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Proteomic characterisation of mutant RAS signalling and oncogene-cooperating activity of wild-type p53

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Abstract

The RAS proteins belong to the superfamily of monomeric GTPases mediating the transmission of extracellular signals from cell-surface receptors to other parts of the cell. Upon activation, RAS induces a wide range of downstream signaling pathways that control fundamental cellular processes, such as proliferation, growth, motility and stress response. Activating point mutations in RAS are known drivers of cancer development in epithelial cells. The tumorigenic effects of the mutant RAS activity are predominantly associated with an impairment of p53 functions. However, the interplay of p53 and oncogenic RAS mutations during transformation and tumor progression remains understudied.

In this work, I aimed to characterise alterations driven by the expression of the mutant (G12V) HRAS in non-malignant human mammary gland MCF10A cells on proteomic, transcriptomic, and phenotypic levels. Furthermore, I investigated the influence of wild-type p53 on the outcome of the aberrant RAS signaling.

First of all, I implemented the nascent proteome analysis to accurately quantify the alterations in protein expression upon the constitutive RAS signalling. This analysis indicated activation of RAS downstream effectors the metabolic shift towards glycolysis as well as upregulation of YAP-activation signatures and EMT markers upon the mutant HRAS expression. These observations were further confirmed by transcriptome analysis. Importantly, some of the effect of the mutant HRAS were shown to be dependent on p53. Particularly, the expression of EMT markers and signatures of YAP activation was reduced upon p53 silencing exclusively in the mutant HRAS expressing MCF10A cells. Yet, the p53 knockdown in wild-type cells caused minor and dissimilar effect on gene and protein expression. The following phenotypic characterisation supported that migration and invasion properties of the cells depend on both the mutant-RAS and p53 expression. Therefore, my results indicate that p53 functions are altered upon the constitutive RAS signalling, and thus the wild-type p53 can act in an oncogene-cooperative manner in the given cellular system.

Along this line, I applied ChIP-SICAP, a method for the selective isolation of the chromatin-associated proteins to identify the on-chromatin interactors of p53 in MCF10A cells. To study the mutant RAS-driven alterations in the p53 interactome and elucidate the mechanisms of the oncogene-dependent p53 activity, the ChIP-SICAP was applied to WT and HRAS mutant MCF10A cells. Although, the comparison on their interactomes revealed high conformity, a few proteins were found to be differentially represented depending on the mutant HRAS expression. Therefore, these pivotal p53 interactors could play a mechanistic role in mediating the oncogenic signaling-dependent alterations in the activity of p53.

Finally, I analysed the mutant HRAS-driven alterations in the composition of secreted proteins. This analysis revealed the upregulation of the secretion of growth factor receptor ligands. Therefore, the outcome of the constitutive HRAS activity might be partially determined by autocrine stimulation. The analysis also confirmed the up-regulation of EMT markers in the extracellular space and showed the down-regulation of ECM remodelling proteins.

Overall, I reported the alterations driven by mutant HRAS in the activity of signalling pathways and transcription factors and showed that wild-type p53 cooperates with the oncogene to promote effects of the constitutive HRAS signaling in the epithelial cells. These results were confirmed by independent methods of proteomic, transcriptomic, and phenotypic analysis. The presented data can serve as a source of quantitative information for subsequent studies of individual mediators of the mutant RAS-driven tumorigenesis.

Zusammenfassung

Die RAS Proteine gehören einer Superfamilie monomerer GTPasen an, die die Weiterleitung extrazellulärer Signale von Oberflächenrezeptoren zu anderen Kompartimenten der Zelle weiterleiten. Aktiviertes RAS induziert verschiedene nachgeschaltete Signalwege, die fundamentale zelluläre Prozesse wie Proliferation, Wachstum, Migration und Stressantwort kontrollieren. Aktivierende Punktmutationen in RAS Proteinen sind bekannte Auslöser von Karzinogenese in epithelialen Zellen. Die tumorigenen Effekte von mutierten RAS Proteinen sind häufig mit einem Verlust der Funktion von p53 assoziiert. Allerdings wurde das Zusammenspiel zwischen p53 und onkogenen RAS Mutationen im Verlauf der Transformation und Tumorprogression bisher nur unzureichend untersucht.

Im Rahmen dieser Arbeit habe ich das Ziel verfolgt, die proteomischen, transkriptomischen und phänotypischen Veränderungen zu charakterisieren, die durch die Expression von mutiertem (G12V) HRAS in gutartigen menschlichen Brustdrüsenzellen (MCF10A) hervorgerufen werden. Zudem habe ich den Einfluss von wildtyp p53 auf die Effekte von konstitutiver RAS Aktivität untersucht.

Hierzu habe ich zunächst eine Analyse etabliert, die neu synthetisierte Proteine detektiert, um die Veränderungen in der Proteinexpression durch konstitutive Aktivierung von HRAS akkurat zu quantifizieren. Diese Analyse deutete auf die Aktivierung von nachgeschalteten RAS Effektoren eine metabolische Verschiebung hin zu vermehrter Glykolyse sowie eine erhöhte Expression von Proteinen der YAP-Aktivierungssignaturen und EMT Markern als Reaktion auf die Expression von mutiertem HRAS hin. Diese Beobachtungen wurden auf Transkriptomebene bestätigt. Bemerkenswerter Weise konnte ich zeigen, dass einige Effekte des mutierten HRAS Proteins abhängig von p53 waren. Im Speziellen waren die Expression von EMT Markern und Signaturgenen von YAP-Aktivierung im Zellen mit reduzierten p53 Leveln ausschließlich im Kontext von mutiertem HRAS reduziert. Im Gegensatz dazu rief der Knockdown von p53 in wildtyp Zellen geringere und unterschiedliche Effekte auf Gen- und Proteinexpression hervor. Die anschließende phänotypische Charakterisierung bestätigte, dass Migrations- und Invasionsfähigkeit der Zellen sowohl auf mutiertem HRAS als auch auf p53 Expression beruhen. Meine Ergebnisse legen also nahe, dass die Funktion von p53 durch konstitutives RAS verändert wird und dass wildtyp p53 dadurch in diesem Zellsystem kooperativ mit dem aktivierten Onkogen wirken kann.

