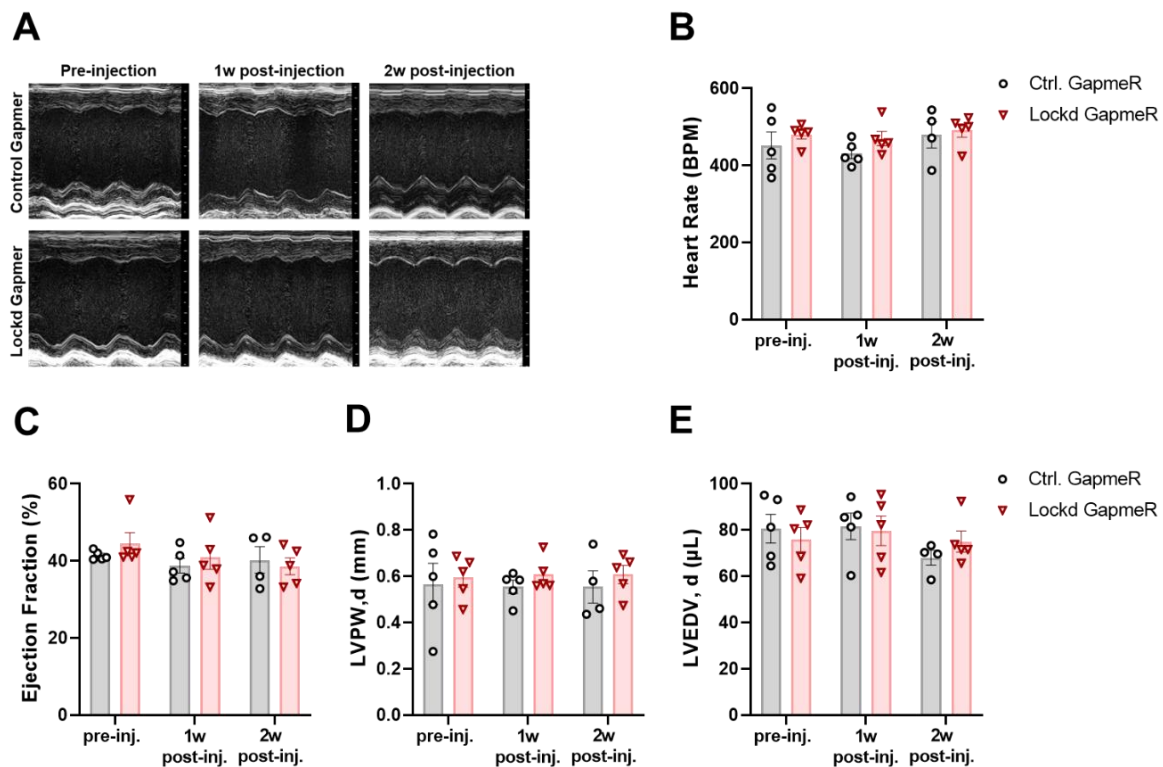


Figure 36: Assessment of heart function following Lockd knock-down using GapmeRs

(A) Experimental timeline and assessment of heart function parameters after Lockd GapmeR or Control GapmeR injections. (B) Analysis of beats per minute (BPM) in Lockd GapmeR-injected mice and Control GapmeR-injected mice. (C) Evaluation of ejection fraction, a measure of cardiac performance, in Lockd GapmeR-injected mice and Control GapmeR-injected mice. (D) Measurement of left ventricular wall thickness in Lockd GapmeR-injected mice and Control GapmeR-injected mice. (E) Assessment of end-diastolic volume, a parameter reflecting the filling capacity of the left ventricle, in Lockd GapmeR-injected mice and Control GapmeR-injected mice. Data represents mean and error bars represent SEM.

5.19 Lockd knockdown post-TAC improves systolic heart function

Considering the significant upregulation of Lockd in the heart tissue, particularly in endothelial cells during the early phase of cardiac remodelling following TAC, further investigation aimed to understand the consequences of inhibiting this upregulation following induced pressure overload. To achieve this, transverse aortic constriction was performed on 9-week-old BL6 mice and subsequently administered four injections of Lockd GapmeRs over a period of two weeks (Figure 37A).

Verification of the surgically induced cardiac pressure overload was confirmed by assessing the flow in the right and left common carotid arteries before and after the surgery, which validated the effectiveness of the TAC procedure in all operated mice (Figure 37B).

Evaluation of Lockd expression in the heart tissue revealed a highly efficient knock-down of Lockd post-TAC of 70 % (Figure 37C). Interestingly, the impact of Lockd GapmeRs on cardiac fibroblasts appeared negligible (Figure 37D), suggesting their resistance to the inhibitory effects. However, in cardiac endothelial cells, Lockd expression was downregulated by nearly 50% two weeks after TAC, similar to that of un-operated mice after GapmeR injections (Figure 37E).

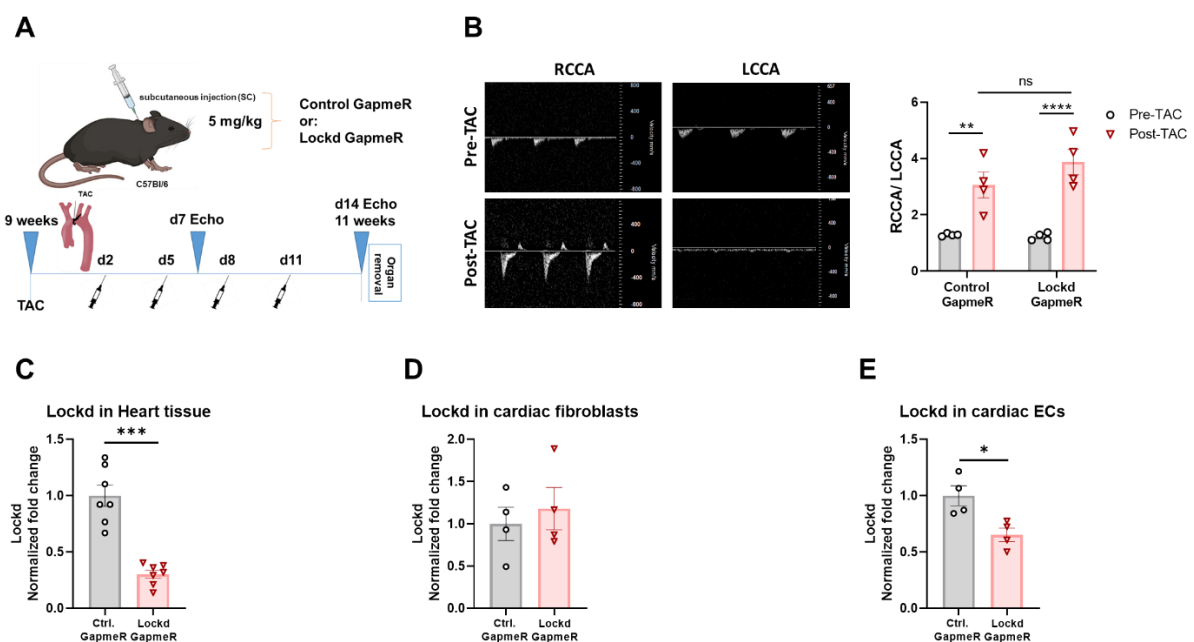


Figure 37: Impact of Lockd knock-down on cardiac remodelling following induced pressure overload

(A) Experimental timeline depicting the transverse aortic constriction (TAC) procedure and subsequent administration of GapmeRs. TAC was performed on 9-week-old BL6 mice, followed by four injections of 5 mg/kg GapmeRs over a two-week period. (B) Assessment of flow in the right and left common carotid arteries before and after TAC surgery, confirming the induction of cardiac pressure overload in all operated mice. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, One-way ANOVA with ns, $P > 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

0.0001. (C) qPCR quantification of Lockd expression in the heart tissue, revealing efficient knock-down of Lockd post-TAC compared to control. (D) Analysis of Lockd expression in cardiac fibroblasts post-TAC, showing no significant impact of Lockd GapmeRs. (E) Analysis of Lockd expression in cardiac endothelial cells post-TAC, showing a significant knockdown of Lockd upon GapmeR injections. qPCR data was normalised to the house keeping gene Hprt. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$.

Analysis of the body weights of the mice two weeks post-TAC indicated no significant differences among Lockd knockdown and control groups (Figure 38A). However, the mice treated with Lockd GapmeRs exhibited a reduced heart-to-body weight ratio compared to the control mice. This reduction suggests a mitigated cardiac hypertrophy in the Lockd GapmeR group compared to the control group (Figure 38B). Conversely, the lung weight-to-body weight ratio was unaffected by GapmeR administration, indicating that Lockd GapmeRs did not influence lung congestion (Figure 38C), nor did it influence liver weight of the mice (Figure 38D).

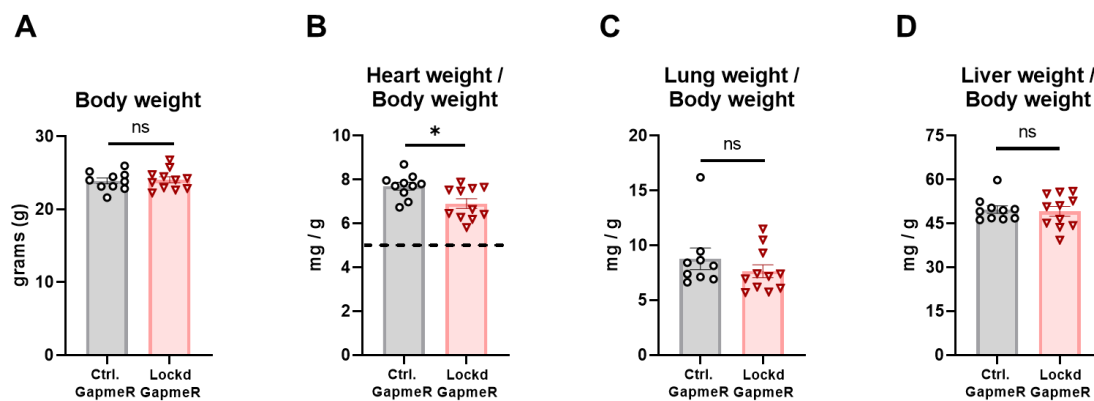


Figure 38: Effects of Lockd GapmeRs on body biometrics following 2 weeks of induced cardiac pressure overload

(A) Body weight measurements of mice two weeks post-TAC, showing comparable weights between the groups treated with Lockd GapmeRs and the control group. (B) Assessment of the heart-to-body weight ratio. (C) Evaluation of the lung weight-to-body weight ratio. (D) Analysis of the liver weight-to-body weight ratio. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with ns, $P > 0.05$ and *, $P < 0.05$.

Assessment of heart function through echocardiography demonstrated that the mice injected with Lockd GapmeRs displayed significantly improved systolic heart function following TAC (Figure 39A), as evidenced by increased ejection fraction (Figure 39B). Moreover, these mice exhibited a decreased left ventricular wall thickness, indicating a reduced propensity for hypertrophy (Figure 39C). These findings were consistent with the observed reduction in heart-to-body weight ratio in the Lockd GapmeR group (Figure 39B). No significant differences in left ventricular end-diastolic volume were observed between the two groups (Figure 39D).

Furthermore, the expression levels of select hypertrophic markers like Nppa, Nppb and Myosin heavy chains were examined. The Lockd GapmeR mice displayed lower expression of Nppa and Nppb compared to the Ctrl GapmeR mice (Figure 39E). Additionally, the ratio of Myosin heavy chain 7 to Myosin heavy chain 6 was also lower in the Lockd GapmeR group than in the control group two weeks after TAC, further indicating a diminished hypertrophic response (Figure 39F).

Overall, the results suggest that inhibiting Lockd upregulation post-TAC leads to a reduction in Lockd expression, particularly in cardiac endothelial cells. This reduction is associated with a decreased hypertrophic response and an improved systolic heart function. These findings highlight the potential role of Lockd in cardiac remodelling.

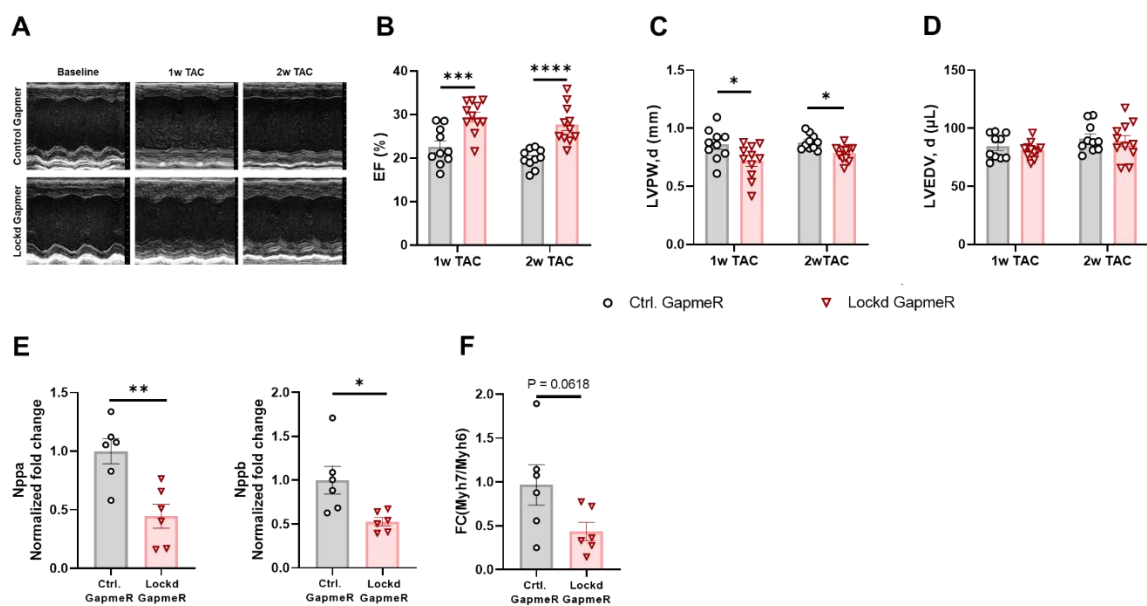


Figure 39: Effects of Lockd knockdown on cardiac function and hypertrophy markers following induced pressure overload

(A) Experimental timeline and assessment of heart function parameters after induced cardiac pressure overload and Lockd GapmeR or Control GapmeR injections. (B) Quantification of systolic heart function by ejection fraction one- and two-weeks post transverse aortic constriction. (C) Evaluation of left ventricular wall thickness, in the Lockd GapmeR group compared to the control group. (D) Assessment of left ventricular end-diastolic volume. Data represents mean and error bars represent SEM. Significance was calculated by One-Way ANOVA with *, $P < 0.05$; ***, $P < 0.001$ and ****, $P < 0.0001$. (E) Quantitative analysis of Nppa and Nppb expression levels. (F) Analysis of the ratio of Myosin heavy chain 7 mRNA to Myosin heavy chain 6 mRNA ratio in the Lockd GapmeR group compared to the control group. qPCR data was normalised to the house keeping gene Hprt. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$ and **, $P < 0.01$.

5.20 Lockd knock-down post-TAC increases capillary density and reduces cardiomyocyte hypertrophy

Fibrosis is a critical pathological process associated with various cardiovascular diseases, including cardiac hypertrophy. Given the potential role of Lockd in cardiac remodelling and its implication in endothelial function, its impact on fibrosis development was investigated. Therefore, fibrotic changes in the Lockd knockdown mice two weeks after transverse aortic constriction were assessed.

Following Sirius red staining of heart tissues, a trend towards decreased fibrosis was observed in the Lockd-GapmeR mice compared to the control-GapmeR mice, two weeks after TAC induction (Figure 40A). A similar trend was observed Col1a1 immuno-staining on whole heart tissue sections (Figure 40B). However, qPCR analysis of whole heart tissue demonstrated a decrease in Col1a1 transcription in the Lockd knock-down hearts (Figure 40C).

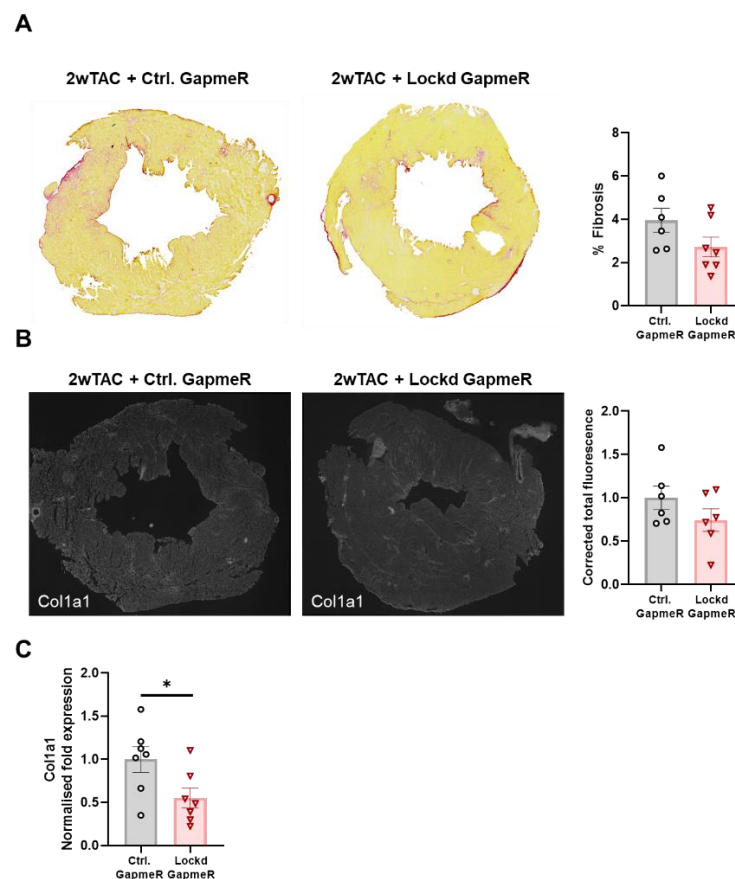


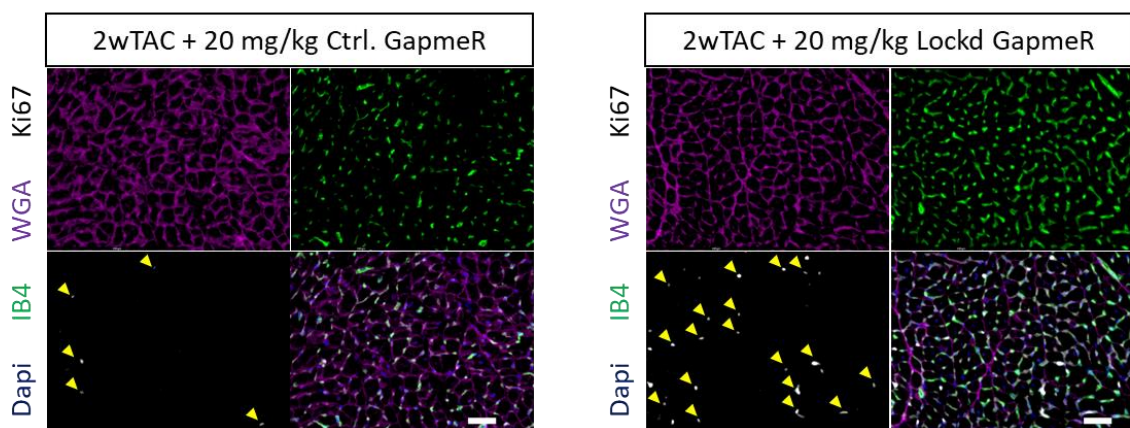
Figure 40: Effects of Lockd GapmeRs on cardiac fibrosis following induced pressure overload

(A) Sirius red staining of heart tissue sections, showing the percentage area of fibrosis in the Lockd GapmeR group compared to the control-GapmeR group. (B) Col1a1 immuno-staining of whole heart tissue sections, showing collagen I deposition in the Lockd-GapmeR group compared to the control-GapmeR group. (C) Quantitative analysis of Col1a1 gene expression levels in whole heart tissue, showing expression in the Lockd-GapmeR group compared to the control-GapmeR group. qPCR data was normalised to the house keeping gene Hprt. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$.

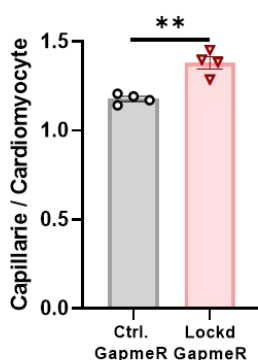
Capillary density and cardiomyocyte hypertrophy were evaluated through fluorescence staining using Wheat germ agglutinin (WGA), used to label cell membranes and Isolectin B4 (IB4) as a marker for endothelial cells in response to Lockd knockdown. Ki67 staining was employed to assess the proliferation ratio of endothelial cells within the capillaries (Figure 41A). The findings revealed that Lockd knockdown mice exhibited a significantly higher capillary density per cardiomyocyte (Figure 41B) and an increased ratio of Ki67-positive proliferating capillaries (Figure 41C). Importantly, a reduction in cross-sectional area per cardiomyocyte was observed in the Lockd knockdown mice, indicating protection against cardiomyocyte hypertrophy (Figure 41D).

Collectively, these results suggest that the improved systolic function observed in the Lockd knockdown mice during cardiac pressure overload may be attributed to a combination of increased capillary density and reduced hypertrophy. The enhanced capillary network likely contributes to improved myocardial perfusion, while the reduction in cardiomyocyte hypertrophy may preserve cardiac function and prevent adverse remodeling. This highlights the potential importance of Lockd as a therapeutic target to modulate cardiac vascularization and remodeling in the context of cardiac stress and dysfunction.

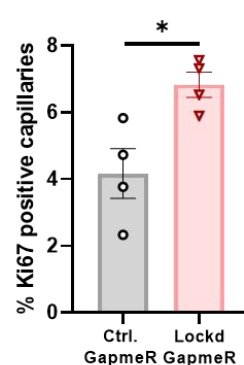
A



B



C



D

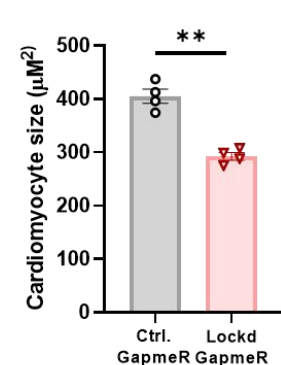


Figure 41: Effects of Lockd knockdown on capillary density and cardiomyocyte hypertrophy

(A) Immunofluorescence staining of heart tissue sections 2 weeks post TAC using WGA, IB4 and Ki67. Scale bar = 50 μ M. (B) Quantification of capillary density per cardiomyocyte in Lockd knockdown mice compared to control mice. (C) Immunofluorescence staining of heart tissue sections using Ki67, showing the ratio of Ki67-positive proliferating capillaries in the Lockd knockdown mice compared to control mice. (D) Immunofluorescence staining of heart tissue sections using WGA, revealing quantified cross-sectional area per cardiomyocyte in Lockd knockdown mice compared to control mice. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$ and ** $P < 0.01$.

5.21 Differentially regulated genes in cardiac remodelling revealed by Lockd knockdown

To gain a comprehensive understanding of the biological processes effected by Lockd, bulk RNA sequencing from whole heart tissue and isolated cardiac endothelial cells two weeks after transverse aortic constriction were conducted. The comparison between control and Lockd knockdown mice revealed numerous differentially regulated genes in both the global heart tissue (Figure 42A and Figure 42B) and cardiac endothelial cells.

In the whole heart tissue, a robust GapmeR induced knockdown of Lockd by 70% was confirmed two weeks after TAC, validating the effectiveness of the knockdown on the tissue level (Figure 37C). Gene ontology analysis of the up-regulated genes ($p\text{-Value} < 0.05$) highlighted the most enriched GO term as glycerolipid catabolic process. Notably, other highly enriched GO terms included regulation of angiogenesis. Conversely, the downregulated genes ($p\text{-Value} < 0.05$) were associated with muscle structure development, as well as genes involved in extracellular matrix organization (Figure 42C).

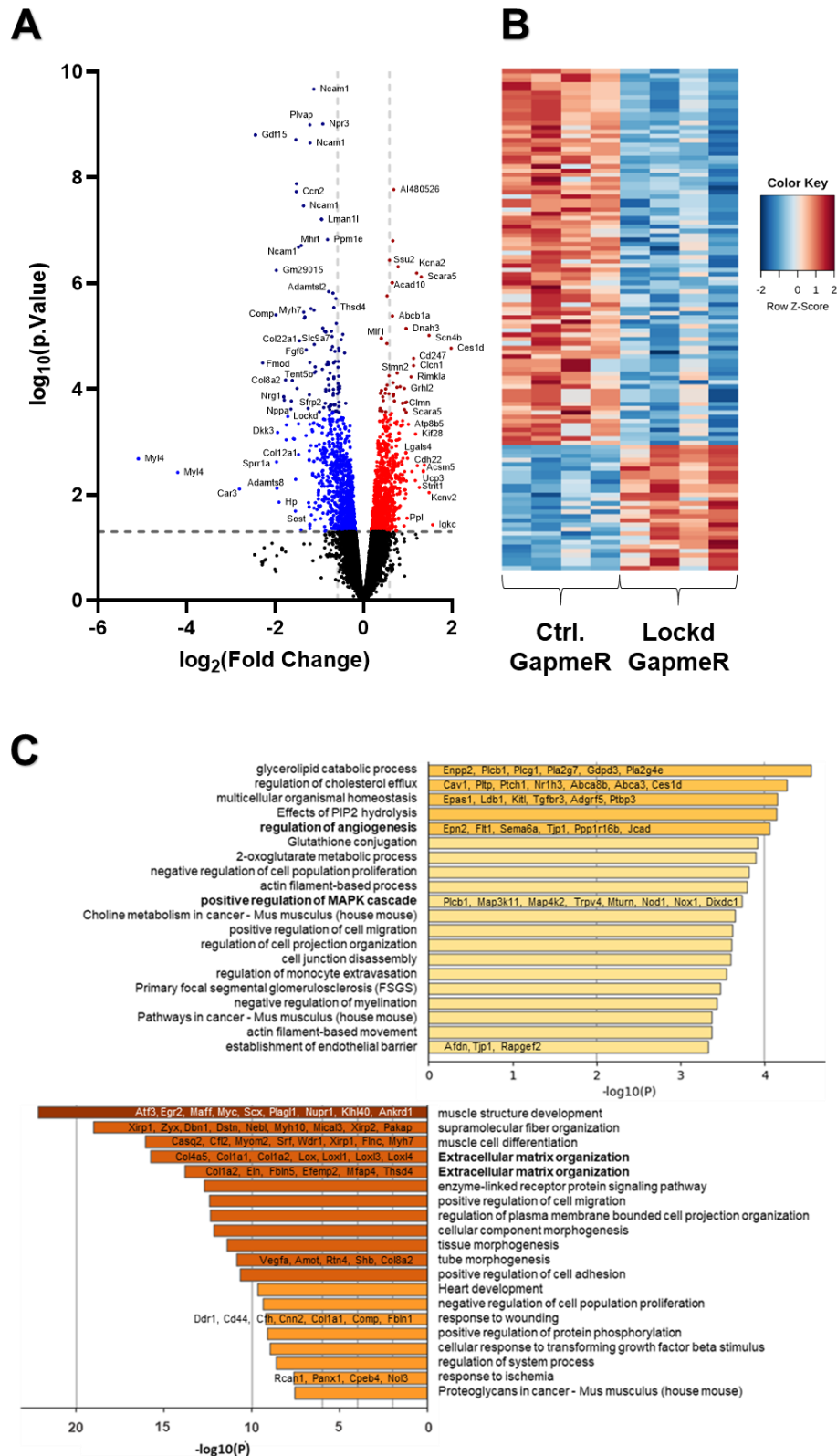


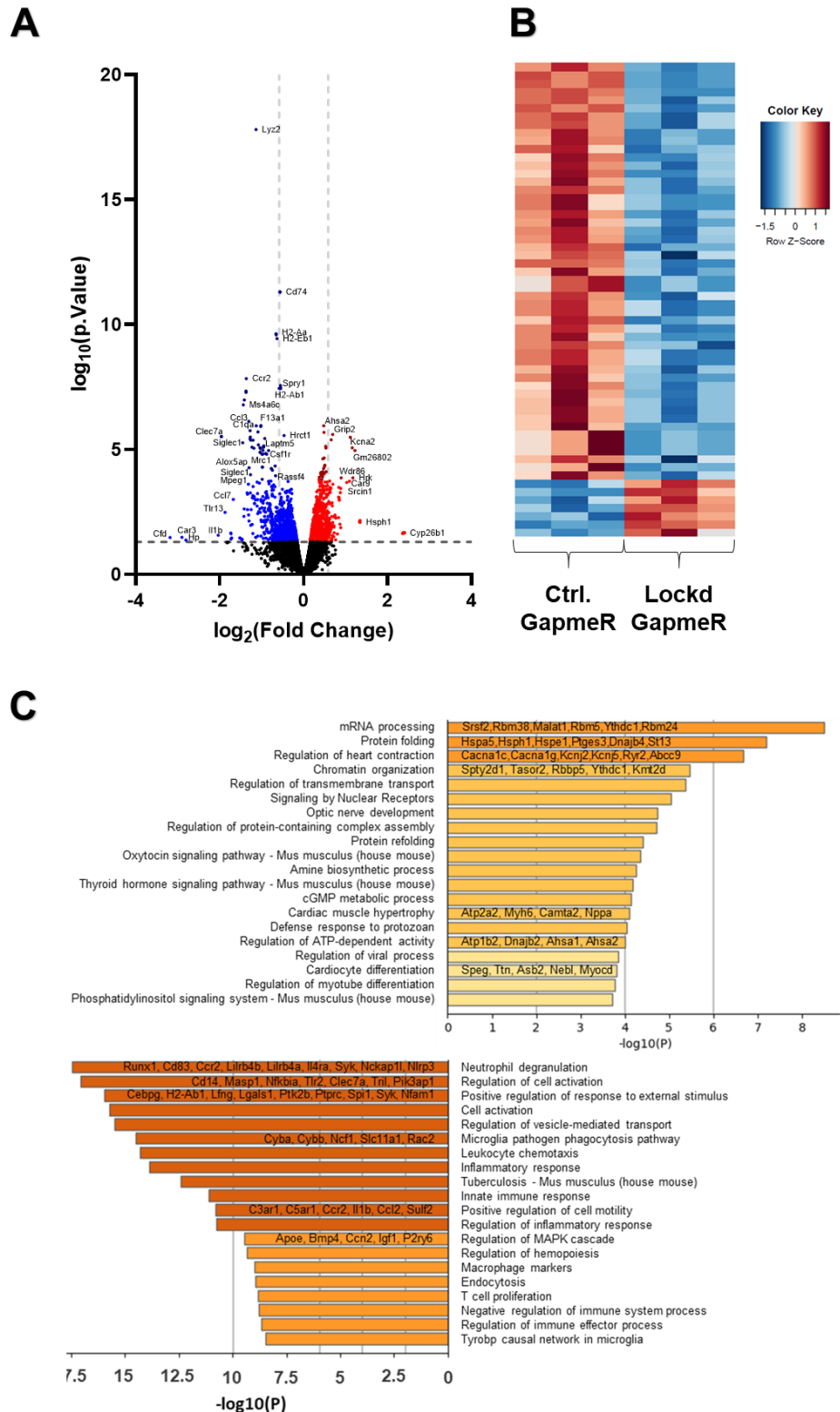
Figure 42: Transcriptomic analysis of Lockd knockdown in whole heart tissue after 2 weeks after transverse aortic constriction

(A) Volcano plot depicting the results of bulk RNA-seq analysis comparing gene expression profiles in whole heart tissue two weeks after transverse aortic constriction (TAC) between control and Lockd knockdown mice. The log₂ fold change (log₂FC) of each gene is plotted on the x-axis against

the negative log₁₀ of the p-value on the y-axis. Red dots represent genes that are significantly upregulated in Lockd knockdown samples ($\log_2\text{FC} > 0$, p-value < 0.05), while blue dots represent genes that are significantly downregulated ($\log_2\text{FC} < 0$, p-value < 0.05). The volcano plot highlights the differentially regulated genes and their statistical significance. (B) Heatmap displaying the expression patterns of differentially regulated genes in whole heart tissue two weeks after TAC between control and Lockd knockdown mice. Genes with altered expression levels ($\log_2\text{FC} > 0.6$ or < -0.6 , FDR < 0.05) are represented in rows, and samples from different experimental conditions are depicted in columns. Red and blue colours indicate upregulated and downregulated genes, respectively, while white represents no change in gene expression. (C) Gene Ontology (GO) analysis results for the upregulated genes and downregulated genes in whole heart tissue upon Lockd knockdown. The bar graph presents the enrichment score ($\log_{10}$ p-value) for each GO term. (B) Volcano plot depicting the results of bulk RNA-seq analysis comparing gene expression profiles in cardiac ECs two weeks after transverse aortic constriction (TAC) between control and Lockd knockdown mice. The $\log_2$ fold change ($\log_2\text{FC}$) of each gene is plotted on the x-axis against the negative $\log_{10}$ of the p-value on the y-axis. Red dots represent genes that are significantly upregulated in Lockd knockdown samples ($\log_2\text{FC} > 0$, p-value < 0.05), while blue dots represent genes that are significantly downregulated ($\log_2\text{FC} < 0$, p-value < 0.05). The volcano plot highlights the differentially regulated genes and their statistical significance. Heatmap displaying the expression patterns of differentially regulated genes in cardiac ECs two weeks after TAC between control and Lockd knockdown mice. Genes with altered expression levels ($\log_2\text{FC} > 0.6$ or < -0.6 , FDR < 0.05) are represented in rows, and samples from different experimental conditions are depicted in columns. Red and blue colours indicate upregulated and downregulated genes, respectively, while white represents no change in gene expression. Gene Ontology (GO) analysis results for the upregulated genes and downregulated genes in cardiac ECs upon Lockd knockdown. The bar graph presents the enrichment score ($\log_{10}$ p-value) for each GO term.

In contrast, in cardiac endothelial cells two weeks after TAC, the Lockd knockdown did not yield as many differentially regulated genes compared to the whole heart tissue (Figure 43A and Figure 43B). (2221 genes in cardiac endothelial cells and 2813 in the whole heart tissue). Gene ontology analysis in cardiac endothelial cells revealed the most up-regulated genes to be associated with mRNA processing, protein folding, and interestingly, heart contraction. The most significantly up-regulated gene was Cyp26b1. On the other hand, the most down-regulated genes were associated with processes such as neutrophil degranulation and cell activation, with Cfd and Car3 being the most prominently down-regulated genes (Figure 43C).

These findings indicate that Lockd knockdown leads to significant changes in gene expression profiles in both whole heart tissue and cardiac endothelial cells following TAC. The enrichment of GO terms related to glycerolipid catabolic process and regulation of angiogenesis in the whole heart tissue suggests Lockd's involvement in metabolic processes and angiogenic responses during cardiac remodeling. In cardiac endothelial cells, Lockd knockdown seems to impact mRNA processing, protein folding, and heart contraction, while potentially affecting neutrophil degranulation and cell activation.



upregulated in Lockd knockdown samples ($\log_2\text{FC} > 0$, $p\text{-value} < 0.05$), while blue dots represent genes that are significantly downregulated ($\log_2\text{FC} < 0$, $p\text{-value} < 0.05$). The volcano plot highlights the differentially regulated genes and their statistical significance. (B) Heatmap displaying the expression patterns of differentially regulated genes in cardiac endothelial cells two weeks after TAC between control and Lockd knockdown mice. Genes with altered expression levels ($\log_2\text{FC} > 0.6$ or < -0.6 , $\text{FDR} < 0.05$) are represented in rows, and samples from different experimental conditions are depicted in columns. Red and blue colours indicate upregulated and downregulated genes, respectively, while white represents no change in gene expression. (C) Gene Ontology (GO) analysis results for the upregulated genes and downregulated genes in endothelial cells upon Lockd knockdown. The bar graph presents the enrichment score ($\log_{10} p\text{-value}$) for each GO term. (B) Volcano plot depicting the results of bulk RNA-seq analysis comparing gene expression profiles in cardiac ECs two weeks after transverse aortic constriction (TAC) between control and Lockd knockdown mice. The $\log_2$ fold change ($\log_2\text{FC}$) of each gene is plotted on the x-axis against the negative $\log_{10}$ of the p-value on the y-axis. Red dots represent genes that are significantly upregulated in Lockd knockdown samples ($\log_2\text{FC} > 0$, $p\text{-value} < 0.05$), while blue dots represent genes that are significantly downregulated ($\log_2\text{FC} < 0$, $p\text{-value} < 0.05$). The volcano plot highlights the differentially regulated genes and their statistical significance. Heatmap displaying the expression patterns of differentially regulated genes in cardiac ECs two weeks after TAC between control and Lockd knockdown mice. Genes with altered expression levels ($\log_2\text{FC} > 0.6$ or < -0.6 , $\text{FDR} < 0.05$) are represented in rows, and samples from different experimental conditions are depicted in columns. Red and blue colours indicate upregulated and downregulated genes, respectively, while white represents no change in gene expression. Gene Ontology (GO) analysis results for the upregulated genes and downregulated genes in cardiac ECs upon Lockd knockdown. The bar graph presents the enrichment score ($\log_{10} p\text{-value}$) for each GO term.

5.22 Early Lockd knockdown improves cardiac response to pressure overload at one-week after transverse aortic constriction

Due to the significantly improved systolic heart function observed in Lockd mice one-week after TAC surgery, the aim was to determine if a similar phenotype, observed at the two-week TAC time-point, could be identified at the one-week TAC time-point. Additionally, given that Lockd exhibits its highest expression in the heart tissue and cardiac endothelial cells at this time, it was crucial to ascertain if the GapmeRs efficiently knocked down Lockd, considering its peak expression and potential impact on the heart.

To assess this, subcutaneous injections of GapmeRs were administered to the mice. The dosing schedule included three injections: the first dose of 5 mg/kg was administered one day after the TAC surgery, followed by the second and third injections of 7.5 mg/kg at the 3rd and 5th days after TAC surgery, respectively. This dosing regimen totalled 20 mg/kg GapmeRs, consistent with the dosage administered to the two-week TAC cohorts. One-week after TAC surgery, the mice were sacrificed for organ removal and further analysis (Figure 44A).

At the one-week time point after transverse aortic constriction, there was no significant difference in body weight between the mice injected with Lockd GapmeRs and the mice injected with Control

GapmeRs (Figure 44B). However, intriguingly, at this early stage post-TAC, there was also a notable reduction in the heart-weight to body-weight ratio in the Lockd knockdown group compared to the control group as in the 2 week stage post-TAC (Figure 44C). This observation suggests that Lockd knockdown at this early time-point may already have a beneficial impact on the heart's response to pressure overload-induced stress.

Furthermore, there was also a trend towards a reduced lung-weight to body-weight ratio in the Lockd knockdown mice at one-week after TAC (Figure 44D).

qPCT analysis, showed an efficient knockdown of Lockd in cardiac endothelial cells, approximately 50% (Figure 43E), while the knockdown in the whole heart tissue was around 30% (Figure 44F). Interestingly also at one-week post GapmeR injections, Lockd was not knock down in cardiac fibroblasts (Figure 44G).

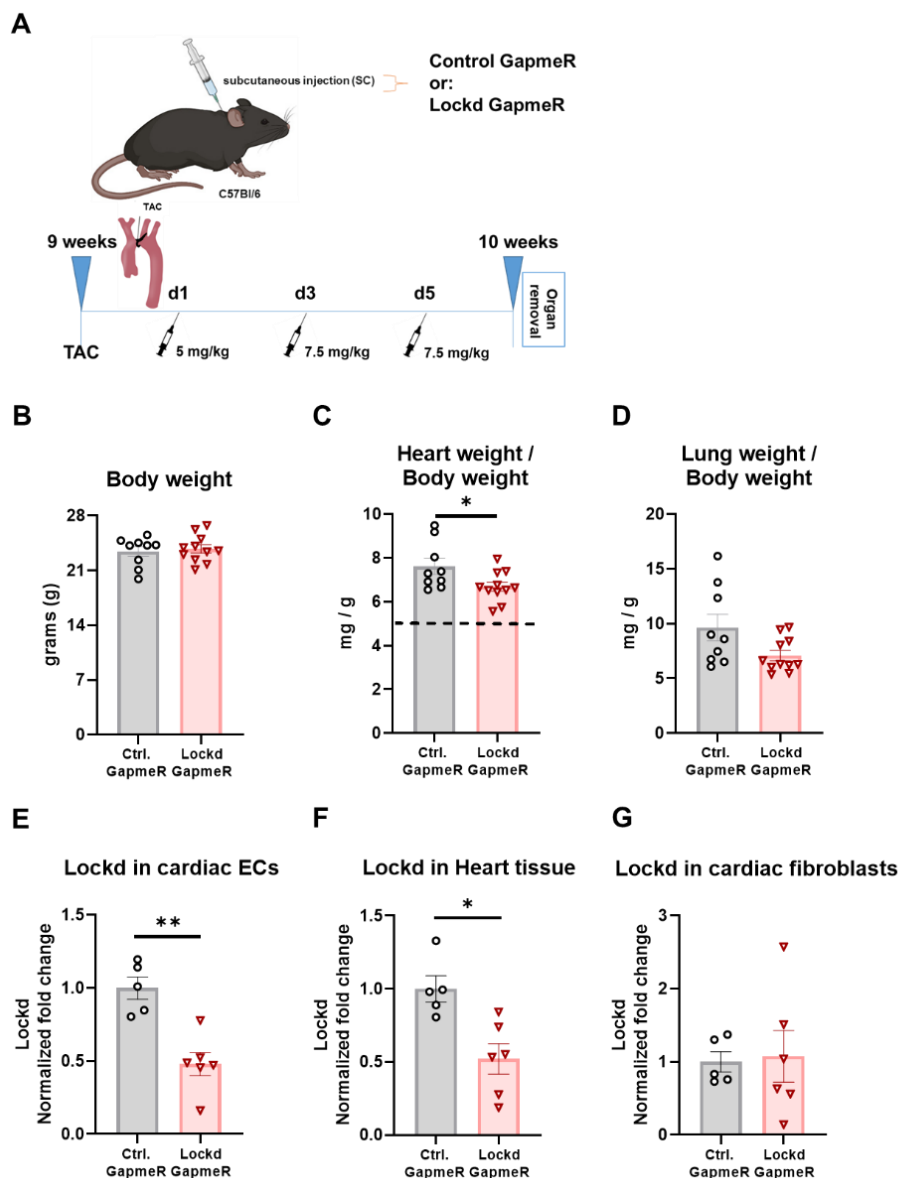


Figure 44: Effects of Lockd knockdown one week after induced cardiac pressure overload

(A) Experimental timeline depicting the transverse aortic constriction (TAC) procedure and subsequent administration of GapmeRs. TAC was performed on 9-week-old BL6 mice, followed by three injections of GapmeRs over a one-week period. (B) Body weight measurements of mice two weeks post-TAC, showing comparable weights between the groups treated with Lockd GapmeRs and the control group. (C) Assessment of the heart-to-body weight ratio. (D) Evaluation of the lung weight-to-body weight ratio. (E) Analysis of the liver weight-to-body weight ratio. (F) Analysis of Lockd expression in cardiac endothelial cells post-TAC, showing a significant knockdown of Lockd upon GapmeR injections. (G) qPCR quantification of Lockd expression in the heart tissue, revealing efficient knock-down of Lockd post-TAC compared to control. (H) Analysis of Lockd expression in cardiac fibroblasts post-TAC, showing no significant impact of Lockd GapmeRs. Significance was calculated by unpaired, two-tailed t-test with ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$.

5.23 Early Effects of Lockd Knockdown on Capillary Density and Cardiomyocyte

Hypertrophy in Pressure Overloaded Hearts

At the one-week post TAC time-point WGA, IB4 and Ki67 staining was also conducted (Figure 46A). Very interestingly, at this early time-point an increased capillary density was visible (Figure 46B), and more proliferating Ki67 positive capillaries than in the 2wTAC group for both the control and the Lockd knockdown groups, however, there was no difference between the Lockd knockdown and the control hearts at the 1w TAC time-point (Figure 45C). At the one-week TAC time-point, reduced hypertrophy of cardiomyocytes could already be quantified. (Figure 45D).

At the one-week time point after transverse aortic constriction my investigation did not reveal any significant difference in the percentage of fibrosis between the Lockd knockdown group and the control group (Figure 45E). This finding suggests that, at this early stage, Lockd knockdown does not exert a significant effect on the development of fibrosis in the heart.

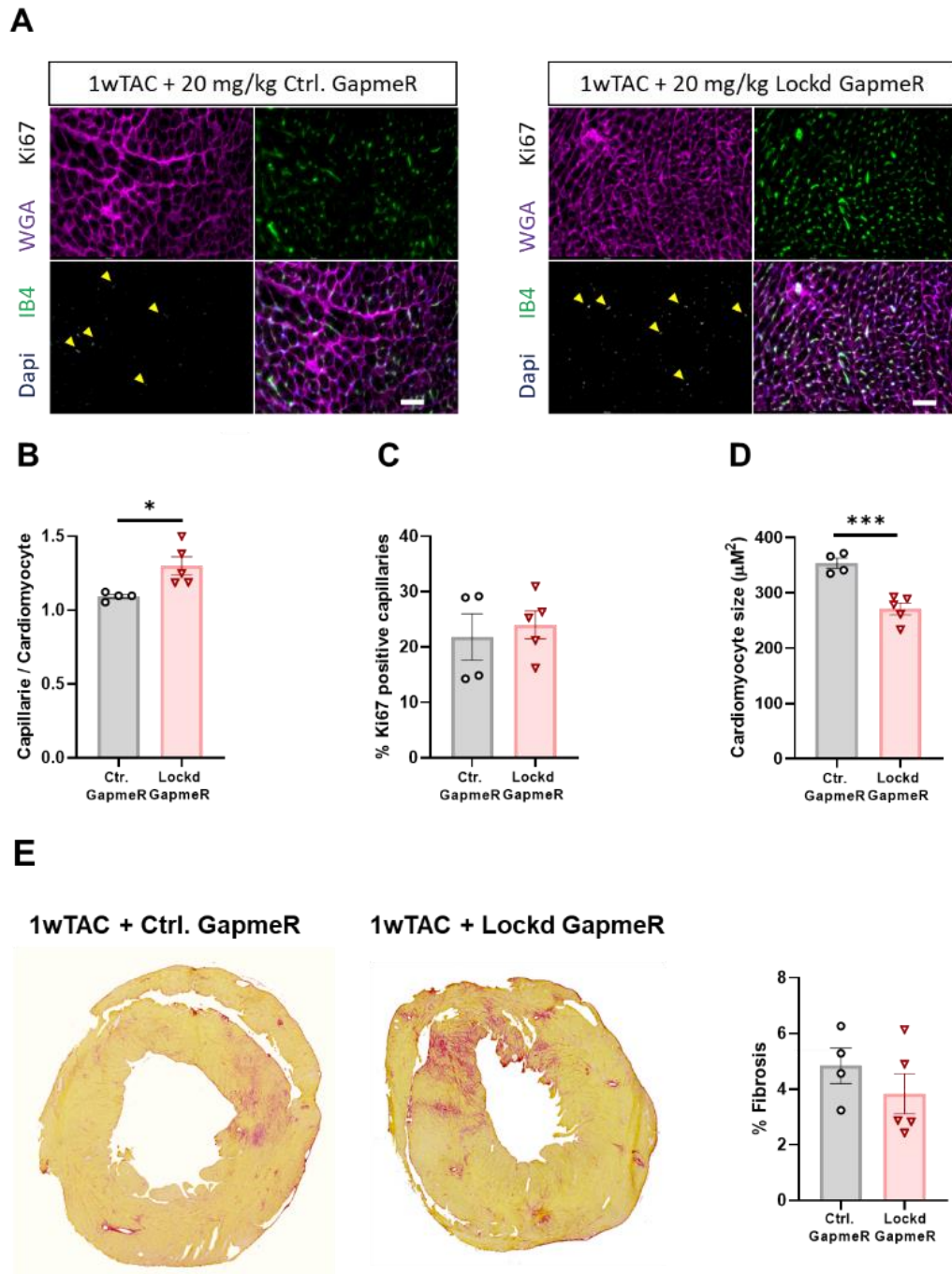


Figure 45: Effects of Lockd knockdown one-week post TAC on capillary density, cardiomyocyte hypertrophy, and fibrosis

(A) Immunofluorescence staining of heart tissue sections 2 weeks post TAC using WGA, IB4 and Ki67. (B) Quantification of capillary density per cardiomyocyte in Lockd knockdown mice compared to control mice. (C) Immunofluorescence staining of heart tissue sections using Ki67, showing the ratio of Ki67-positive proliferating capillaries in the Lockd knockdown mice compared to control mice. (D) Immunofluorescence staining of heart tissue sections using WGA, revealing quantified cross-sectional area per cardiomyocyte in Lockd knockdown mice compared to control mice. (E) Sirius red staining of heart tissue sections, showing the percentage area of fibrosis in the Lockd-GapmeR group compared to the control-GapmeR group. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$ and ** $P < 0.01$.

5.24 Lockd knockdown in one-week after induced pressure overloaded hearts causes the dysregulation of cell-cycle related genes

To comprehensively assess the consequences of Lockd knockdown at an early disease time-point, bulk RNA sequencing on isolated endothelial cells one-week after TAC and on whole heart tissue was conducted.

One week after Lockd knockdown post-TAC, significant changes in the gene expression profile of the whole heart tissue (Figure 46A and Figure 46B) and cardiac endothelial cells were observed. In the whole heart tissue, there was a notable upregulation of genes associated with angiogenesis, endothelial cell differentiation, response to growth factors, and regulation of endothelial cell proliferation. Conversely, several genes related to neutrophil degranulation and negative regulation of immune system processes were downregulated. Additionally, genes involved in extracellular matrix (ECM) organization exhibited decreased expression, consistent with the findings observed in the whole heart tissue at two weeks after TAC (Figure 46C).

upregulated in Lockd knockdown samples ($\log_2\text{FC} > 0$, $p\text{-value} < 0.05$), while blue dots represent genes that are significantly downregulated ($\log_2\text{FC} < 0$, $p\text{-value} < 0.05$). The volcano plot highlights the differentially regulated genes and their statistical significance. (B) Heatmap displaying the expression patterns of differentially regulated genes in whole heart tissue two weeks after TAC between control and Lockd knockdown mice. Genes with altered expression levels ($\log_2\text{FC} > 0.6$ or < -0.6 , $\text{FDR} < 0.05$) are represented in rows, and samples from different experimental conditions are depicted in columns. Red and blue colours indicate upregulated and downregulated genes, respectively, while white represents no change in gene expression. (C) Gene Ontology (GO) analysis results for the upregulated genes and downregulated genes in whole heart tissue upon Lockd knockdown. The bar graph presents the enrichment score ($\log_{10} p\text{-value}$) for each GO term. (B) Volcano plot depicting the results of bulk RNA-seq analysis comparing gene expression profiles in cardiac ECs one-week after transverse aortic constriction (TAC) between control and Lockd knockdown mice. The $\log_2$ fold change ($\log_2\text{FC}$) of each gene is plotted on the x-axis against the negative $\log_{10}$ of the p-value on the y-axis. Red dots represent genes that are significantly upregulated in Lockd knockdown samples ($\log_2\text{FC} > 0$, $p\text{-value} < 0.05$), while blue dots represent genes that are significantly downregulated ($\log_2\text{FC} < 0$, $p\text{-value} < 0.05$). The volcano plot highlights the differentially regulated genes and their statistical significance. Heatmap displaying the expression patterns of differentially regulated genes in cardiac ECs one-week after TAC between control and Lockd knockdown mice. Genes with altered expression levels ($\log_2\text{FC} > 0.6$ or < -0.6 , $\text{FDR} < 0.05$) are represented in rows, and samples from different experimental conditions are depicted in columns. Red and blue colours indicate upregulated and downregulated genes, respectively, while white represents no change in gene expression. Gene Ontology (GO) analysis results for the upregulated genes and downregulated genes in cardiac ECs upon Lockd knockdown. The bar graph presents the enrichment score ($\log_{10} p\text{-value}$) for each GO term.

In cardiac endothelial cells (Figure 47A and Figure 47B), there was an upregulation of genes associated with the regulation of angiogenesis and genes involved in the regulation of macrophage activation, Rap1 signalling pathway and response to retinoic acid signalling.

Surprisingly, a significant number of genes related to the cell cycle were downregulated in cardiac endothelial cells one-week post TAC. This downregulation affected key processes such as the regulation of cell cycle progression, DNA replication, cell cycle checkpoint regulation, DNA-templated DNA replication, cell cycle phase transition, chromosome maintenance, and kinetochore organization. These findings strongly indicate that Lockd knockdown exerts a notable impact on the cell cycle machinery of cardiac endothelial cells, potentially influencing their proliferation and function (Figure 47C).

genes that are significantly downregulated ($\log_2\text{FC} < 0$, $p\text{-value} < 0.05$). The volcano plot highlights the differentially regulated genes and their statistical significance. Heatmap displaying the expression patterns of differentially regulated genes in whole heart tissue two weeks after TAC between control and Lockd knockdown mice. Genes with altered expression levels ($\log_2\text{FC} > 0.6$ or < -0.6 , $\text{FDR} < 0.05$) are represented in rows, and samples from different experimental conditions are depicted in columns. Red and blue colours indicate upregulated and downregulated genes, respectively, while white represents no change in gene expression. Gene Ontology (GO) analysis results for the upregulated genes and downregulated genes in endothelial cells upon Lockd knockdown. The bar graph presents the enrichment score ($\log_{10} p\text{-value}$) for each GO term. (B) Volcano plot depicting the results of bulk RNA-seq analysis comparing gene expression profiles in cardiac ECs one-week after transverse aortic constriction (TAC) between control and Lockd knockdown mice. The $\log_2$ fold change ($\log_2\text{FC}$) of each gene is plotted on the x-axis against the negative $\log_{10}$ of the p-value on the y-axis. Red dots represent genes that are significantly upregulated in Lockd knockdown samples ($\log_2\text{FC} > 0$, $p\text{-value} < 0.05$), while blue dots represent genes that are significantly downregulated ($\log_2\text{FC} < 0$, $p\text{-value} < 0.05$). The volcano plot highlights the differentially regulated genes and their statistical significance. Heatmap displaying the expression patterns of differentially regulated genes in cardiac ECs one-week after TAC between control and Lockd knockdown mice. Genes with altered expression levels ($\log_2\text{FC} > 0.6$ or < -0.6 , $\text{FDR} < 0.05$) are represented in rows, and samples from different experimental conditions are depicted in columns. Red and blue colours indicate upregulated and downregulated genes, respectively, while white represents no change in gene expression. Gene Ontology (GO) analysis results for the upregulated genes and downregulated genes in cardiac ECs upon Lockd knockdown. The bar graph presents the enrichment score ($\log_{10} p\text{-value}$) for each GO term.

5.25 Lockd knockdown affects cardiomyocyte hypertrophy

Upon Lockd knockdown, one-week and 2 weeks after transverse aortic constriction, a reduction in hypertrophy and individual cardiomyocyte area size was observed. To directly investigate the impact of Lockd knockdown on cardiomyocyte hypertrophy, neonatal rat cardiomyocytes were transfected with siRNA targeting rat-Lockd. The transfection resulted in an efficient knockdown of approximately 55% of rat-Lockd expression in neonatal rat cardiomyocytes (Figure 48A).

Troponin T staining of the transfected cells was performed to specifically identify and visualize cardiomyocytes. The cells were cultured in serum-free M199 medium without or with 25 μM phenylephrine to induce cardiomyocyte hypertrophy. Stimulation with phenylephrine for 24 hours is well-established to promote hypertrophic responses in cardiomyocytes (Figure 48B).

The Troponin T staining allowed for the selective identification of cardiomyocytes within the cell population, enabling the assessment of cardiomyocyte-specific changes in response to phenylephrine-induced hypertrophy. The use of phenylephrine as a hypertrophic stimulus allowed for the examination of how Lockd knockdown may modulate the molecular pathways involved in cardiomyocyte growth and hypertrophy under hypertrophic conditions.

Area size quantification of uni-nucleated cardiomyocytes was performed to assess the impact of Lockd knockdown on cardiomyocyte hypertrophy. The results demonstrated that the Lockd knockdown cardiomyocytes exhibited a reduced area size compared to the control cells, indicating a potential regulatory role of Lockd in cardiomyocyte growth.

Upon stimulation with phenylephrine, both siCtrl and siLockd transfected cells exhibited an increased cardiomyocyte size, as expected due to the hypertrophic stimulus. However, even under hypertrophic conditions, the Lockd knockdown cells displayed a lower area size compared to the siCtrl cells, suggesting a dampened hypertrophic response in the absence of Lockd (Figure 48C).

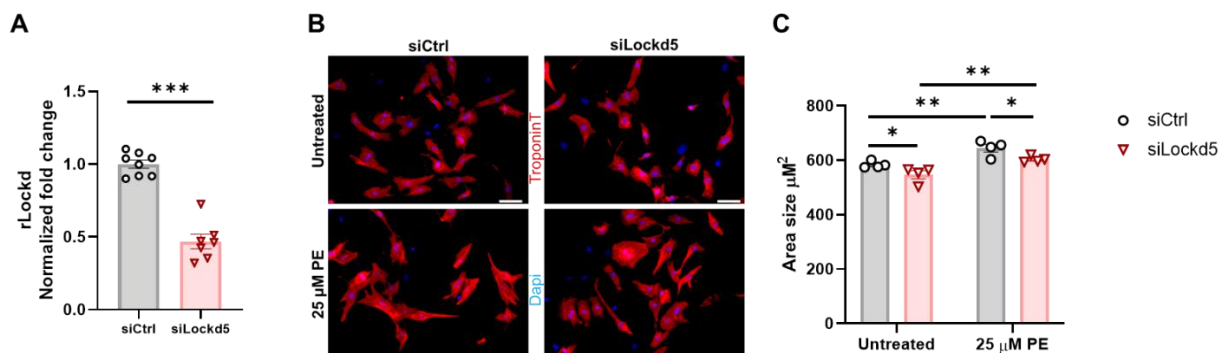


Figure 48: Lockd knockdown affects neonatal rat cardiomyocyte area size

(A) qPCR analysis of neonatal rat cardiomyocytes transfected with siCtrl and siLockd5. qPCR data was normalised to the house keeping gene Gapdh. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *** and $P < 0.001$. (B) Staining of unstimulated and phenylephrine-stimulated cardiomyocytes with TroponinT and Dapi. Scale bar = 50 μm (C) Quantification of area size of uni-nucleated single cardiomyocytes. Data represents mean and error bars represent SEM. Significance was calculated by 2way ANOVA with *, $P < 0.05$ and ** $P < 0.01$.

5.26 Lockd interacts with ribosomal proteins

To investigate the mechanism of action of Lockd in endothelial cells, the potential protein binding partners of Lockd needed to be determined. To achieve this, two distinct RNA pulldown assays were performed, namely RNA antisense purification (RAP-MS) and ChIRP-Mass spectrometry (ChIRP-MS). RAP-MS uses UV light to cross-link RNA to proteins, whereas ChIRP-MS uses formaldehyde to cross-link the RNA to their target molecules. These methodologies enabled a comprehensive examination of the functional associations between Lockd and proteins within a specific cellular context. The protocols involved the selective enrichment of lncRNA-protein complexes using biotinylated antisense oligonucleotides specifically designed to target and capture Lockd. Subsequently, these antisense

oligonucleotides were hybridized to the target lncRNA, facilitating the isolation of the associated lncRNA-protein complexes from cellular lysates. The captured complexes were then purified using streptavidin-coated magnetic beads, followed by mass-spectrometry, facilitating the systematic identification of protein binding partners of Lockd (Figure 49A).

In the RNA antisense purification coupled with mass spectrometry (RAP-MS) experiment, a comparison was made between non-cross-linked and UV-cross-linked samples. Cross-linking RNA to proteins is done to stabilize and preserve RNA-protein interactions, allowing to isolate and identify the specific proteins that bind to the RNA of interest through pull-down. The analysis revealed the identification of numerous proteins that were specifically pulled down in the cross-linked sample, indicating their potential interaction with Lockd (Figure 49B). To gain further insights into the specific binding partners of Lockd, ChIRP-MS was employed. This involved a comparison between formaldehyde-cross-linked samples treated without and with RNase A as a control and non-formaldehyde-cross-linked samples. Through this approach, a total of 47 enriched candidates were identified, out of which 21 exhibited a high degree of enrichment ($\text{Log}_2\text{FC} > 1$ & $\text{FDR} < 0.05$) (Figure 49C). To assess the specificity of the biotinylated antisense probes in pulling down Lockd from cell lysate, qPCR on extracted RNA from the ChIRP experiments was performed. The non-RNaseA treated ChIRP samples exhibited a significant enrichment of Lockd pulldown compared to the non-RNaseA treated Input samples, indicating the successful capture of Lockd RNA. The RNaseA control in ChIRP-MS experiments involves treating ChIRP samples with RNaseA, an enzyme that degrades RNA, to ensure that the observed enrichments of Lockd pulldown are RNA-dependent and not due to non-specific interactions. In contrast, the RNaseA treated samples showed no detectable Lockd pulldown, confirming the RNA-dependent nature of the interaction. To further demonstrate the specificity of the antisense probes for Lockd, their ability to pull down Hprt mRNA, a negative control target was tested. The Hprt mRNA was not detected in the Lockd antisense probe pulldown, confirming the specificity of the probes for binding Lockd and excluding non-specific interactions (Figure 49D).

Subsequently, an overlap analysis was conducted to compare the interaction partners identified by RAP-MS with the highly enriched candidates from ChIRP-MS. Out of the 21 highly enriched candidates, 14 proteins demonstrated concurrence with the proteins obtained from the RAP-MS pull-down experiment, thus establishing their classification as Lockd-interacting proteins. Notably, all 12 of these 14 identified proteins were ribosomal proteins that collectively formed a complex, suggesting a potential interaction between Lockd and the ribosome complex (Figure 49E). This finding is particularly intriguing considering Lockd's status as a non-coding RNA with a coding probability of only 3% (Figure 8).

String analysis of the most highly and significantly enriched binding partners of lockd revealed, that indeed Lockd primarily binds to a whole complex of ribosomal proteins that are tightly interconnected (Figure 49F)

The identification of these ribosomal proteins as potential binding partners of Lockd highlights the intriguing possibility of Lockd's involvement in ribosome-related processes, despite its classification as a non-coding RNA.

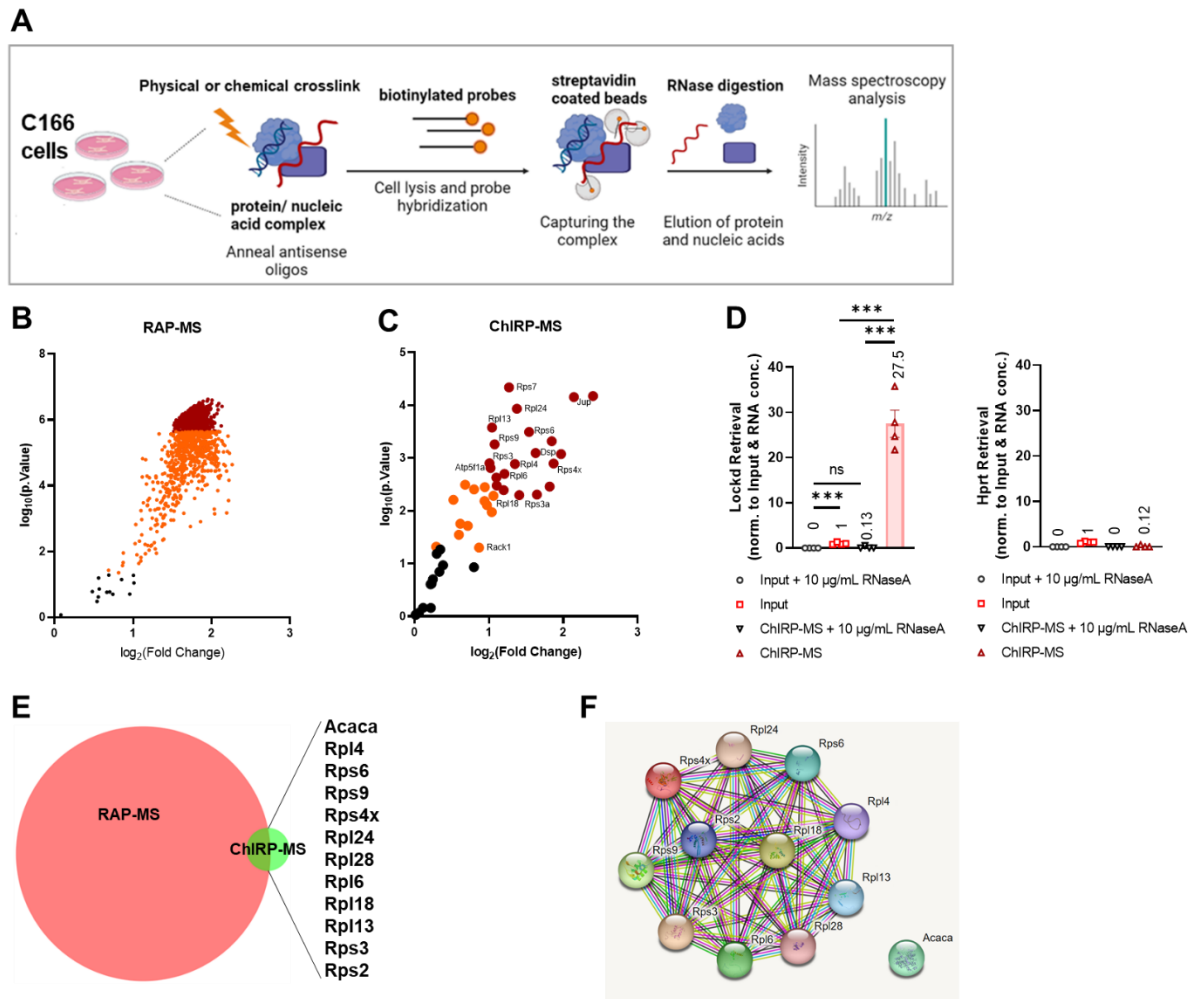


Figure 49: Identification of Lockd protein binding partners using RNA pulldown assays

(A) Simplified experimental setup of RNA pulldown assays. (B) Volcano Plot depicting enriched binding partners of lncRNA identified through RAP-MS. Each data point on the plot represents an individual protein identified through mass spectrometry analysis. The x-axis represents the $\log_2$ fold change, indicating the change in protein abundance between the RAP-MS samples and the control samples. The y-axis represents the negative logarithm (base 10) of the adjusted p-value, reflecting the statistical significance of the protein enrichment in the RAP-MS samples. Proteins with lower p-values are considered more significantly enriched. (C) Volcano Plot depicting enriched binding partners of lncRNA identified through ChIRP-MS. Each data point on the plot represents an individual protein identified through mass spectrometry analysis. The x-axis represents the $\log_2$ fold change, indicating the change in protein abundance between the ChIRP samples and the control samples. The y-axis represents the negative logarithm (base 10) of the adjusted p-value, reflecting the statistical significance of the protein enrichment in the ChIRP samples. Proteins with lower p-values

are considered more significantly enriched. (D) qPCR demonstrating the specificity of biotinylated antisense probes in capturing the target long non-coding RNA (lncRNA) Lockd from cell lysate. qPCR of Hprt was used as a negative control. Data represents mean and error bars represent SEM. Significance was calculated by One-Way ANOVA, with ns, $P > 0.05$; ***, $P < 0.001$. (E) Venn Diagram showing the overlap of binding partners identified by ChIRP-MS and RAP-MS for Lockd.

To validate the interactions identified through RAP-MS and ChIRP-MS screens, RNA immunoprecipitation (RIP) was performed. Rps6 and Rack1 were selected as candidate binding partners of Lockd, as they exhibited significant enrichment in both screens.

Formaldehyde-treated cell lysate was incubated with a Rps6-specific antibody and the resulting protein-RNA complexes were pulled down using Protein A magnetic beads. Following five washes, the beads were treated with Proteinase K to remove proteins, and the RNA fraction was extracted. As a negative control, an equivalent concentration of IgG from the same species (rabbit) was used. Notably, a strong enrichment of Lockd binding to Rps6 was observed, with an average three-fold enrichment compared to IgG control (Figure 50A). This demonstrates the specific interaction between Lockd and Rps6.

Additionally, Rack1 was investigated as another candidate binding partner of Lockd. While Rack1 was identified as an enriched candidate, but not as highly enriched as Rps6, the interaction between Lockd and Rack1 was successfully validated through RNA immunoprecipitation. An average two-fold enrichment of Lockd binding to Rack1 was observed compared to the IgG control (Figure 50B).

These results confirm the specific interaction of Lockd with Rps6 and Rack1, as demonstrated by the enrichment of Lockd in the immunoprecipitates.

In previous experiments (Figure 27B), it was observed that Lockd predominantly localizes to the perinuclear region of the cell, with additional localization in the nucleus and cytoplasm. Similarly, Rps6 predominantly exhibits perinuclear subcellular localization with some cytoplasmic distribution. Notably, Rps6 is entirely absent from the nucleus. In this study, the subcellular co-localization of Lockd and Rps6 was investigated further by smFISH (single molecule fluorescence in situ hybridization). The results revealed co-localization of Lockd and Rps6 in the perinuclear region of the cells, confirming a potential interaction between these two molecules in that cellular region. This observation supports the hypothesis of their functional association. Surprisingly, an overall co-localization of Lockd and Rps6 was observed in mitotic cells, suggesting their potential involvement in mitosis and highlighting their significance during cellular proliferation (Figure 50C).

Of interest, Lockd exhibited a similar subcellular localization pattern as 18S, a component of the ribosome complex. This finding further strengthens the hypothesis that Lockd may interact with the

ribosome complex, potentially influencing its function and cellular processes. Interestingly, in the case of 18S, an overall co-localization with Rps6 was also observed in mitotic cells (Figure 50D).

In contrast, the subcellular localization pattern of polyadenylated RNA, as visualized using polyA staining, differed from that of Lockd and 18S. Polyadenylated RNA displayed increased accumulation in specific regions of the nucleolus, as well as a pronounced perinuclear localization and scattered cytoplasmic distribution (Figure 50E).

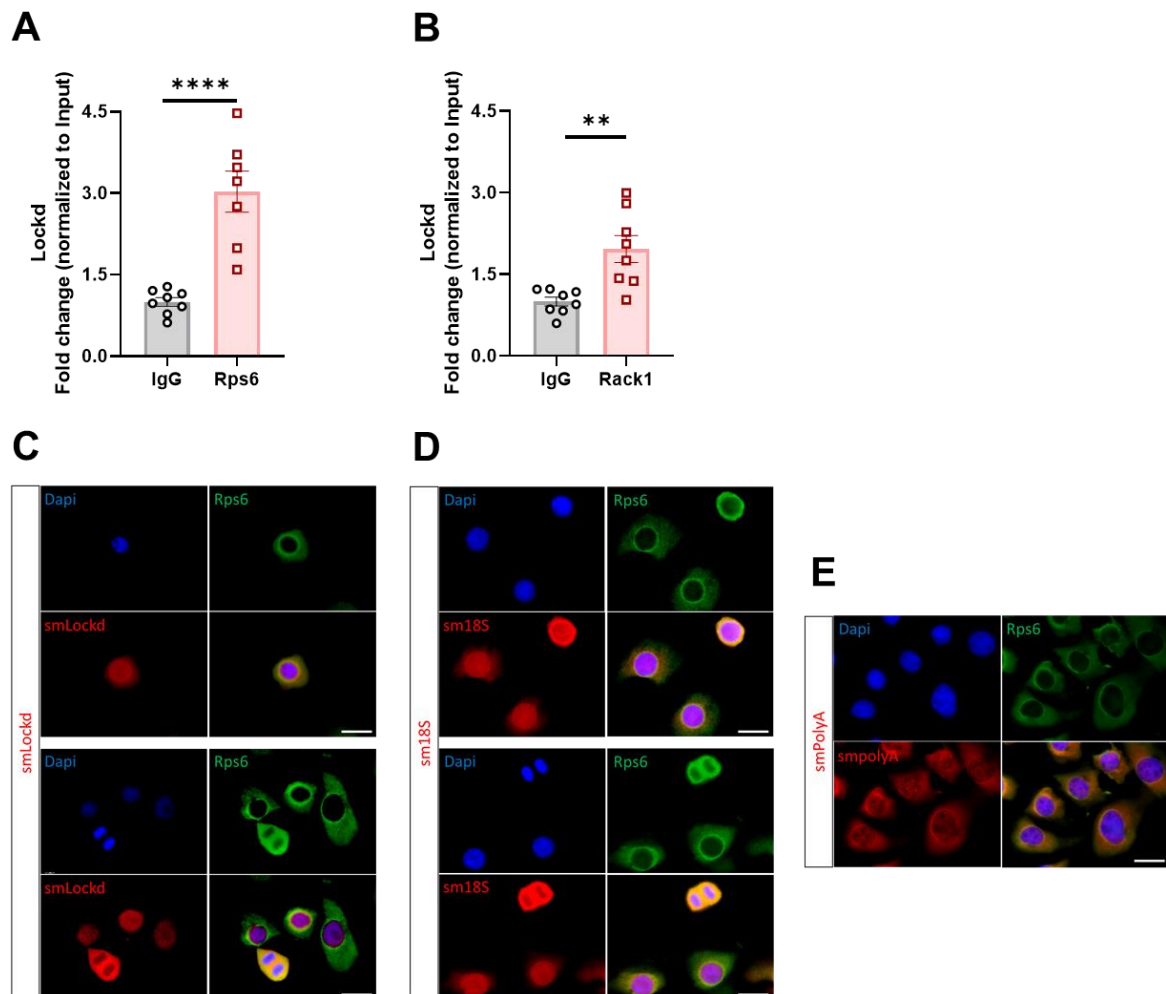


Figure 50: Protein binding partners of Lockd in cellular context

(A) RNA immunoprecipitation (RIP) analysis of Lockd binding to Rps6 compared to the IgG control. (B) RIP analysis of the interaction between Lockd and Rack1 compared to the IgG control. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with **, $P < 0.01$; ****, $P < 0.0001$. (C) Immunofluorescence staining of Rps6 and Fluorescence *in situ* hybridization of Lockd in C166 cells. (D) Immunofluorescence staining of Rps6 and Fluorescence *in situ* hybridization of 18S. (E) Immunofluorescence staining of Rps6 and Fluorescence *in situ* hybridization of polyadenylated RNA (polyA) in C166 cells. Scale bar = 25 μ M

5.27 Lockd overexpression affects *de novo* protein synthesis

The impact of Lockd on translation was investigated in light of its identified binding to ribosomal proteins. After discovering the interaction between Lockd and Rps6, the effect of Lockd knockdown on Rps6 phosphorylation in C166 cells was examined. Rps6 phosphorylation is known to promote protein translation of ribosomes thereby playing a role in cell size [81, 282]. Western-Blot of Rps6 and Phospho-Rps6 did not reveal any differences in the ratio of phospho-Rps6 to total Rps6 (Figure 51A). Similarly, adenoviral overexpression of Lockd did not affect the ratio of phosphorylated Rps6 to total Rps6 (Figure 52B). As protein synthesis can be regulated by many different pathways, the puromycin sunset assay, a technique employed to evaluate *de novo* protein synthesis, was employed. C166 cells were subjected to puromycin treatment, inducing premature termination of protein synthesis, and subsequent quantification of puromycin-labeled nascent polypeptides provided an assessment of ongoing protein synthesis activity. Puromycin is a structural analog of aminoacyl-tRNA that gets incorporated into nascent polypeptide chains during translation [283]. To explore this further, Lockd was overexpressed or knocked down, and cell lysates were analysed via SDS-PAGE and subsequent Western blot analysis. The results demonstrated that Lockd knockdown had no significant effect on total *de novo* protein synthesis (Figure 51C). However, the overexpression of Lockd revealed an increase in total *de novo* protein synthesis.(Figure 51D).

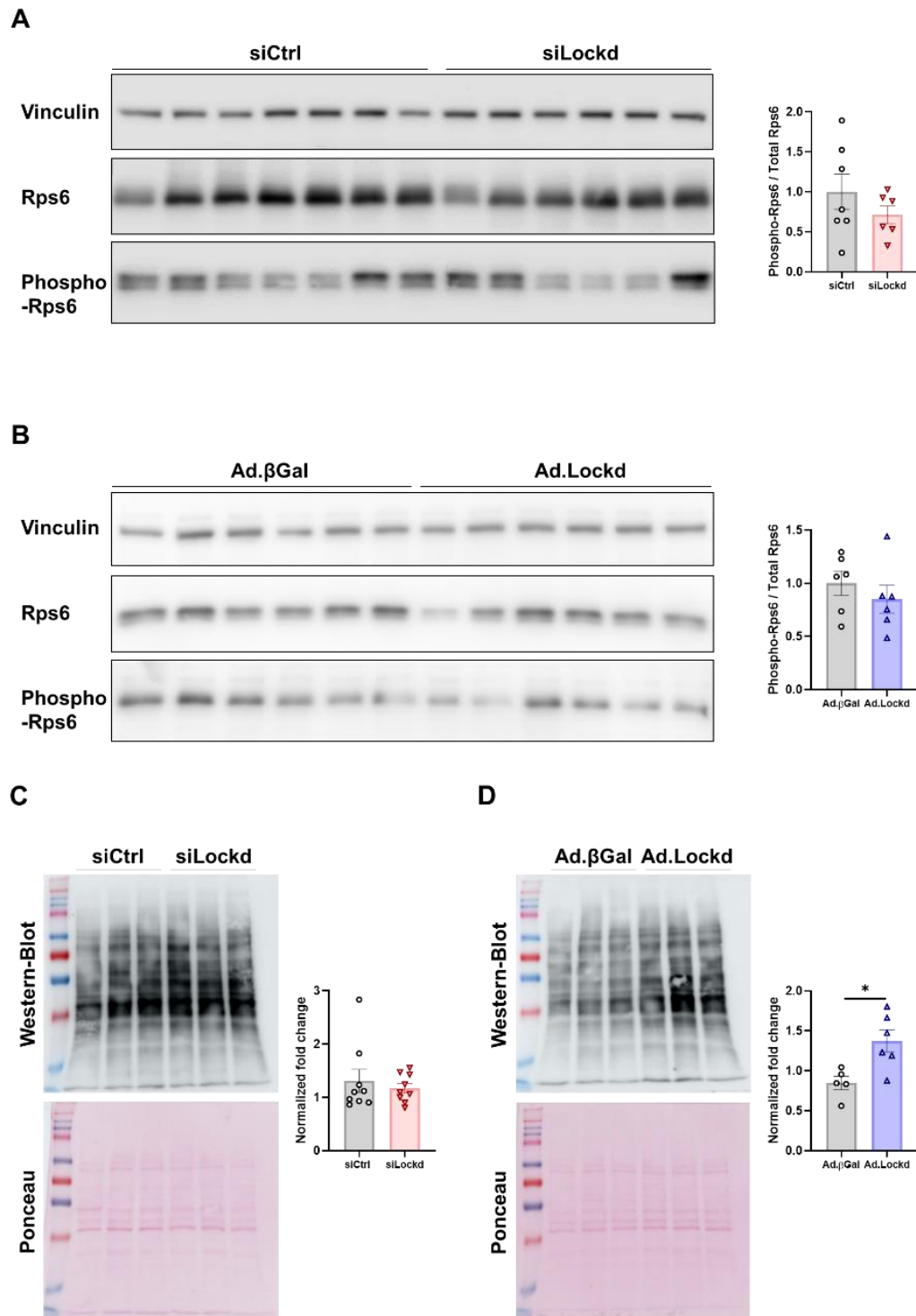


Figure 51: Assessment of Rps6 phosphorylation and protein synthesis

(A) Western blot analysis of Rps6 and Phospho-Rps6 under the siRNA induced Lockd knockdown conditions. And calculated ratio of ratio of phosphorylated Rps6 to total Rps6 (B) Western blot analysis of Rps6 and Phospho-Rps6 post adenoviral overexpression of Lockd. Adenoviral overexpression of Lockd did not alter the ratio of phosphorylated Rps6 to total Rps6. (C) Puromycin sunset assay for the evaluation of *de novo* protein synthesis upon Lockd knockdown. (D) Puromycin sunset assay for the evaluation of *de novo* protein synthesis after adenoviral induced Lockd overexpression. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$.

5.28 Proteomic analysis of Lockd knockdown and overexpression endothelial cells

Our comprehensive investigation into the functional role of Lockd has revealed specific binding interactions with key components of the ribosome complex, namely Rps6 and Rack1. Rps6 is a crucial protein involved in protein translation [282], while Rack1 plays significant roles in both cell cycle progression and translational repression [284]. The specific interaction of Lockd with these ribosome-associated proteins suggests its potential involvement in modulating translation processes and cellular responses to stress.

Moreover, the study demonstrated that Lockd overexpression led to a notable increase in *de novo* protein synthesis, as evidenced by the puromycin treatment experiment. This observation further supports Lockd's involvement in translation regulation and its potential impact on cellular protein synthesis.

To gain deeper insights into Lockd's influence on translation and various biological processes, proteomic analyses on C166 cells with Lockd knockdown and overexpression was conducted. Notably, Lockd knockdown resulted in significant changes in the cellular proteome, indicating its role in regulating protein expression, as shown by the Volcano-plot (Figure 52A).

Gene Ontology (GO) enrichment analysis of the top up-regulated proteins (39 proteins, Table 16) upon Lockd knockdown ($\log_2\text{FC} > 0.2$ & $p\text{-Value} < 0.05$) revealed notable enrichment of proteins associated with the G13 signalling pathway. The G13 pathway is known to play a crucial role in actin polymerization and cytokinesis. The up-regulation of proteins such as Calm1, Pik3a, Myo19, and Dynl1 indicates that Lockd may influence cytokinesis and actin dynamics through its regulatory role in the G13 signalling pathway.

In addition to the impact on cytokinesis, Lockd knockdown also led to significant up-regulation of proteins involved in cilium assembly, including centrosomal proteins such as Cep350, Cep162, and Cep164. These centrosome-associated proteins are essential for proper centrosome organization, microtubule dynamics, spindle assembly, and accurate chromosome segregation during cell division, thereby influencing cell cycle progression.

On the other hand GO analysis revealed a set of top downregulated proteins (42 proteins, Table 17) upon Lockd knockdown, with $\log_2\text{FC}$ values less than -0.15 and statistically significant p -values ($p\text{-Value} < 0.05$). Among these downregulated proteins, notable candidates were Rrm1, Mettl13, and Atrip, which are key players in the regulation of cell cycle phase transitions. Specifically, Rrm1, Mettl13, and Atrip are crucial for controlling the transition from the G1 to the S phase of the cell cycle, a critical checkpoint in cell proliferation [285].

These findings collectively indicate that Lockd plays a multifaceted role in cellular processes. It interacts specifically with ribosome-associated proteins, potentially affecting translation processes. Moreover, Lockd knockdown influences the proteome, leading to alterations in the expression of proteins involved in the G13 signalling pathway and cilium assembly, thereby influencing cytokinesis and cell cycle progression (Figure 52B).

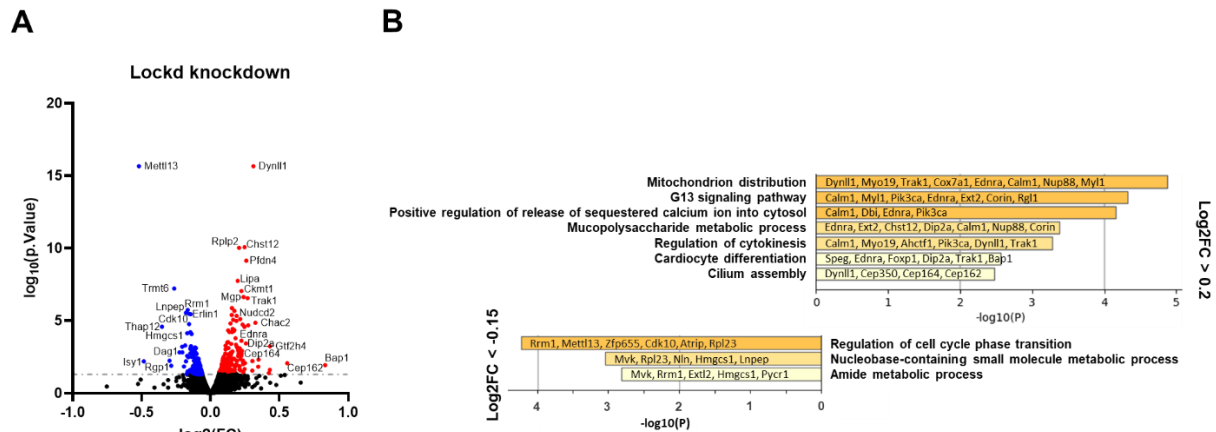


Figure 52: Impact of Lockd knockdown on cellular proteome and biological processes

(A) Volcano plot depicting the impact of Lockd knockdown on the cellular proteome in C166 cells. The log₂ fold change (log₂FC) of each protein is plotted on the x-axis against the negative log₁₀ of the p-value on the y-axis. Red dots represent significantly up-regulated proteins (log₂FC > 0, p-value < 0.05), while blue dots represent significantly down-regulated proteins (log₂FC < 0, p-value < 0.05). The Volcano plot highlights the differential protein expression upon Lockd knockdown. (B) Gene Ontology (GO) analysis of differentially regulated proteins upon Lockd knockdown of the top up-regulated proteins (log₂FC > 0.2, p-value < 0.05) and the top downregulated proteins (log₂FC < -0.15, p-value < 0.05).

Due to Lockd's increased expression in the heart during cardiac pressure overload, the consequences of Lockd overexpression on the endothelial cell proteome was investigated (Figure 53A). The proteomic analysis identified a set of upregulated proteins with Log₂FC values exceeding 0.2 and statistically significant p-values (p-value < 0.05) (51 proteins, Table 18). These upregulated proteins were found to be associated with crucial gene ontology terms, particularly "Metaphase plate congression." Notable genes falling within this category, such as Mlh1, Ska1, and Llg1, play pivotal roles during metaphase, ensuring the accurate alignment of chromosomes along the metaphase plate. This alignment is a critical step for precise chromosome segregation during cell division, safeguarding genomic stability and proper inheritance of genetic material.

In contrast, downregulation of proteins upon Lockd overexpression, characterized by Log₂FC values less than -0.1 and statistically significant p-values (p-value < 0.05) were observed (93 proteins in total,

Table 19). These downregulated proteins were primarily involved in crucial cellular processes such as mRNA transcription and mitotic sister chromatid segregation. The reduction in proteins associated with mRNA transcription suggests a potential role of Lockd in modulating transcriptional activity, which may have broader implications for gene expression regulation and cellular function. Furthermore, the downregulation of proteins linked to mitotic sister chromatid segregation, exemplified by Kif11 and Kif18b, is of particular interest as these motor proteins are vital for the accurate separation of sister chromatids during mitosis, contributing to faithful chromosome distribution to daughter cells (Figure 53B).

Moreover, proteins involved in the regulation of protein folding were also downregulated, pointing towards the potential influence of Lockd upregulation on cellular protein homeostasis and folding processes. This finding may indicate a broader impact of Lockd on cellular proteostasis, which could have important consequences for endothelial cell function and cardiac adaptation.

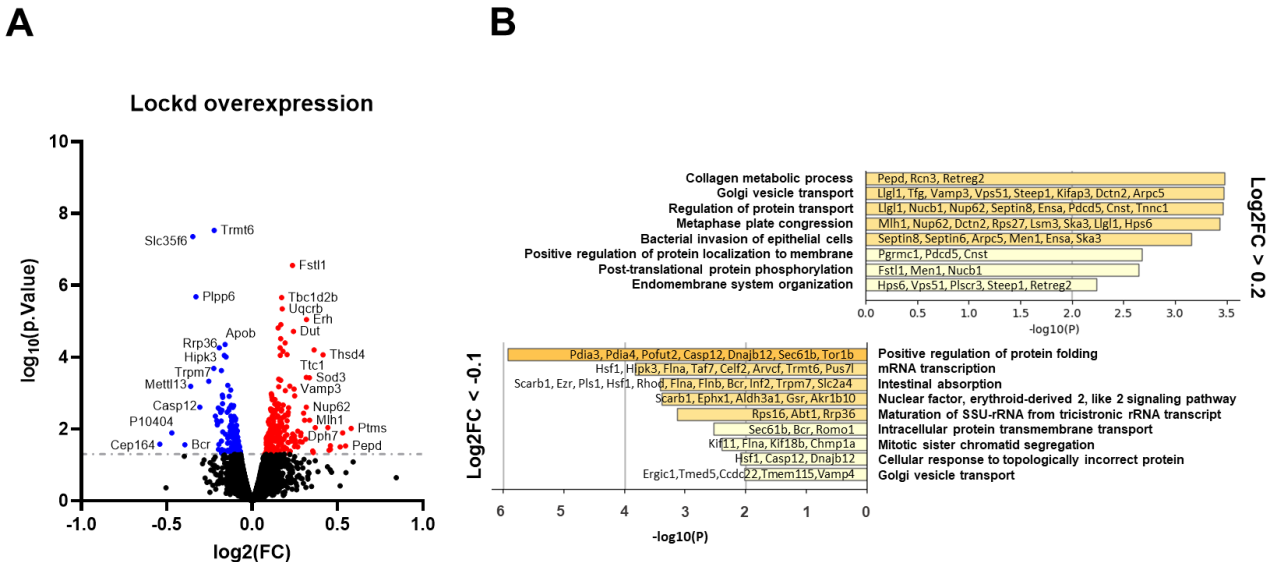


Figure 53: Impact of Lockd overexpression on cellular proteome and biological processes
(A) Volcano plot depicting the impact of Lockd overexpression on the cellular proteome in C166 cells. The log₂ fold change (log₂FC) of each protein is plotted on the x-axis against the negative log₁₀ of the p-value on the y-axis. Red dots represent significantly up-regulated proteins (log₂FC > 0, p-value < 0.05), while blue dots represent significantly down-regulated proteins (log₂FC < 0, p-value < 0.05). The Volcano plot highlights the differential protein expression upon Lockd overexpression. (B) Gene Ontology (GO) analysis of differentially regulated proteins upon Lockd knockdown of the top up-regulated proteins (log₂FC > 0.2, p-value < 0.05) and the top downregulated proteins (log₂FC < -0.1, p-value < 0.05).

5.29 ChIRP Analysis Reveals Lockd's Specific Genomic Binding Sites and Differential Gene Expression Impact

The investigation of Lockd's potential role in gene expression modulation through direct interactions with chromatin structures was prompted by its observed presence in the nucleoplasm. To address this aspect, the Chromatin Immuno-RNA precipitation (ChIRP) technique to identify the genomic binding sites of Lockd in C166 endothelial cells was utilized. The ChIRP method selectively captures the target lncRNA-chromatin complex using a set of odd (Probe 1 and Probe 3) and even (Probe 2 and Probe 4) antisense oligos, which cover different part of the Lockd RNA, allowing for the identification of enriched Lockd peaks. As a control, RNaseA treated cell lysate was included, which primarily degrades Lockd (and other RNAs). Quantitative PCR analyses of input and pull-down samples provided compelling evidence of Lockd enrichment in the non-RNase A-treated ChIRP samples, confirming the successful capture of Lockd-associated chromatin regions. Interestingly, a higher enrichment of Lockd using the odd pair of probes (Probe 1 and Probe 3) compared to the even pair (Probe 2 and Probe 4) was observed, suggesting variations in probe efficiency. Nevertheless, both even and odd probe pulldown ChIRP samples showed higher Lockd enrichment compared to the RNase A-treated control, where Lockd pulldown was negligible (Figure 54A). To assess the specificity of the ChIRP probes, the retrieval of housekeeping mRNA Hprt was also examined. qPCR validation demonstrated that no Hprt mRNA was pulled down with either the even or odd pair of Lockd probes (Figure 54B).

Next, an overlap analysis of the identified peaks from the even and odd probe ChIRP experiments within the genomic locations of genes was conducted. The results revealed a high degree of overlap, with 82% of the even identified gene peaks also present within the odd identified gene peaks. This finding indicates that both even and odd probes target Lockd and capture similar DNA fragments (Figure 54C).

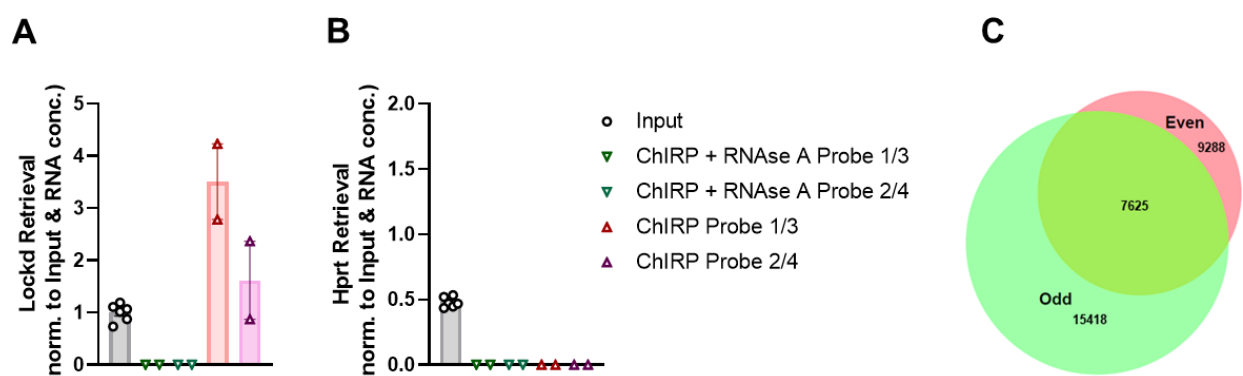


Figure 54: ChIRP analysis of Lockd probe specificity

(A) Quantitative PCR analyses of input and pull-down samples showing enrichment of Lockd in non-RNase A-treated ChIRP-MS samples. (B) Quantitative PCR analyses of mRNA Hprt was examined for

RNA retrieval using either the even or odd set of Lockd probes. Data represents mean and error bars represent SEM. (C) Overlap analysis of even and odd identified peaks within gene locations indicated a substantial overlap of 82% of the even identified gene peaks within the odd identified gene peaks.

Subsequently, the odd and even peaks were merged to create a unified dataset. Comparative analysis of the peak distribution with the merged RNaseA control peaks indicated a clear enrichment in peak height. Additionally, a notable concentration of peaks at the transcription start site (TSS) of genes was observed (Figure 55A).

To gain further insights into the functional relevance of Lockd-bound genomic regions, the gene-specific peaks, including those within the 5' untranslated region (UTR), the promoter-transcription start site, exons, and 3' UTR were overlapped with the significantly differentially regulated genes identified from the siRNA bulk RNA sequencing data (p -value < 0.05). Intergenic and intronic DNA binding sequences were excluded from this analysis, as the binding of Lockd to these sequences does not provide direct evidence of regulatory involvement in the expression of nearby genes. Chromatin dynamics can lead to the action of enhancers that are located far upstream or downstream from the target genes. Consequently, genes with potential direct regulatory links to Lockd by potential binding to the 5' untranslated region (UTR), the promoter-transcription start site, exons, and 3' UTR were focussed on.

The analysis of overlapping gene sets revealed a mere 44 genes that exhibited differential regulation at both the RNA level (Figure 30) and potential binding by Lockd on the DNA level, indicating a notably limited convergence between these two regulatory aspects. (Figure 55B). Gene Ontology (GO) analysis of these genes shed light on potential pathways directly impacted by Lockd. Particularly, Lockd appears to exert its influence on gene expression related to MAPK signalling and the regulation of mitotic cell cycle (Figure 55C).

These findings highlight the specific genomic regions where Lockd may play a direct regulatory role in gene regulation and may potentially impact key cellular pathways related to cell cycle control and intracellular signalling.

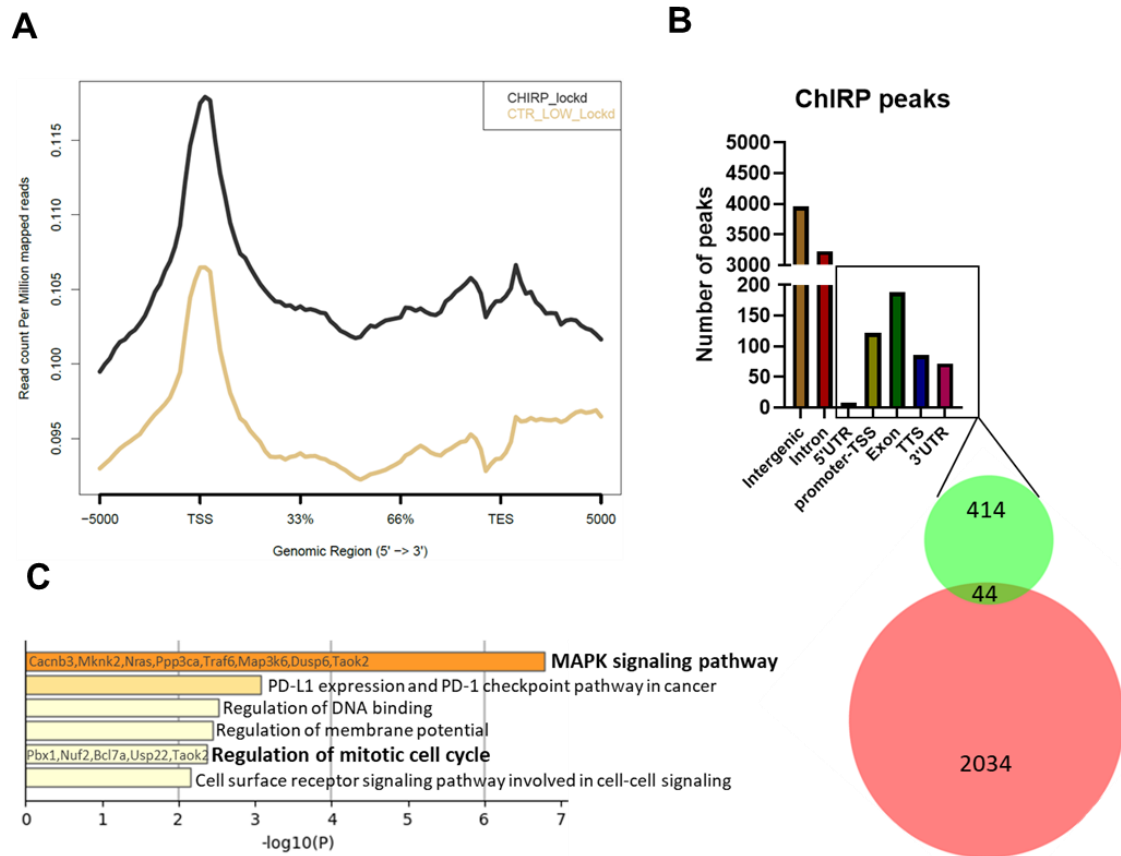


Figure 55: Lockd's genomic binding sites and differential gene expression impact

(A) Merged odd and even ChIRP peaks in comparison to RNaseA control peaks. Peaks are shown and concentrated at the transcription start site (TSS) of genes. (B) Overlapping gene-specific ChIRP peaks (green circle) with siRNA bulk RNA sequencing data (red circle, see Figure 30) revealed 44 genes potentially bound by Lockd and differentially regulated. (C) Gene Ontology (GO) analysis of the 44 genes highlights Lockd's potential impact on MAPK signalling and mitotic cell cycle regulation.

Interestingly, an overlap analysis between the 44 genes potentially bound by Lockd on the genome and the significantly differentially regulated genes identified in the siRNA knockdown proteomics samples revealed only three gene names that were differentially regulated at both the RNA and protein levels and were directly targeted by Lockd, which are Slc29a1, Edf1 and Cep164 .

Subsequent qPCR analysis of several of these genes confirmed that the ones directly bound by Lockd, such as Dusp6, Nras, and Pbx1, displayed differential expression upon Lockd knockdown. On the other hand, for other genes to which Lockd was presumed to bind, such as Taok2, Nif2, and Usp22, no significant differential expression was observed (Figure 56).

These findings provide intriguing insights into the specific regulatory role of Lockd on a select subset of genes. The limited overlap between the differentially regulated genes at the RNA and protein levels suggests that Lockd's regulatory actions may be complex and multifaceted, suggesting that Lockd may

exert its regulatory actions through various mechanisms, including post-transcriptional modifications, or interactions with other molecules, leading to diverse effects on gene expression and protein abundance.

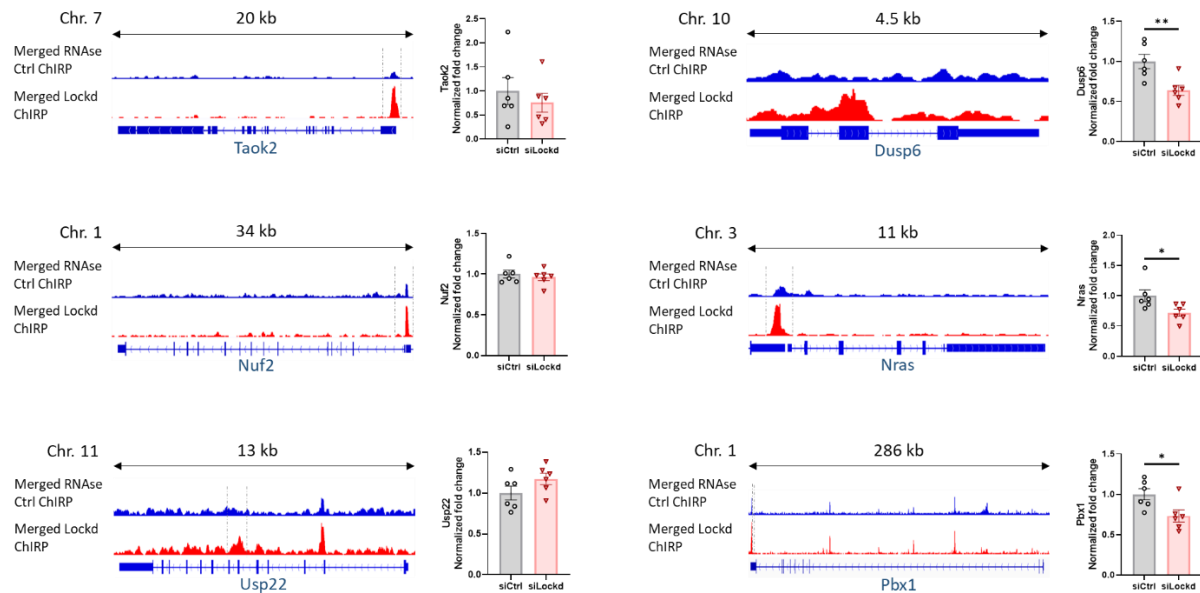


Figure 56: qPCR validation of gene expression changes upon Lockd knockdown

ChIRP-seq peaks for merged control samples, representing the genomic regions where Lockd is potentially bound, are shown in blue. The merged Lockd ChIRP-seq peaks are displayed in red, indicating the regions enriched for Lockd binding. The dashed grey lines represent a significantly enriched peak in the merged Lockd samples. The peak height represents the enrichment level. qPCR analysis of gene expression changes in response to Lockd knockdown. The expression levels of genes directly bound by Lockd. qPCR data have been normalised to the house keeping gene Gapdh. Data represents mean and error bars represent SEM. Significance was calculated by unpaired, two-tailed t-test with *, $P < 0.05$; **, $P < 0.01$.

6. Discussion

Long non-coding RNAs have emerged as important regulators of gene expression and cellular processes. In this study, the involvement of the lncRNA Lockd in endothelial cells in the early phase of cardiac response to stress and induced by cardiac pressure overload and its impact on cardiovascular function was explored. Endothelial cells play a crucial role in cardiac function as they contribute to angiogenesis, inflammation and vascular tone regulation, all of which are essential processes in cardiac tissue repair and remodelling. During the early phase of cardiac remodelling, endothelial cells respond to various stimuli, such as mechanical stress and pro-inflammatory or angiogenic factors by altering their gene expression profile and releasing signalling molecules that can influence other cell types in the heart, including cardiomyocytes and fibroblasts. Angiogenesis is a critical process during cardiac remodelling, as it provides essential nutrients and oxygen to the expanding heart tissue. Dysregulated angiogenesis can lead to inadequate vascular supply and worsen cardiac function. Identifying factors that influence angiogenesis, such as Lockd, could promote proper vascularization in the remodelling heart. These findings indicate that Lockd participates in various endothelial cell responses. Modulating Lockd's expression or activity might influence angiogenesis and endothelial function, ultimately affecting the progression of cardiac remodelling and improving cardiac function.

6.1 Lockd: A potential regulator of cardiac endothelial cells during cardiac remodelling and angiogenesis

The investigation began with a comprehensive analysis of multiple RNA sequencing datasets to identify differentially expressed long non-coding RNAs in cardiac endothelial cells following transverse aortic constriction (TAC). Through this filtering process, a highly upregulated lncRNA specifically in cardiac endothelial cells one-week after TAC, compared to Sham-operated mice was identified. Interestingly, within this dataset, Lockd was the 4th most up-regulated lncRNA one-week post TAC in cardiac ECs. Furthermore, Lockd's expression was also significantly higher in endothelial cells than in cardiomyocytes post-TAC. Additionally, through this rigorous filtering process, another lncRNA, Pvt1, which is already known for being up-regulated in response to cardiac pressure overload and for its involvement in promoting cardiomyocyte hypertrophy was identified, which is further supporting the validity of this filtering criteria [260, 261].

Studying lncRNAs located near important protein-encoding genes, such as *Lockd*, can be intriguing due to their potential cis-acting regulatory roles. Cis-acting lncRNAs can influence neighbouring protein-coding genes through various mechanisms, including recruiting chromatin-modifying complexes, impacting transcriptional elongation, or affecting RNA splicing. The close proximity of lncRNAs to important genes suggests the possibility of coordinated transcriptional regulation, where changes in lncRNA expression could directly influence the neighbouring genes [250, 286, 287].

Characterization of *Lockd* revealed its genomic positioning downstream of the protein-encoding gene *Cdkn1b* and upstream of the protein-encoding gene *Apold1*. The positioning of *Lockd* between these two important protein-coding genes was particularly interesting due to the significance of *Cdkn1b* in cardiac remodelling. *Cdkn1b* functions as a crucial negative regulator of the cell cycle progression, inhibiting cell proliferation by controlling cyclin-dependent kinase (CDK) activity and halting cell cycle progression. Upregulation of *Cdkn1b* has been shown to protect against pathological cardiac hypertrophy. *Cdkn1b* helps to limit the growth of individual cardiomyocytes and thereby prevent excessive enlargement of the heart [288], while downregulation or inactivation of *Cdkn1b* is necessary for endothelial cell proliferation during tissue repair and angiogenesis. Especially growth factors and pro-angiogenic cytokines, such as *Vegf*, stimulate the downregulation of *Cdkn1b*, relieving its inhibitory effect on the cell cycle and promoting endothelial cell proliferation [289, 290]. The balance of *Cdkn1b* expression and activity is crucial for maintaining cellular homeostasis and controlling proliferation and cell cycle entry capacity [160].

Apold1, on the other hand, is a relatively unstudied gene, but it is known to play a role in ischemia-induced revascularization and tumour angiogenesis by regulating endothelial cell proliferation [291].

Previous studies have shown that the *Cdkn1b* gene is positively regulated by a nearby cis element at the *Lockd* promoter, while the transcribed *Lockd* lncRNA itself does not play a role in the regulation of *Cdkn1b* expression. Understanding the function of lncRNA loci requires distinguishing between the activities of the RNA transcript and its underlying DNA [250].

Interestingly, *Lockd* exhibits tissue and cell-specific expression patterns, and it was found to be particularly high in primary mouse foetal liver erythroblasts and the mouse erythroid cell line G1E and its derivative, G1E-ER4. Molecular characteristics support *Lockd*'s active transcriptional status, including the presence of RNA sequencing peaks, DNase I hypersensitive sites indicative of open chromatin, elevated levels of H3K4 methylation associated with active promoters, and reduced methylation of H3K36 linked to active transcriptional elongation within these cells [250]. The transcriptional activity of *Lockd* in endothelial cells has not been studied so far, so whether the *Lockd* gene is actively being transcribed into RNA molecules within endothelial cells

However, the overall identification of Lockd's genomic positioning and convergence molecular features that strongly indicate the active transcriptional state of the genomic region housing Lockd opens up intriguing avenues for understanding Lockd's impact in endothelial cells.

6.2 Cell type-specific upregulation of Lockd and early response in pressure overload-induced cardiac remodelling

To confirm the upregulation of Lockd after transverse aortic constriction, qPCR on heart tissue from mice subjected to TAC surgery was conducted. The results validated a substantial upregulation of Lockd in one-week TAC hearts compared to Sham-operated hearts. To understand the cellular specificity of Lockd upregulation, cardiac cell types, including cardiomyocytes, endothelial cells, and fibroblasts were isolated from TAC and Sham-operated hearts. Interestingly, Lockd showed the highest upregulation in cardiomyocytes, a 14-fold increase, and significant upregulation in endothelial cells with an average 6-fold increase one-week after TAC. Cardiac endothelial cells exhibited the highest overall Lockd expression one-week post-TAC. However, no significant change in Lockd expression was observed in cardiac fibroblasts.

This discovery suggests a cell type-specific regulatory roles of Lockd in response to cardiac stress. The observation of differential upregulation of Lockd in cardiomyocytes and endothelial cells, despite fibroblasts being the cell type having the highest baseline (Sham) expression, suggests that Lockd's function may be more pronounced in these cell types under stress conditions induced by cardiac pressure overload. Conversely, the lack of significant change in fibroblasts indicates that Lockd may not play a key role in fibroblast stress responses, despite their high responsiveness to cardiac stress during cardiac pressure overload. Fibroblasts are known to produce increased amounts of extracellular matrix proteins, contributing to fibrosis and cardiac remodelling in response to stress [64, 91, 292, 293]. However, their specific stress response mechanisms and regulatory pathways might differ from those of cardiomyocytes and endothelial cells, leading to the distinct upregulation of Lockd in the latter cell types.

To gain further insights into Lockd's regulatory landscape and its implications for cardiac remodelling, the co-expression of its neighboring gene, Cdkn1b, known to play a role in cardiac remodelling was investigated. Surprisingly, while Lockd showed upregulation after TAC, Cdkn1b exhibited mild downregulation, indicating independent regulation of these genes. This implies that Lockd and Cdkn1b, as already described previously [250], have independent promoters governing their

differential expression. The experimental results highlight the influence of Lockd expression on Cdkn1b at the protein level. Enhanced cellular proliferation resulting from Lockd knockdown leads to the degradation of Cdkn1b, as demonstrated. Conversely, the upregulation of Lockd, associated with proliferation inhibition, leads to the accumulation of Cdkn1b.

Examining the temporal expression pattern of Lockd during cardiac remodelling through a TAC time course on mice revealed that Lockd expression peaked one-week after TAC and then gradually decreases in whole heart tissue and cardiac endothelial cells, whilst still being mildly up-regulated compared to the unoperated control mice. No differential regulation of Lockd was observed in cardiac fibroblasts during the TAC time course. These results suggest that Lockd may play a role in the early response to pressure overload-induced cardiac remodelling, particularly in cardiomyocytes and endothelial cells. This underscores Lockd's potential role in regulating cellular processes in both cardiac endothelial cells and cardiomyocytes during the onset of cardiac remodelling.

Collectively, these findings suggest that Lockd likely plays a role in the initial response to the pathological stimulus. Its early and robust upregulation suggests involvement in mechanisms activated during pressure overload-induced cardiac remodelling. For example, the initial responses that coincide with Lockd upregulation may include hypertrophic growth of cardiomyocytes, regulated angiogenesis, and remodelling of the cardiac vasculature.

As the pressure overload induced stress persists or subsides, Lockd's expression gradually returns to basal levels. This decline could indicate that the initial response of Lockd has achieved its intended effect or that Lockd's effective response is more specific in the early response.

6.3 Evolutionary Conservation, Functional Significance, and Potential as a Biomarker in Cardiac Remodelling

The investigation into the functional relevance of the long non-coding RNA Lockd in human biology began with an analysis of evolutionary conservation. By comparing the mouse Lockd gene with the human genome, two conserved regions corresponding to the first and second exons of mouse Lockd were identified in humans. The high degree of conservation at the Lockd locus between mice and humans was validated, and alignment analysis revealed up to 73% identity and 80.9% similarity between the murine and potential human Lockd transcripts. This is a high level of evolutionary conservation, considering that *Homo sapiens* and *Mus musculus* have diverged from a common

ancestor approximately 75 million years ago [294]. This high level of conservation indicates a potential conserved biological function.

Interestingly, the human *Lockd* locus shares a genomic context similar to mice, being located downstream of the *Cdkn1b* gene, but with the first exon potentially overlapping with the first exon of an *Apold1* transcriptional variant, which is not the case in mice. Intron-spanning qPCR in human cell lines did not yield PCR products, but specific qPCR only targeting the second exon of human *Lockd* produced an amplicon, suggesting active transcription of at least exon 2 of human *Lockd*, which is contains a longer sequence than exon 1. The lack of transcriptional activity of exon 1 in *Lockd* or the presence of an overlapping region with *Apold1* might present challenges in generating an amplicon specific to the exon-spanning *Lockd* transcript.

Given the high conservation of *Lockd* in humans, its potential involvement in human heart failure was investigated using tissue samples from individuals diagnosed with heart failure and individuals that did not die due to a cardiac disease. The results indicated significant upregulation of *Lockd* in the hearts of individuals with heart failure, suggesting an association between *Lockd* expression and human cardiac dysfunction.

Notably, the upregulation of *Lockd* in human heart failure tissue was not as prominent as observed in the 1-week TAC-induced heart tissue of mice. This discrepancy may be attributed to the more advanced stage of cardiac dysfunction in human heart failure samples compared to the initial phase of pathological cardiac remodelling in mice.

However, long-term TAC in mice did maintain a milder yet noticeable upregulation of *Lockd*, consistent with the findings in human heart failure samples. This suggests that prolonged pressure overload in mice also leads to increased *Lockd* expression, reflecting the results observed in humans.

The overall trend of *Lockd* upregulation in both human heart failure and long-term TAC mouse models supports its potential involvement in pathological cardiac remodelling across species.

The upregulation of *Lockd* in human heart failure samples suggests its potential as a biomarker for the onset of pathological cardiac disease development, and therefore elevated *Lockd* expression in a patient could be potentially used as a marker for the onset for the development of a pathological cardiac condition.

These findings provide insights into *Lockd* as a potential biomarker, however a comprehensive understanding of its molecular mechanisms and functional significance in human cells requires further investigation. High-throughput sequencing data and specific experiments in human cells with *Lockd* overexpression and knockdown are crucial to understand *Lockd*'s relevance and therapeutic potential.

Comparing Lockd's regulatory mechanisms and functional consequences in human cells to those in animal models can validate its translational relevance.

6.4 Lockd as a regulator of cardiac endothelial cell function with implications for angiogenesis and vascular remodelling

The investigation into Lockd expression during cardiac development revealed a dynamic pattern, with the highest expression observed in new born mice and a gradual decline as mice transitioned into adulthood. This age-dependent expression pattern of Lockd during cardiac development is consistent with observations in other organs, as shown in liver, lung and brain, suggesting that Lockd may play a role in the early phases of organ growth and development.

Pathological hypertrophy often involves changes in gene expression patterns reminiscent of the foetal gene program observed during cardiac development. Certain genes highly expressed during foetal stages are downregulated as the heart matures into adulthood. However, in pathological hypertrophy, some foetal genes are re-expressed, contributing to heart remodelling and impaired function [295, 296]. One example of a foetal gene that is up-regulated during cardiac hypertrophy is Nppa (Natriuretic Peptide A). Nppa is primarily produced in the heart's atria and is involved in regulating blood pressure and fluid balance. During cardiac development and hypertrophy, Nppa expression is increased in the ventricular myocardium as a compensatory response to the increased workload on the heart, where it acts as a cardiac hormone, promoting vasodilation and increasing sodium excretion, thereby helping to reduce blood volume and pressure [297]. Lockd's high expression in newborn mice suggests its potential role in early cardiac development when the heart is actively growing and maturing. It may be part of the regulatory network influencing the development of proper heart formation and function.

As the heart transitions into adulthood, foetal gene expression is typically downregulated to maintain proper cardiac function. The downregulation of Lockd with aging may be essential to prevent the detrimental effects of sustained foetal gene expression, which could lead to pathological hypertrophy and compromised cardiac performance. It is plausible that Lockd's influence on the heart is more crucial during the critical phases of heart growth and less necessary in adulthood when the heart is in a relatively stable state.

The stability of Lockd RNA was explored through a time-course experiment using Actinomycin D on cultured endothelial cells. The results revealed remarkable stability of Lockd RNA with a half-life of

approximately 24 hours, indicating that it resists degradation and remains relatively stable over time. This stability implies that Lockd may act as a long-lasting regulator in endothelial cells, suggesting a potential enduring role in endothelial cell function. One possibility is that Lockd may interact with specific RNA-binding proteins, such as ribosomal proteins, or form stable secondary structures that protect it from degradation by cellular RNases [298]. Additionally, certain modifications, such as 5' caps and 3' polyadenylation, commonly found in stable RNAs, could also contribute to Lockd's stability [299]. The capacity of Lockd to withstand degradation over time implies that it could function as a long-lasting regulatory molecule, persistently influencing endothelial cell responses to various cellular signals. The mechanisms underlying Lockd's stability is yet to be fully elucidated, but various factors may contribute to its resistance to degradation. Based on the findings presented, it is plausible to suggest that the observed up-regulation of Lockd during induced cardiac pressure overload is predominantly a consequence of enhanced transcription rather than increased RNA stability.

Next it was of interest to see which factors influence Lockd expression. The regulatory factors influencing Lockd expression in endothelial cells were investigated through stimulating cells in serum-free media with different cytokines. Pro-angiogenic factors including Vegf, Fgf2, and Angpt2 significantly upregulated Lockd expression. Vegf, Fgf2, and Angpt2 are well-known growth factors that stimulate blood vessel formation and angiogenesis, essential processes for example during tissue development, wound healing. [180, 300, 301]. Lockd's responsiveness to these pro-angiogenic cytokines suggests that it may be actively involved in orchestrating the changes necessary for endothelial, proliferation, and angiogenesis to regulate the formation of new blood vessels. The fact that Lockd is also upregulated by the presence of foetal calf serum (FCS), which contains a mix of growth factor suggests that Lockd is not limited to specific signalling pathways but rather can be influenced by a broad range of factors that promote endothelial cell proliferation.

TGF- β on the other hand is known to inhibit blood vessel formation and promote vascular regression, important processes for tissue remodelling, wound healing resolution, and the prevention of excessive angiogenesis [86, 87, 107, 293]. Lockd's downregulation in response to TGF- β suggests that Lockd may act as a negative regulator, potentially counterbalancing the pro-angiogenic signals induced by growth factors like Vegf and Fgf2.

The opposing regulation of Lockd by both pro-angiogenic and anti-angiogenic factors indicates its potential role as a versatile player in endothelial cell physiology and angiogenesis. Its responsiveness to various growth factors implies that Lockd may be actively involved in changes necessary for endothelial cell proliferation and angiogenesis. Additionally, its downregulation in response to anti-angiogenic signals may help maintain vascular homeostasis and prevent uncontrolled angiogenesis.

Lockd's differential responsiveness to cytokines suggests it may act as a dynamic regulator, adjusting the balance between vascular growth and regression.

Overall, these results indicate that Lockd is tightly regulated during cardiac development and endothelial cell function, with potential implications for angiogenesis and vascular remodelling.

6.5 Lockd as a negative regulator of angiogenesis and endothelial cell proliferation

The exploration of Lockd expression during the cardiac overload phase unveiled a predominant upregulation, particularly evident in the early stage, precisely one-week after transverse aortic constriction. This was corroborated by its highest expression levels observed in cardiac endothelial cells and heart tissue. This finding aligns with the activation of endothelial cells for neovascularization during the compensatory phase of cardiac remodelling [302, 303]. The differential regulation of Lockd by angiogenic cytokines in endothelial cells further supports the hypothesis that Lockd may play a specific role in angiogenesis during this phase.

To test the function of Lockd in endothelial cells sprouting assay was performed. The knockdown of Lockd resulted in increased sprouting capacity of endothelial spheroids, especially when stimulated with Fgf2, a potent inducer of angiogenesis [266]. This indicates that Lockd may act as a negative regulator of angiogenesis, inhibiting endothelial cell sprouting and angiogenic processes. Conversely, Lockd overexpression inhibits endothelial angiogenesis, and upon Fgf2 stimulation, the increased angiogenesis is much less than in control conditions, further supporting its inhibitory role in angiogenesis. The results indicate that the knockdown of Lockd enhances the pro-angiogenic effects of Fgf2 on endothelial cells, promoting angiogenesis. Conversely, Lockd overexpression appears to counteract the pro-angiogenic effect of Fgf2, inhibiting endothelial cell sprouting and angiogenesis.

Proliferation and angiogenesis are two interconnected processes that play essential roles in establishing functional blood vessels. Endothelial cell proliferation is a fundamental component of angiogenesis, ensuring the expansion and elongation of new blood vessels. To explore the link between Lockd's effect on sprouting angiogenesis and endothelial cell proliferation, BrdU incorporation assays were performed. The knockdown of Lockd resulted in enhanced endothelial cell proliferation, while Lockd overexpression led to reduced proliferation. Immunostaining of proliferation markers such as phospho-Histone 3 and Aurkb further supported these findings, showing increased populations of proliferating cells during the knockdown of Lockd and decreased proliferation during the overexpression of Lockd. Interestingly, no significant effect on endothelial cell

migration was observed in the presence or absence of Lockd, indicating that its primary influence is on endothelial cell proliferation and the resulting angiogenesis.

The intriguing aspect of these results lies in the conservation of Lockd's inhibitory role in human endothelial cells (HUVECs). Overexpression of the murine Lockd in HUVECs also resulted in reduced sprouting capacity and reduced proliferation capacity, supporting the hypothesis that Lockd's regulatory role in angiogenesis is conserved across species. Knockdown of the human Lockd transcript in HUVECs also increased cell proliferation, further confirming its involvement in regulating angiogenic processes in a human context.

The collective evidence from these experiments points to Lockd as an important factor in regulating angiogenesis and endothelial cell proliferation. Its inhibitory role in angiogenesis suggests that Lockd plays a part in adjusting the balance between vascular growth and regression during cardiac remodelling. By inhibiting angiogenesis, Lockd may help prevent excessive and uncontrolled vessel formation, maintaining proper vascular homeostasis. The identification of Lockd as a negative regulator of angiogenesis provides a potential target for therapeutic interventions in vascular diseases and conditions involving abnormal angiogenesis.

Overall, these results provide strong evidence for Lockd's involvement in regulating angiogenesis and endothelial cell proliferation. The findings suggest that Lockd acts as a negative regulator of angiogenesis, inhibiting endothelial cell sprouting and proliferation. This role may be critical during the development of the cardiovascular system, however, this might be detrimental for the early phase of cardiac remodelling when endothelial cell activation and angiogenesis are crucial for compensatory mechanisms. Additionally, the conservation of Lockd's inhibitory role in human endothelial cells indicates its potential significance in human cardiovascular biology and angiogenic processes.

6.6 Lockd's dynamic role in inhibiting endothelial cell proliferation via cell cycle regulation

The investigation of Lockd's subcellular localization yielded valuable insights into its potential functions within the cell. Subcellular fractionation revealed the presence of Lockd in both the nucleus and cytoplasm, suggesting its involvement in various cellular processes. Additionally, fluorescence in situ hybridization (FISH) experiments further supported these findings, with visualisation that a high proportion of Lockd is predominantly localised around and in the nucleus.

An intriguing observation during the FISH experiments was the notably higher Lockd fluorescence signals in mitotic cells, indicating dynamic expression during cell cycle progression. To delve deeper into this, the impact of cell cycle perturbations on Lockd expression was assessed. Cells treated with Mitomycin C, which arrests the cell cycle at the G1 phase, displayed a significant down-regulation of Lockd expression, suggesting its tight link to the active cell cycle, possibly controlling cell proliferation. Conversely, short-term Nocodazole treatment, blocking cells in the G2/M phase, led to the upregulation of Lockd expression, further supporting its dynamic regulation during cell cycle phase transitions and suggesting its involvement in cellular processes related to cell cycle progression.

Curiously, a prior transcriptomic investigation involving NIH/3T3 cells subjected to Nocodazole treatment to induce arrest in the G2/M cell cycle phase similarly identified Lockd as one of the prominently upregulated long non-coding RNAs [251]. Moreover, single-cell sequencing data from adult cardiac endothelial cells highlighted Lockd's prominent expression in a cluster associated with cell cycle and cell division.

The prominence of Lockd as the most up-regulated lncRNA within the cluster of single-cell sequencing data linked to cell cycle and cell division strongly suggests its pivotal role in regulating these processes in endothelial cells. The specific up-regulation of Lockd in this cluster indicates its active involvement in controlling and coordinating endothelial cell proliferation, a key aspect of neovascularization during cardiac development and remodelling [103, 149]. This finding also suggests that Lockd may function as a dynamic regulator, responding to signals and cues that trigger endothelial cells to enter the cell cycle and undergo mitosis. Tight control over endothelial cell proliferation and cell cycle progression is crucial for vascular integrity.

Functional implications of Lockd were explored through bulk RNA sequencing analysis on endothelial cells treated with siLockd and siCtrl. The data revealed significant alterations in gene expression, particularly upregulation of cell cycle-related genes and downregulation of genes linked to the regulation of blood vessel development, ECM organization, and heart development.

The elevation of differentially regulated cell cycle-related genes following Lockd knockdown underscores Lockd's notable upregulation in mitotic endothelial cells, providing evidence for its involvement in the modulation of cell cycle progression and division within endothelial cells during cardiac development and remodelling.

The identification of Uhmk1 as the most significantly upregulated protein-coding gene upon Lockd knockdown in C166 endothelial cells was intriguing for several reasons. Despite limited knowledge about Uhmk1's specific function in endothelial cell biology, it is known as a serine/threonine protein

kinase involved in facilitating cell cycle progression from G1 to S phase [187]. This finding suggests that Lockd may indirectly influence crucial cell cycle processes through its regulatory effects on Uhmk1 *in vitro*. Adding to the significance of the Lockd-Uhmk1 relationship is the role of Cdkn1b as a cyclin-dependent kinase inhibitor in maintaining cells in the G1 phase.

In normal conditions, Uhmk1 phosphorylates Cdkn1b at position Ser10, leading to its nuclear export and subsequent degradation, which is essential for promoting cell cycle progression and cellular proliferation [193, 274]. The observed increase in Cdkn1b phosphorylation upon Lockd knockdown suggests that Lockd may normally act as a negative regulator of Uhmk1 or the downstream signalling pathways that lead to Cdkn1b phosphorylation and degradation. This indicates an indirect regulation of Cdkn1b by Lockd.

By influencing Uhmk1 expression, Lockd can indirectly impact the levels and phosphorylation status of Cdkn1b, ultimately affecting cell cycle progression and cellular proliferation. Subsequently, an attempt was made to counteract the up-regulation of Uhmk1 prompted by Lockd knockdown by simultaneously employing siRNA targeting Uhmk1 and Lockd. This approach aimed to discern whether attenuating Uhmk1 mRNA levels could mitigate the augmented cellular proliferation attributed to Lockd knockdown. Indeed, the finding that combined knockdown of both Lockd and Uhmk1 can rescue the phenotype of increased cell proliferation *in vitro* further strengthens the link between Lockd and Uhmk1 in regulating endothelial cell behaviour. It implies that the dysregulation of Uhmk1 upon Lockd knockdown is, in part, responsible for the observed increase in cell proliferation and that these two molecules may function in a coordinated manner to control cell cycle progression and maintain cellular homeostasis.

Collectively, these observations indicate that Lockd might indirectly support its neighbouring gene Cdkn1b to potentiate the inhibitory effect on cell cycle progression and cellular proliferation by triggering a regulatory loop involving its reduced posttranslational modification and protection from proteasomal degradation.

Furthermore, the downregulation of genes associated with ECM organization upon Lockd knockdown suggests Lockd's involvement in modulating the synthesis or remodelling of the ECM in endothelial cells. Proper ECM organization is vital for tissue integrity and function. Alterations in the ECM can influence endothelial cell behaviour, affecting processes such as angiogenesis and tissue repair [304, 305].

These results highlight Lockd's potential role in various biological processes, including cell cycle regulation, apoptosis, ECM organization, and angiogenesis. Its dynamic expression during cell cycle progression suggests involvement in promoting cell proliferation and maintaining cellular quiescence.

Considering these new findings along with the previous results, Lockd appears to be a dynamic regulator modulating endothelial cell function. It is upregulated in mitotic endothelial cells, potentially controlling cell cycle progression and proliferation during angiogenesis. On the other hand, Lockd knockdown results in increased cell proliferation and upregulation of cell cycle-related genes, whereas Lockd overexpression results in decreased cell proliferation, suggesting it may restrain or balance cell proliferation during angiogenesis.

The specific up-regulation of Lockd in mitotic endothelial cells, both *in vitro* and *in vivo* within cardiac endothelial cells and the differential expression of cell cycle related genes is a compelling observation. This suggests a close link between Lockd expression and the active cell cycle, particularly during cell division, further emphasizing its role in promoting cell proliferation and division.

To delve deeper into Lockd's functional implications in cell cycle regulation, knockdown and overexpression experiments were conducted. Flow cytometry analysis of propidium iodide stained cells further supported that Lockd knockdown resulted in increased endothelial cell proliferation, evidenced by a higher number of cells entering the S phase of the cell cycle. This indicates that Lockd may actively inhibit cell proliferation, and its down-regulation could lead to a more active cell cycle with increased cell division.

Conversely, Lockd overexpression showed the opposite effect, with fewer cells in the G2/M phase and more in the G0/G1 phase of the cell cycle. This suggests that Lockd overexpression may inhibit cell cycle progression, potentially leading to fewer cells undergoing division. Additionally, Lockd overexpression was associated with reduced apoptotic activity, indicating a potential role for Lockd in inhibiting apoptosis and promoting cell survival in the quiescent phase.

These findings are consistent with the regulation of Cdkn1b, where increased Cdkn1b levels promote cell cycle arrest in the quiescent G0/G1 phase, while Cdkn1b degradation facilitates the entry of more cells into the S phase of the cell cycle [185, 271, 273].

6.7 Role of Lockd in Cardiac Remodelling: Insights from *In Vivo* Knockdown Studies

To investigate Lockd's impact on heart function and cardiac remodelling, Antisense LNA GapmeRs were utilized to knockdown Lockd expression *in vivo*. Under normal heart conditions without induced cardiac stress, Lockd knockdown did not significantly alter heart function or other physiological parameters, suggesting limited influence under normal physiological conditions in adult hearts. However, the scenario changed when Lockd knockdown was performed following 2 weeks of transverse aortic constriction.

In the two week TAC-induced stress setting, the mice with Lockd knockdown exhibited several positive outcomes. They displayed a reduced heart-to-body weight ratio, improved systolic heart function, at one-week and two weeks post-TAC, and an attenuated hypertrophic response compared to control mice. These findings imply that Lockd may specifically play a role in cardiac remodelling during stress conditions, such as pressure overload.

Furthermore, the results indicated a trend towards decreased fibrosis in the Lockd knockdown mice following TAC induction. Additionally, Lockd knockdown led to increased capillary density, reduced cardiomyocyte hypertrophy, and altered expression levels of hypertrophic markers such as Nppa and Nppb. These findings suggest that Lockd knockdown may offer protection against adverse cardiac remodelling, potentially through its effects on vascularization and hypertrophy.

Integrating these new findings with the previous results, it becomes evident that Lockd is intricately involved in cardiac remodelling and function, particularly under stress conditions. The earlier findings of Lockd's upregulation in cardiomyocytes and endothelial cells after TAC support its relevance in the cardiac response to stress signals. The current results complement this understanding by demonstrating the beneficial effects of Lockd knockdown on systolic heart function, reduced hypertrophic response, increased capillary density, and altered hypertrophic markers.

To delve deeper into the biological processes involving Lockd bulk RNA sequencing on whole heart tissue and isolated cardiac endothelial cells two weeks after transverse aortic constriction induction was conducted.

In whole heart tissue, Lockd knockdown triggered significant changes in gene expression, particularly related to the glycolipid catabolic process and regulation of angiogenesis. These findings reinforce the idea that Lockd may have a substantial impact on various aspects of heart physiology and vascular development.

Similarly, in cardiac endothelial cells, Lockd knockdown affected mRNA processing, protein folding, heart contraction, and potentially neutrophil degranulation and cell activation.

To directly investigate Lockd's impact on cardiomyocyte hypertrophy neonatal rat cardiomyocytes stimulated with phenylephrine to induce hypertrophy were utilized [306]. The knockdown of Lockd in these cardiomyocytes resulted in reduced cell area, implying that Lockd may play a regulatory role in cardiomyocyte growth and hypertrophy.

The collective evidence from the study supports the notion that Lockd plays a significant role in cardiac remodelling and the response to stress conditions. It seems to modulate various biological processes, including vascularization, cardiomyocyte hypertrophy, and gene expression in both cardiac endothelial cells and cardiomyocytes.

Echocardiography analysis revealed an improved heart function upon Lockd knockdown as early as 1 week after induced pressure overload. My objective was to investigate the consequences of Lockd knockdown at its highest expression level, which coincides with the one-week time-point after transverse aortic constriction. The knockdown of Lockd one-week after transverse aortic constriction surgery led to several interesting observations. qPCR analysis demonstrated an efficient knockdown of Lockd in cardiac ECs (> 50%) at the time-point when Lockd expression was highest during induced cardiac pressure overload. This level of knockdown in cardiac endothelial cells was notably higher than the overall knockdown observed in the heart tissue (30%). Considering this, it is reasonable to speculate that the improved heart function at this time-point is primarily attributable to Lockd knockdown in cardiac ECs. However, it is essential to bear in mind that knockdown in other cell types could potentially impact result interpretation.

Interestingly, no knockdown of Lockd was observed in cardiac fibroblasts at both time-points. If a similar phenotype to what was observed in endothelial cells, such as increased proliferation upon Lockd knockdown, were also seen in fibroblasts, one would expect that knockdown of Lockd in fibroblasts would lead to increased fibroblast proliferation under the stress stimuli induced by cardiac pressure overload. However, since the GapmeRs used did not effectively knock down Lockd in cardiac fibroblasts, there were no significant differences in fibrosis between the Lockd knockdown and control groups at one and two weeks after induced cardiac pressure overload as shown by the Sirius red staining. A trend indicating reduced fibrosis was discerned at the two-week TAC time point. It is plausible that with extended Lockd knockdown over an extended period after TAC, a notable decrease in fibrosis could be observed. This presumption is based by the observed downregulation in the expression of extracellular matrix-associated genes across the heart tissue at both the one-week and two-week TAC time points.

The increased capillary density and reduced cardiomyocyte hypertrophy observed at one-week post TAC in Lockd knockdown mice were interesting. These changes in cardiac structure and vasculature

were already visible at an early time-point, indicating that Lockd knockdown may have rapid effects on vascular remodelling and cardiomyocyte hypertrophy. This is significant as it suggests that Lockd plays a role in these processes during the early phase of cardiac remodelling, and its knockdown, when its expression would otherwise peak, can exert rapid effects on cardiac morphology and vasculature.

Furthermore, the bulk RNA sequencing analysis revealed significant changes in gene expression profiles in both whole heart tissue and cardiac endothelial cells one-week after Lockd knockdown. Notably, the downregulation of cell cycle-related genes in cardiac endothelial cells was of particular interest. The downregulation of genes involved in cell cycle progression, DNA replication, cell cycle checkpoint regulation, and other cell cycle processes suggests that Lockd knockdown may influence the cell cycle machinery of cardiac endothelial cells. This finding implies that Lockd may have a potential role in modulating endothelial cell proliferation and function, which is critical for angiogenesis and vascular remodelling during this early stage of cardiac development and remodelling.

The contrasting effects observed when knocking down Lockd in different cellular contexts raise intriguing possibilities for understanding its diverse roles in cell-cycle regulation. *In vitro* knockdown of Lockd in C166 cells resulted in upregulation of cell-cycle-related genes, suggesting a potential role of Lockd as a suppressor of cell cycle progression or an inhibitor of specific cell cycle regulators. Conversely, *in vivo* knockdown of Lockd in cardiac endothelial cells one-week after transverse aortic constriction led to downregulation of cell-cycle-related genes. This response might represent a compensatory mechanism in response to increased capillary density, as sufficient capillary density might already have been achieved, signifying a state of reduced proliferation or transition to a more quiescent cellular state.

The observed variations could also be influenced by temporal dynamics, as gene expression and cellular responses are known to fluctuate over time, a pattern also seen in Lockd expression during pressure overload. The timing of Lockd knockdown and the progression of pressure overload-induced changes in cardiac endothelium could influence gene expression of cell cycle related genes. Cardiac endothelial cells one-week after TAC may be at a distinct phase of the cell cycle or exhibit different cellular responses compared to non-Lockd-knockdown endothelial cells [302].

Furthermore, the contrasting effects might stem from compensatory mechanisms activated in response to Lockd knockdown. In C166 cells, increased endothelial proliferation and upregulation of cell cycle genes could compensate for the loss of Lockd function. Conversely, in cardiac endothelial cells, the increased capillary density could arise from alternative compensatory mechanisms independent of cell cycle regulation.

In contrast, we observed a significant upregulation of genes involved in key processes such as the regulation of angiogenesis, response to retinoic acid, and the Rho GTPase cycle.

At the one-week time-point following Lockd knockdown in the whole heart tissue, we observed a noteworthy upregulation of genes involved in angiogenesis, endothelial cell differentiation, and the regulation of endothelial cell proliferation. Additionally, genes associated with the Rho GTPase cycle and response to retinoic acid were also upregulated, implying a potential influence of Lockd on endothelial cell migration and cytoskeletal rearrangements, which could contribute to the observed effects on angiogenesis as well as modulating signalling pathways associated with cell differentiation and proliferation.

Conversely, there was a downregulation of genes related to neutrophil degranulation, extracellular matrix organization, and the regulation of MAPK signalling in the whole heart tissue at the one-week TAC Lockd knockdown time-point.

Interestingly in the whole heart and in cardiac endothelial cells, one-week after TAC and in cardiac endothelial cells 2 weeks after TAC the gene Cyp26b1 was found to be by far the most upregulated gene upon Lockd knockdown ($\log_2FC > 2$). This is interesting because Cyp26b1 is primarily responsible for the breakdown and clearance of retinoic acid from cells and tissues [307]. By controlling the levels of retinoic acid (RA), Cyp26b1 indirectly influences the transcriptional regulation of numerous target genes. RA acts as a ligand for specific nuclear receptors, known as retinoic acid receptors (RARs), which bind to specific DNA sequences, thereby affecting gene expression [308, 309]. During development it is involved in regulating cell proliferation, differentiation, and apoptosis during organogenesis [310, 311]. For the future direction of this project, it would be interesting to study the link between Lockd knockdown and the resulting up-regulation of Cyp26b1.

Furthermore, we could not detect a different expression of Uhmk1 following Lockd knockdown post-TAC in any of the bulk RNA sequencing data. From this, it can be concluded that perhaps the specific time point by which the differential regulation of Uhmk1 is caused by Lockd knockdown in the one and two week TAC time point could not be grasped. Alternatively, it is plausible that the regulatory mechanism governing Uhmk1 regulation following Lockd knockdown in continuously proliferating immortalized cells differs from that operating in the context of cardiac stress-induced proliferation in cardiac endothelial cells.

Utilizing antisense LNA GapmeRs to achieve cardiac endothelial-specific knockdown of long non-coding RNAs, such as Lockd, in mice provides a valuable research tool, but it presents certain limitations and challenges.

One important consideration is the possibility of off-target effects on genes with partial complementarity to the GapmeRs. These off-target effects may unintentionally lead to knockdown of unrelated genes, potentially confounding the experimental results [312, 313].

Efficient and targeted delivery of LNA GapmeRs to cardiac endothelial cells represents a significant challenge. Ensuring effective uptake by the intended cells is crucial for the success of the knockdown procedure.

For LNA GapmeRs to remain effective throughout the experimental duration, they need to be stable in the *in vivo* environment. Rapid degradation or clearance by endogenous nucleases could limit their efficacy.

Additionally, determining the appropriate dosage of LNA GapmeRs is critical. An excessively high dose might lead to off-target effects or unintended toxicity, while a too low dose may not achieve sufficient knockdown, limiting the ability to observe the desired effects. Based on these observations, a total dosage higher than 20 mg/kg did not result in a more efficient Lockd knockdown in cardiac ECs, whereas 5 mg/kg per injection was the minimum GapmeR dosage used in other GapmeR studies [314-318].

Ensuring the specificity of LNA GapmeRs to cardiac endothelial cells is also crucial. An efficient Lockd knockdown in both the heart and cardiac ECs was achieved by injecting four doses of 5 mg/kg GapmeRs over a two-week period. However, a significant knockdown of Lockd in cardiomyocytes was also observed, raising the possibility that the improved heart function upon pressure overload may, in part, be attributed to Lockd knockdown in cardiomyocytes. Indeed, a very efficient knockdown of Lockd in cardiomyocytes of 80 % was quantified, probably contributing most to the overall Lockd knockdown in the whole heart tissue of around 70 % at the 2-week TAC time-point.

LNA GapmeRs typically induce transient knockdown of the target lncRNA [319, 320]. Exploring the long-term effects of Lockd knockdown in pathophysiological cardiac remodelling upon induced cardiac pressure overload would be intriguing. It would be interesting to see how long term Lockd knockdown would influence pathological cardiac remodelling. It would be interesting to see if the beneficial effects upon Lockd knockdown would still persist at these longer time-points. However, the prolonged use of LNA GapmeRs requires careful assessment, as it may lead to potential off-target effects or unintended alterations in cellular processes.

6.8 Potential role of Lockd in translational regulation, cellular proliferation and signalling pathways in endothelial cells

To explore Lockd's potential protein binding partners, two distinct RNA pulldown assays were utilized: RNA antisense purification (RAP-MS) and ChIRP-Mass spectrometry (ChIRP-MS) [256, 257]. These assays enabled the identification of proteins that interact with Lockd. Notably, the overlap between the results from both RAP-MS and ChIRP-MS experiments revealed a set of proteins that potentially interact with Lockd. The pulldown approach identified ribosomal proteins as the most significantly enriched proteins. This intriguing observation suggests that Lockd, despite being a non-coding RNA, may be involved in ribosome-related processes, potentially influencing translation processes.

To confirm the interactions identified in the pulldown experiments, RNA immunoprecipitation (RIP) were then conducted which validated the specific interaction of Lockd with ribosomal protein Rps6 and Rack1. Moreover, subcellular co-localization experiments demonstrated that Lockd and the ribosomal protein Rps6 primarily co-localized in the perinuclear region of the endothelial cells, similar to the co-localisation of Rps6 and 18S, suggesting their potential functional association. This suggests that Lockd's interactions with ribosomal proteins may occur in specific subcellular compartments actively involved in translation processes.

The identification of Lockd's interactions with the ribosomal protein Rps6 and the kinase Rack1, despite being a non-coding RNA, is particularly intriguing. Ribosomes are essential for protein synthesis, and their proper functioning is crucial for cellular homeostasis [200, 282, 321]. While lncRNAs were once considered transcriptional noise, growing evidence indicates their dynamic roles in gene regulation and cellular processes including the regulation of translation/protein synthesis (?)

The specific binding of Lockd to Rps6 is a compelling finding with potential implications for proliferation, given the crucial role of Rps6 in regulating protein synthesis, cell growth, and proliferation [81, 322]. Rps6 specifically interacts with RNAs that have a TOP (Terminal Oligopyrimidine) motif, a regulatory element found in the 5' untranslated region (UTR) of certain mRNAs [282, 323-327]. The presence of the TOP motif in the UTRs of specific mRNAs allows for their selective translation [328, 329]. Despite not having a TOP motif itself, it is intriguing that Lockd binds to Rps6.

Rps6 is a known target of the mTOR pathway, a central regulator of cell growth and proliferation. When activated, mTOR initiates the phosphorylation of Rps6, initiating protein synthesis and promoting cell cycle progression from G1 to S phase. This phase is critical for DNA replication and cell division, making the regulation of Rps6 essential for proper cell cycle control and cellular proliferation [81, 85, 322]. Interestingly, mTor mRNA was found to be significantly up-regulated upon Lockd

knockdown. Furthermore, the observation that Lockd is upregulated in mitotic endothelial cells and that its knockdown leads to increased cell proliferation further supports the hypothesis that Lockd-Rps6 interaction may play a pivotal role in endothelial cell proliferation machinery.

The interaction between Lockd and Rps6 therefore suggests a potential regulatory mechanism where Lockd may influence Rps6 and modulate the activation of the mTOR pathway.

Rack1, also known as Small Ribosomal Subunit Protein RACK1, is an integral component of the ribosome. Its presence within the ribosomal complex emphasizes its significance in cellular translation processes and underscores its potential role in influencing translation-related mechanisms [330]. The interaction between Lockd and Rack1 is particularly interesting due to Rack1's involvement in various cellular processes including cell cycle regulation and cell proliferation. Rack1 serves as a scaffolding protein and is a key component of several signalling pathways that govern cell growth, proliferation, and survival [330, 331].

Rack1's role in regulating the cell cycle is notable, as it interacts with cyclin-dependent kinases (CDKs) and plays a part in controlling cell cycle checkpoints, ensuring proper progression through different phases. By modulating CDK activity, Rack1 helps regulate DNA replication, cell division, and cell cycle exit [162, 284].

Furthermore, Rack1's role in modulating apoptosis is noteworthy. Its interaction with various signalling proteins contributes to these effects [332-334]. The reduced Caspase3/7 activity upon Lockd overexpression suggests that Lockd may play a role in inhibiting apoptosis, potentially through its association with Rack1 or other apoptosis-related signaling pathways.

The specific binding of Lockd to Rps6 and Rack1 suggests that Lockd may influence both proteins activity and modulate downstream signalling pathways related to cell cycle regulation. Consequently, Lockd might have a direct impact on the cell cycle progression of endothelial cells during cardiac remodelling, which could be vital for processes like angiogenesis and overall cardiac function.

Together, Lockd's association with ribosomal proteins Rps6 and Rack1 could potentially influence the translation of specific mRNAs involved in angiogenesis, and cellular proliferation. Through such influence on protein synthesis.

The identification of Lockd's binding to ribosomal proteins, particularly Rps6, hinted at its potential involvement in regulating translation processes. Investigating Lockd's impact on translation and *de novo* protein synthesis is essential for understanding its functional role. To delve deeper into this aspect, Puromycin Sunset assay was conducted, which assesses ongoing protein synthesis activity.

The results of the Puromycin Sunset assay were intriguing. Lockd overexpression led to a significant increase in *de novo* protein synthesis, despite being a non-coding RNA incapable of coding proteins itself. This finding further indicates that Lockd may function as a regulatory molecule, influencing the translation of mRNAs.

Conversely, Lockd knockdown did not result in significant changes in *de novo* protein synthesis, despite its interactions with ribosomal proteins like Rps6. This may seem counterintuitive at first given Lockd's potential role in translation regulation. However, protein synthesis is controlled by complex pathways involving multiple factors and signalling cascades. The lack of significant changes after Lockd's lockdown could be due to specific populations of RNAs being differentially translated rather than the entire proteome decreasing or decreasing.

Although Lockd interacts with Rps6, Western blot analysis of Lockd knockdown or overexpression did not significantly alter the ratio of phospho-Rps6 to total Rps6. This implies that Lockd's influence on translation might not involve direct modulation of Rps6 phosphorylation.

Taken together, the interactions between Lockd and ribosomal proteins Rps6 and Rack1 suggest its potential involvement in translation-related processes, however not via the modulation of Rps6 phosphorylation. It could be that Lockd might selectively influence the translation of specific mRNAs or function in a context-dependent manner.

In order to gain a more comprehensive understanding of Lockd's potential mechanism of action on translation, proteomic analysis on endothelial cells was conducted. This involved both knocking down Lockd using siRNA and overexpressing Lockd using an Adenovirus.

Lockd knockdown resulted in notable changes in the cellular proteome, with several proteins showing significant upregulation and downregulation. Gene Ontology (GO) enrichment analysis of the upregulated proteins upon Lockd knockdown revealed an enrichment of proteins associated with the G13 signalling pathway. The G13 pathway is known for its role in actin polymerization and cytokinesis, which are crucial for cell division and cellular architecture [335, 336]. The upregulation of proteins related to the G13 signalling pathway, such as Calm1, Pik3ca, Myo19, and Dynll1, suggests that Lockd may influence cytokinesis and actin dynamics, potentially impacting cellular proliferation [337].

Additionally, Lockd knockdown led to an upregulation of proteins involved in cilium assembly, including centrosomal proteins like Cep350, Cep162, and Cep164 [258]. Centrosomes are essential for proper cell division, spindle assembly, and chromosome segregation during cell division, and their dysregulation can have implications for cell cycle progression and genomic stability [338]. The

upregulation of these centrosomal proteins suggests that Lockd might play a role in ensuring accurate cell division and maintaining cellular homeostasis.

Conversely, the downregulation of specific proteins upon Lockd knockdown indicates its involvement in regulating key cell-cycle related processes in endothelial cells. Notably, Rrm1, Mettl13, Cdk10, and Atrip, which were downregulated upon Lockd knockdown, are involved in the regulation of cell cycle phase transitions, specifically controlling the transition from the G1 to the S phase [258, 339-342]. This finding suggests that Lockd might play a role in modulating endothelial cell proliferation and cell cycle progression.

Lockd's specific binding interactions with ribosomal proteins indicate its potential involvement in modulating translational processes. The observed upregulation of proteins associated with the G13 signaling pathway and cilium assembly upon Lockd knockdown aligns with its specific upregulation in mitotic endothelial cells, indicating a potential role in cytokinesis and cell cycle regulation during active cell division. Moreover, the downregulation of proteins involved in the negative control of cell cycle phase transition upon Lockd knockdown further supports its potential role in modulating endothelial cell proliferation and cell cycle progression.

The proteomic analysis of Lockd overexpression in endothelial cells also revealed significant changes, providing further insights into Lockd's functional role in cellular processes which are potentially happening during cardiac pressure overload in cardiac endothelial cells.

Lockd overexpression led to the upregulation of proteins associated with Metaphase plate congression, such as Mlh1, Ska1, Nup62, Rps27, Lsm3, Hps6, Dctn2, and Llg1 [258]. These proteins play pivotal roles during metaphase, ensuring the accurate alignment of chromosomes along the metaphase plate, which is essential for proper chromosome segregation during cell division [343]. The upregulation of these proteins suggests that Lockd overexpression might influence the precise distribution of genetic material during cell division, potentially impacting genomic stability and cellular inheritance.

Conversely, Lockd overexpression resulted in the downregulation of proteins associated with mitotic sister chromatid segregation, such as Hsf1, Casp12, Dnajb12, Kif11, Kif18b, Flna, and Chmp1a [258]. These proteins are crucial for the accurate separation of sister chromatids during mitosis, contributing to faithful chromosome distribution to daughter cells [344]. The downregulation of these proteins upon Lockd overexpression suggests that Lockd might inhibit or retard the machinery involved in chromosome segregation during cell division, potentially affecting cell proliferation and genomic integrity. Prior research has demonstrated that kinesins, essential proteins involved in various cellular

processes like mitosis and intracellular transport of organelles and vesicles, including Kif11, exhibit distinct patterns of regulation in response to Lockd expression [251].

Additionally, Lockd overexpression caused the upregulation of proteins related to post-translational protein phosphorylation, including Fstl1, Men1, and Nub1. Post-translational protein phosphorylation plays a regulatory mechanism that modulates protein activity and function, affecting various cellular processes [345].

Furthermore, Lockd overexpression resulted in the downregulation of proteins associated with positive regulation of protein folding, such as Pdia3, Pdia4, Pofut2, Casp12, Dnajb12, Sec61b, and Tor1b. Proper protein folding is essential for cellular function and health, and the downregulation of these proteins indicates that Lockd overexpression may impair cellular protein homeostasis and folding processes, potentially influencing cellular function and adaptation [346].

Additionally, proteins associated with mRNA transcription, including Hsf1, Hipk3, Flna, Taf7, Celf2, Arvcf, Trmt6, and Pus7l, were downregulated upon Lockd overexpression. This suggests that Lockd overexpression may have broader implications for gene expression regulation and cellular function, potentially impacting transcriptional activity.

The comparison of differentially regulated proteins and genes upon Lockd knockdown revealed that there was a surprisingly low overlap between the two datasets. Among the top differentially regulated proteins, only Cep164 showed upregulation at both the mRNA and protein levels, while Gpsm1, Sccpdh, Hmgcs1, Dag1, Lnpep, and Nln were found to be downregulated at both levels. This discrepancy suggests that a significant portion of the observed changes in protein expression is likely regulated at the post-transcriptional level through differential translation upon Lockd modulation.

The significant number of differentially regulated proteins, in contrast to the limited overlap with the results from bulk RNA sequencing implies that Lockd may exert its influence on protein expression by modulating translation rather than solely through changes in gene expression at the transcriptional level.

We observed several important regulator proteins that exhibited differential regulation solely at the protein level, one of which was Pik3ca (Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha). Pik3ca was found to be upregulated in endothelial cells upon Lockd knockdown ($\text{Log}_2\text{FC} = 0.22$; $p\text{-value} = 0.027$). Elevated Pik3ca levels can lead to the hyperactivation of the Pi3k-Akt-mTOR (Mechanistic Target of Rapamycin) signalling pathway [347]. This pathway plays a crucial role in regulating various cellular processes, and its dysregulation can significantly impact endothelial cell behaviour and function. The Pi3k-Akt-mTOR pathway, promotes crucial endothelial cell processes,

including proliferation, migration, and tube formation, all of which are essential for angiogenesis [340, 348]. Additionally, the upregulation of *Pik3ca* may influence endothelial cell differentiation and specialization, potentially affecting the differentiation of endothelial progenitor cells into mature endothelial cells, a process critical for vessel maturation and stabilization [160].

Another protein that showed upregulation in endothelial cells upon *Lockd* knockdown was *Dynll1* (Dynein Light Chain LC8-type 1) ($\log_2FC = 0.31$; $p\text{-value} = 2e^{-16}$). *Dynll1* has been associated with cell cycle regulation and cellular proliferation. Its upregulation in endothelial cells might influence cell cycle progression and cellular proliferation rates, thereby potentially impacting endothelial cell turnover and tissue homeostasis, ultimately promoting angiogenesis [349-351].

Additionally, a significant downregulation of *Mettl13* (Methyltransferase-like protein 13) in endothelial cells upon both *Lockd* knockdown ($\log_2FC = -0.51$; $p\text{-value} = 2e^{-16}$) and *Lockd* overexpression ($\log_2FC = -0.36$; $p\text{-value} = 0.0007$) was observed. *Mettl13* is a methyltransferase enzyme involved in post-transcriptional RNA modification. *Mettl13* is known to catalyse the addition of methyl groups to specific RNA molecules [352]. Down-regulation of *Mettl13* could lead to altered RNA methylation patterns in endothelial cells, potentially affecting RNA stability, splicing, and translation [353]. Changes in RNA methylation could impact gene expression by affecting RNA processing and translation efficiency. Down-regulation of *Mettl13* might lead to differential expression of genes involved in endothelial cell function, potentially influencing cellular phenotype.

Through a comparative analysis of all proteins that were specifically significantly up-regulated upon *Lockd* knockdown and significantly downregulated upon *Lockd* overexpression, an observation was made. A significant number of proteins related to translation and ribosome biogenesis processes displayed up-regulation upon *Lockd* knockdown, whereas their expression was downregulated upon *Lockd* overexpression. Specifically, *Etf1*, *Rpl14*, *Cyp51a1*, *Larp4*, *Nob1*, *Dimt1*, and *Ddx49* are involved in translation regulation and ribosome assembly [258], adding further complexity to the potential influence of *Lockd* on cellular protein synthesis machinery.

The comparative analysis of proteins specifically down-regulated upon *Lockd* knockdown and upregulated upon *Lockd* overexpression revealed interesting patterns associated with DNA damage response, mRNA processing, and Rho GTPase signalling. Proteins related to the DNA damage response, including *Plk1*, *Hltf*, *Zfp385a*, *Cep164*, and *Fam174b*, showed upregulation upon *Lockd* overexpression and downregulation upon *Lockd* knockdown [258].

The upregulation of DNA damage response proteins, upon Lockd overexpression suggests that Lockd might be involved in regulating DNA repair mechanisms. DNA damage is a common cellular stress that can lead to genomic instability and potentially contribute to the development of various diseases, including cancer. The differential regulation of these proteins upon Lockd knockdown and Lockd overexpression implies that Lockd might play a role in coordinating the cellular response to DNA damage, ensuring genomic integrity, and maintaining cell viability.

Additionally, upon Lockd overexpression and downregulation, certain proteins involved in mRNA processing exhibited altered expression levels. Specifically, *Arvcf*, *Hltf*, and *Taf7* were upregulated upon Lockd overexpression and downregulated upon Lockd knockdown [258]. mRNA processing is a crucial step in controlling gene expression, influencing mRNA stability, splicing, transport, and translation. Dysregulation of mRNA processing can lead to aberrant gene expression patterns and contribute to disease pathogenesis [354].

Furthermore, the upregulation of *Rhod*, *Gja1*, and *Plk1* upon Lockd overexpression and downregulation suggests a potential role for Lockd in modulating Rho GTPase signaling. Rho GTPases are critical regulators of various cellular processes, including proliferation, cytoskeletal dynamics, and gene transcription. The differential expression of these proteins upon Lockd modulation indicates that Lockd might influence Rho GTPase-mediated signalling pathways, thereby affecting endothelial cell behavior and function.

6.9 Chromatin interaction analysis and gene expression modulation

Considering the significant presence of Lockd in the nucleoplasm and the well-established role of various long non-coding RNAs in directly influencing gene expression through chromatin interactions, the possibility that Lockd might exert its effects on endothelial cell function as well through direct binding to genomic sequences within gene regions became intriguing [211, 216, 217, 219, 221, 230, 286]. Therefore, the presence of Lockd in the nucleoplasm raises the possibility that it may function as a regulatory molecule that directly modulates gene expression in endothelial cells.

The ChIRP technique revealed that Lockd has the ability to bind directly to specific chromatin regions in endothelial cells. The enrichment of Lockd in non-RNase A-treated ChIRP samples confirmed the successful capture of Lockd-associated chromatin regions, and the high degree of overlap between the even and odd probe peaks indicates the specificity of the ChIRP probes for targeting Lockd-bound DNA fragments. Furthermore, the concentration of Lockd peaks at the transcription start site (TSS) of

genes is intriguing, as it suggests that Lockd may play a direct regulatory role in initiating transcription or modulating the expression of nearby genes.

The overlap analysis of Lockd-bound genomic regions with differentially regulated genes identified from siRNA bulk RNA sequencing data revealed a relatively low level of overlap, with only 44 genes being both differentially regulated and potentially bound by Lockd. The Gene Ontology (GO) analysis of these genes points to potential cellular pathways impacted by Lockd, particularly MAPK signalling and the regulation of mitotic cell cycle. This suggests that Lockd may have a direct role in modulating cell cycle progression and intracellular signalling pathways.

Interestingly, only three genes (*Slc29a1*, *Edf1*, and *Cep164*) were found to be differentially regulated at both the RNA and protein levels and were directly targeted by Lockd, indicating that Lockd's regulatory actions may not always lead to differential expression of its target genes. This observation suggests that Lockd's regulatory role may be more complex and multifaceted, involving various mechanisms beyond direct gene expression modulation.

Subsequent qPCR analysis confirmed the differential expression of genes directly bound by Lockd, such as *Dusp6*, *Nras*, and *Pbx1*, upon Lockd knockdown. However, for other genes where Lockd was presumed to bind, such as *Taok2*, *Nuf2*, and *Usp22*, no significant differential expression was observed. This further supports the idea that Lockd's regulatory actions are selective and context-dependent, impacting specific subsets of genes in endothelial cells.

6.10 Conclusion

The initial study of Lockd's involvement revealed that its transcript is dispensable for directly regulating the adjacent *Cdkn1b* gene's transcription, while the Lockd genomic locus contains regulatory elements influencing *Cdkn1b* expression. Furthermore, in this study evidence was found indicating that the Lockd transcript indirectly affects *Cdkn1b* degradation, confirming Lockd's multiple mechanism of action [250].

Interestingly, a previous transcriptomic analysis of Nocodazole-treated NIH/3T3 cells, inducing G2/M cell cycle arrest, identified Lockd as one of the most highly upregulated lncRNAs. Similarly, my investigation identified Lockd's upregulation in mitotic endothelial cells, implying a conserved regulatory role for Lockd within cell cycle progression. However, their study found kinesin downregulation upon Lockd knockdown, including *Kif11* [251], while C166 cell analysis did not exhibit *Kif11* transcriptomic changes post-Lockd knockdown. Intriguingly, proteomic analysis indicated Lockd

overexpression causing Kif11 downregulation, potentially signifying a divergent effect in C166 cells compared to NIH/3T3 cells, underscoring Lockd's potential involvement in mitotic spindle dynamics.

In a more recent study, Lockd was revealed to promote myoblast proliferation and muscle regeneration by binding with Dhx36 to facilitate 5' UTR rG4 unwinding and Anp32e translation [252]. This finding aligns with our observations of Lockd's potential influence on the translation of specific protein subsets. However, our pull-down assays did not identify DHX36 as a Lockd interaction partner, and no differential Anp32e levels were detected during proteomic analysis. Additionally, this study demonstrated Lockd's promotion of myoblast proliferation, contrasting with our finding of Lockd inhibiting endothelial cell proliferation. This highlights diverse mechanisms of Lockd action in myoblasts and endothelial cells, potentially emphasizing cell-specific effects of Lockd and lncRNAs in general.

In the latest research that studied Lockd, an investigation into its mechanistic involvement in the regulation of autophagy via the mTOR signaling pathway was conducted. The study found that maternal obesity induced heightened expression of p-mTOR protein within offspring liver tissues. Notably, transfection with siRNA-lockd yielded augmented p-mTOR protein expression, thus implying Lockd's plausible role in autophagy modulation through the mTOR pathway. Furthermore, my experimentation revealed that Lockd knockdown in C166 endothelial cells elicited an up-regulation of the mTor transcript. The intriguing interaction between Lockd and Rps6, alongside discernible discrepancies in protein translation, could potentially underscore Lockd's influence on the mTOR signalling cascade. However, more research is needed in order to study this hypothesis.

In this investigation, it is deduced that upon the initiation of cardiac hypertrophy, there is a notable upregulation of Lockd expression in the heart, specifically observed in cardiomyocytes and cardiac endothelial cells. The increased expression of Lockd in response to hypertrophic signals suggests its potential role in the cellular response to stress stimuli. In particular, Lockd upregulation seems to modulate the response of cardiac endothelial cells to angiogenic cytokines to proliferate by delaying progression through the cell cycle.

It is plausible that Lockd acts as a modulator of gene expression via directly binding to regulatory elements in gene regions and by affecting translation, regulating key genes involved in endothelial cell cycle progression and angiogenic responses. The modulation of these processes by Lockd could slow down the proliferative endothelial cell behaviour, ultimately leading to impaired angiogenesis and adverse cardiac remodelling.

This inhibition of endothelial cell proliferation during the initial phase of cardiac remodelling may contribute to reduced angiogenesis, which is critical for vascular growth and adaptation during hypertrophy. Consequently, impaired angiogenesis may negatively impact the overall heart function during cardiac hypertrophy.

All in all, the current data suggest that Lockd may act as a negative feedback regulator of cell proliferation following a cardiac stress response, thus exacerbating cardiac dysfunction during pressure overload. Therefore, lockd inhibition might be considered as therapeutic strategy to counteract maladaptive cardiac remodelling and to promote angiogenesis.

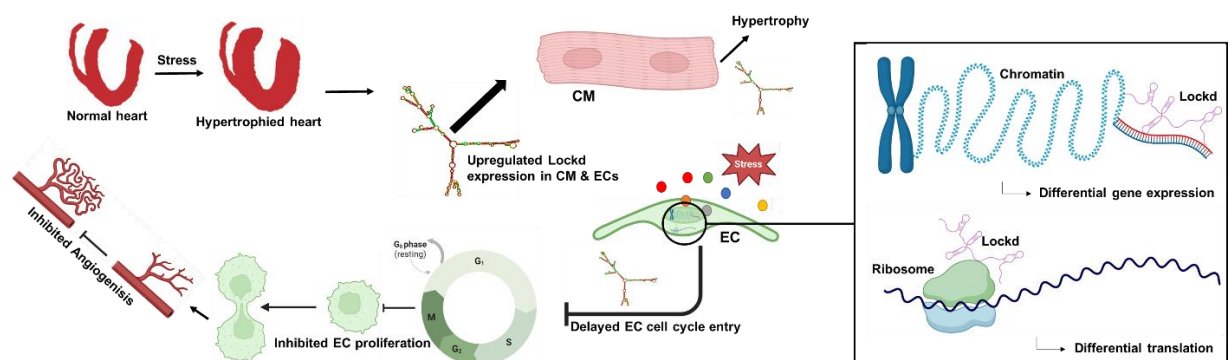


Figure 57: Proposed mechanism of action of Lockd

The onset of cardiac hypertrophy leads to the upregulation of Lockd in the heart, specifically in cardiomyocytes and in cardiac endothelial cells. The up-regulation of Lockd causes cardiac endothelial cells to have a delayed response from stress-stimuli such as angiogenic cytokines to progress through the cell cycle by differentially regulating gene expression and translation of a subset of genes. This leads to an inhibited endothelial cell proliferation, leading to an inhibited angiogenesis during the initial phase of cardiac remodelling, ultimately leading to a decreased heart function during cardiac hypertrophy. Illustration was created using: BioRender.com.

7. Appendix

Table 16: Top up-regulated proteins upon Lockd knockdown in C166 cells

Gene	Protein Description	logFC	p.Value
Dynll1	Dynein light chain 1, cytoplasmic	0.311	2.22E-16
Chst12	Carbohydrate sulfotransferase 12	0.247	8.43E-11
Rplp2	60S acidic ribosomal protein P2	0.208	9.40E-11
Pfdn4	Prefoldin subunit 4	0.260	7.21E-10
Ckmt1	Creatine kinase U-type, mitochondrial	0.225	9.01E-08
Mgp	Matrix Gla protein	0.240	2.35E-07
Trak1	Trafficking kinesin-binding protein 1	0.269	2.77E-07
Nudcd2	NudC domain-containing protein 2	0.216	7.79E-06
Chac2	Putative glutathione-specific gamma-glutamylcyclotransferase 2	0.325	1.41E-05
Dbi	Acyl-CoA-binding protein	0.233	1.83E-05
Calm1	Calmodulin-1	0.274	2.09E-05
Cox7a1	Cytochrome c oxidase subunit 7A1, mitochondrial	0.246	2.65E-05
Ednra	Endothelin-1 receptor	0.225	2.41E-04
Dip2a	Disco-interacting protein 2 homolog A	0.257	3.67E-04
Gtf2h4	General transcription factor IIH subunit 4	0.432	5.81E-04
Foxp1	Forkhead box protein P1	0.212	8.07E-04
Myo19	Unconventional myosin-XIX	0.229	1.19E-03
Myl1	Myosin light chain 1/3, skeletal muscle isoform	0.214	1.33E-03
Cep350	Centrosome-associated protein 350	0.204	1.85E-03
Hps5	BLOC-2 complex member HPS5	0.226	1.93E-03
Mettl7a1	Methyltransferase-like 7A1	0.224	2.63E-03
Atad2	ATPase family AAA domain-containing protein 2	0.218	4.52E-03
Npc2	NPC intracellular cholesterol transporter 2	0.349	4.89E-03
Ext2	Exostosin-2	0.235	5.64E-03
Cep164	Centrosomal protein of 164 kDa	0.304	6.05E-03
Rgl1	Ral guanine nucleotide dissociation stimulator-like 1	0.236	7.09E-03
Corin	Atrial natriuretic peptide-converting enzyme	0.250	8.31E-03
Cep162	Centrosomal protein of 162 kDa	0.556	8.68E-03

Eepd1	Endonuclease/exonuclease/phosphatase family domain-containing protein 1	0.237	1.01E-02
Bap1	Ubiquitin carboxyl-terminal hydrolase BAP1	0.832	1.17E-02
Nup88	Nuclear pore complex protein Nup88	0.211	1.42E-02
Scap	Sterol regulatory element-binding protein cleavage-activating protein	0.347	1.49E-02
Speg	Striated muscle-specific serine/threonine-protein kinase	0.247	2.17E-02
Zhx2	Zinc fingers and homeoboxes protein 2	0.211	2.32E-02
Vps51	Vacuolar protein sorting-associated protein 51 homolog	0.431	2.45E-02
Pik3ca	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform	0.218	2.68E-02
Igf1bp4	Insulin-like growth factor-binding protein 4	0.303	2.77E-02
Ahctf1	Protein ELYS	0.238	3.46E-02
Ccdc69	Coiled-coil domain-containing protein 69	0.424	4.08E-02

Table 17: Top down-regulated proteins upon Lockd knockdown in C166 cells

Gene	Protein Description	logFC	p.value
Mettl13	eEF1A lysine and N-terminal methyltransferase	-0.520	2.20E-05
Isy1	Pre-mRNA-splicing factor ISY1 homolog	-0.484	6.55E-03
Thap12	52 kDa repressor of the inhibitor of the protein kinase	-0.352	8.79E-04
Rgp1	RAB6A-GEF complex partner protein 2	-0.297	6.24E-03
Bud31	Protein BUD31 homolog	-0.285	9.70E-03
Trmt6	tRNA (adenine(58)-N(1))-methyltransferase non-catalytic subunit TRM6	-0.263	2.49E-04
Dag1	Dystroglycan 1	-0.225	3.35E-03
Trim65	Tripartite motif-containing protein 65	-0.206	2.36E-03
Mvk	Mevalonate kinase	-0.204	3.38E-03
Zfp655	Zinc finger protein 655	-0.193	1.04E-02
Gpsm1	G-protein-signaling modulator 1	-0.184	2.19E-03
Lnpep	Leucyl-cystinyl aminopeptidase	-0.178	5.17E-04
Atrip	ATR-interacting protein	-0.178	6.54E-03

Sccpdh	Saccharopine dehydrogenase-like oxidoreductase	-0.172	2.36E-02
Rpl23	60S ribosomal protein L23	-0.169	4.26E-03
Hmgcs1	Hydroxymethylglutaryl-CoA synthase, cytoplasmic	-0.169	1.17E-03
Extl2	Exostosin-like 2	-0.166	1.76E-02
Rrm1	Ribonucleoside-diphosphate reductase large subunit	-0.166	4.71E-04
Pycr1	Pyrroline-5-carboxylate reductase 1, mitochondrial	-0.161	4.76E-03
Cdk10	Cyclin-dependent kinase 10	-0.155	5.37E-04
Hmcn1	Hemicentin-1	-0.155	7.91E-04
Nln	Neurolysin, mitochondrial	-0.151	1.52E-02

Table 18: Top up-regulated proteins upon Lockd overexpression in C166 cells

Gene	Protein Description	logFC	p.value
Fstl1	Follistatin-related protein 1	0.236	2.81E-07
Erh	Enhancer of rudimentary homolog	0.319	8.94E-06
Dut	Deoxyuridine 5'-triphosphate nucleotidohydrolase	0.242	1.93E-05
Col5a2	Collagen alpha-2(V) chain	0.363	6.34E-05
Supt3	SPT3, SAGA and STAGA complex component	0.204	8.47E-05
Thsd4	Thrombospondin type-1 domain-containing protein 4	0.415	8.65E-05
Ttc1	Tetratricopeptide repeat protein 1	0.317	3.66E-04
Sod3	Extracellular superoxide dismutase [Cu-Zn]	0.334	3.78E-04
Arpc5	Actin-related protein 2/3 complex subunit 5	0.220	6.45E-04
Vamp3	Vesicle-associated membrane protein 3	0.246	7.75E-04
Plgrkt	Plasminogen receptor (KT)	0.249	1.21E-03
Nup62	Nuclear pore glycoprotein p62	0.317	2.49E-03
Pgrmc1	Membrane-associated progesterone receptor component 1	0.213	2.58E-03
Tfg	TFG protein	0.211	3.46E-03
Syap1	Synapse-associated protein 1	0.301	3.67E-03
Ska3	Spindle and kinetochore-associated protein 3	0.208	4.07E-03
Mlh1	DNA mismatch repair protein Mlh1	0.337	5.71E-03
Q8C3W1	Uncharacterized protein C1orf198 homolog	0.306	5.76E-03

Plscr3	Phospholipid scramblase 3	0.204	5.90E-03
Rps27	40S ribosomal protein S27	0.202	8.75E-03
Dph7	Diphthine methyltransferase	0.369	9.15E-03
C130050O18Rik	C130050O18Rik protein	0.443	9.16E-03
Ptms	Parathymosin	0.580	9.62E-03
Apobec3	DNA dC->dU-editing enzyme APOBEC-3	0.267	1.15E-02
Nucb1	Nucleobindin-1	0.529	1.29E-02
Rcn3	Reticulocalbin-3	0.285	1.30E-02
Men1	Menin	0.238	1.39E-02
Vps51	Vacuolar protein sorting-associated protein 51 homolog	0.203	1.46E-02
Tssc4	Protein TSSC4	0.284	1.56E-02
Lsm3	U6 snRNA-associated Sm-like protein LSM3	0.247	1.58E-02
Septin6	Septin-6	0.230	1.80E-02
Llg1	Lethal(2) giant larvae protein homolog 1	0.314	1.92E-02
Sra1	Steroid receptor RNA activator 1	0.250	2.03E-02
Hps6	BLOC-2 complex member HPS6	0.231	2.18E-02
Dctn2	Dynactin subunit 2	0.279	2.26E-02
Grb10	Growth factor receptor-bound protein 10	0.293	2.32E-02
Urod	Uroporphyrinogen decarboxylase	0.210	2.38E-02
Glo1	Lactoylglutathione lyase	0.213	2.39E-02
Pdcd5	Programmed cell death protein 5	0.253	2.53E-02
Ciapi1	Anamorsin	0.458	2.93E-02
Pepd	Xaa-Pro dipeptidase	0.546	2.98E-02
Steep1	STING ER exit protein	0.248	3.08E-02
Ptma	Prothymosin alpha	0.514	3.15E-02
Ensa	Alpha-endosulfine	0.267	3.15E-02
Septin8	Septin-8	0.201	3.53E-02
Zbtb45	Zinc finger and BTB domain-containing protein 45	0.458	3.70E-02
Smap	Small acidic protein	0.450	3.94E-02
Psme3ip1	PSME3-interacting protein	0.355	4.14E-02
Cnst	Consortin	0.223	4.16E-02
Retreg2	Reticulophagy regulator 2	0.358	4.56E-02

Tnnc1	Troponin C, slow skeletal and cardiac muscles	0.214	4.91E-02
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Table 19: Top down-regulated proteins upon Lockd overexpression in C166 cells

Gene	Protein Description	logFC	p.value
Trmt6	tRNA (adenine(58)-N(1))-methyltransferase non-catalytic subunit TRM6	-0.223	2.01E-04
Slc35f6	Solute carrier family 35 member F6	-0.348	2.14E-04
Plpp6	Polyisoprenoid diphosphate/phosphate phosphohydrolase PLPP6	-0.329	4.44E-04
Apob	Apolipoprotein B-100	-0.158	9.42E-04
Rrp36	Ribosomal RNA processing protein 36 homolog	-0.192	9.99E-04
Hmmr	Hyaluronan mediated motility receptor	-0.164	1.15E-03
Taf7	Transcription initiation factor TFIID subunit 7	-0.153	1.19E-03
Hipk3	Homeodomain-interacting protein kinase 3	-0.224	1.50E-03
Pcyox1	Prenylcysteine oxidase 1	-0.181	1.57E-03
Trpm7	Transient receptor potential cation channel subfamily M member 7	-0.254	2.00E-03
Mettl13	eEF1A lysine and N-terminal methyltransferase	-0.360	2.25E-03
Mrpl4	39S ribosomal protein L4, mitochondrial	-0.174	2.80E-03
Flnb	Filamin-B	-0.173	2.97E-03
Tor1b	Torsin-1B	-0.174	3.55E-03
Casp12	Caspase-12	-0.307	3.92E-03
Sec11c	Signal peptidase complex catalytic subunit SEC11C	-0.198	4.01E-03
Ephx1	Epoxide hydrolase 1	-0.189	4.20E-03
Mrpl17	39S ribosomal protein L17, mitochondrial	-0.177	4.30E-03
Ahnak2	AHNAK nucleoprotein 2	-0.185	4.58E-03
Kif18b	Kinesin-like protein KIF18B	-0.215	5.15E-03
Pus7l	Pseudouridylate synthase PUS7L	-0.208	5.95E-03
Fgf1	Fibroblast growth factor 1	-0.182	6.02E-03
Strada	STE20-related kinase adapter protein alpha	-0.202	6.51E-03
Dcxr	L-xylulose reductase	-0.205	6.92E-03

Abhd4	(Lyso)-N-acylphosphatidylethanolamine lipase	-0.173	8.96E-03
P10404	MLV-related proviral Env polyprotein	-0.470	9.24E-03
Gja1	Gap junction alpha-1 protein	-0.156	9.80E-03
Znf385a	Zinc finger protein 385A	-0.180	1.29E-02
Cep164	Centrosomal protein of 164 kDa	-0.541	1.48E-02
Arvcf	Splicing regulator ARVCF	-0.195	1.50E-02
Bcr	Breakpoint cluster region protein	-0.394	1.52E-02
Cnnm3	Metal transporter CNNM3	-0.197	1.90E-02
Cep41	Centrosomal protein of 41 kDa	-0.177	1.92E-02
Ankrd52	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C	-0.163	2.01E-02
Inf2	Inverted formin-2	-0.179	2.05E-02
Slc2a4	Solute carrier family 2, facilitated glucose transporter member 4	-0.178	2.23E-02
Serinc3	Serine incorporator 3	-0.161	2.28E-02
Wdr90	WD repeat-containing protein 90	-0.179	2.30E-02

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DECLARATION

I hereby declare that this doctoral thesis titled, “Stress-induced lncRNA Lockd inhibits proliferation and angiogenesis of myocardial endothelial cells and promotes maladaptive cardiac remodeling during pressure overload,” is the result of my work. This work has been conducted in the department of Cardiovascular Physiology at the Mannheim Medical Faculty of the University of Heidelberg under the guidance of Prof. Dr. Jörg Heineke. Instances where information has been derived from other sources are explicitly indicated.

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