Um dies weiter zu bestätigen, habe ich ChIP-SICAP angewandt, eine Methode zur selektiven Isolation von Chromatin-assoziierten Proteinen, um die Interaktionspartner von p53 am Chromatin in MCF10A Zellen zu identifizieren. Um die Veränderungen im p53 Interaktom durch konstitutives RAS zu untersuchen und die Mechanismen der Onkogen-abhängigen p53 Aktivität zu beleuchten, wurde ChiP-SICAP für wildtyp und HRAS-mutierte MCF10A Zellen angewandt. Obwohl die beiden Interaktome sich sehr ähnlich waren, wurden einige Proteine zwischen beiden Konditionen unterschiedlich stark repräsentiert detektiert. Dementsprechend könnten diese p53 interagierenden Proteine eine mechanistische Rolle in der Vermittlung der Onkogen-abhängigen Veränderungen in der Aktivität von p53 spielen.

Zuletzt habe ich die durch mutiertes HRAS hervorgerufenen Veränderungen in der Zusammensetzung von sezernierten Proteinen untersucht. Diese Analyse zeigte eine vermehrte Sezernierung von Liganden für Wachstumsfaktor-Rezeptoren. Dementsprechend könnten die Effekte von konstitutiver HRAS Aktivität möglicherweise zum Teil durch autokrine Stimulation erklärt werden. Diese Analyse bestätigte zudem die erhöhte Präsenz von EMT Markern im extrazellulären Raum und eine Reduktion von ECM remodellierenden Proteinen.

Zusammenfassend habe ich die Veränderungen, die durch mutiertes HRAS hervorgerufen werden, auf der Ebene von Signalwegen und Transkriptionsfaktoren beschrieben und konnte dabei zeigen, dass wildtyp p53 mit dem Onkogen kooperiert, um die Effekte von konstitutiv aktiviertem HRAS in epithelialen Zellen zu verstärken. Diese Ergebnisse wurden durch unabhängige Methoden auf proteomischer, transkriptomischer und phänotypischer Ebene bestätigt. Die hier präsentierten Daten können zudem als Quelle für quantitative Informationen für Folgestudien an individuellen Mediatoren der durch mutiertes RAS vorangetriebenen Tumorigenese verwendet werden.

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— *Quintus Horatius Flaccus*

Schlage die Trommel und fürchte dich nicht

— *Heinrich Heine*

Contents

1. Introduction

1.1 Overview and objectives

The RAS proteins belong to the RAS superfamily of monomeric GTPases. These small membrane-bound proteins function as GTP/GDP-dependent molecular switches mediating transmission of extracellular signals from cell-surface receptors to other parts of the cell. Activation of RAS induces a wide range of downstream signaling pathways controlling fundamental cellular processes, such as proliferation, growth, and stress responses. Malfunctions in the regulation of the processes can cause oncogenic transformation. Data from cell lines, animal models, and clinical studies have confirmed that RAS proteins harbouring an activating point mutation are able to initiate oncogenesis through stimuli-independent activation of downstream pathways. In normal cells, this RAS-initiated mitogenic stimulation is controlled by checkpoint mechanisms which direct cells towards senescence or apoptosis in response to the aberrant signaling thus, the mutant-RAS activity alone is not sufficient for successful oncogenic transformation [1]. Full transformation requires inactivation of the cell cycle controlling machinery in addition to RAS activating mutations.

A critical element of cell cycle control is p53, a tumor suppressor that orchestrates cell survival and proliferation depending on the cell status and plays a pivotal role in anticancer protection. The loss of the wild-type p53 activity is an essential tumorigenic event in numerous cancers, including those driven by RAS. The cooperation of the p53 deactivation and stimulation of RAS for the epithelial cancer progression has been reported for colon, pancreatic, and breast cancers [2], [3], [4]. Typically, this is caused by somatic missense mutations in p53 itself (~50% of all cancers [5]). Importantly, it has been shown that some p53 mutants, along with the loss of the tumor-suppressive functions, acquire novel activities, non-characteristic for the wild-type p53 (gain-of-function), and beneficial for

tumorigenesis in terms of promoting aberrant proliferation, invasion, migration, etc. The transcriptional activity of p53 and its outcome are mainly regulated through posttranslational modifications (PTM) of the protein, its interaction with other partners, and the resulting differential binding to regulatory elements on DNA. The mutations in p53 lead to malfunction of these mechanisms reflecting in alterations of the PTM acquirement, and the assortment of the p53 on-chromatin partners and controlled genes [6]. The accumulation of data on the p53 network driven by the interest in the role of p53 mutations in cancer elucidated the great complexity of the crosstalk between p53 and other signaling cascades. Despite the stereotyped role of p53 as just a guard of the genomic integrity, the p53 mutants have been shown to modulate a surprisingly wide spectrum of cellular processes, from metabolism to inflammation [7]. At the same time, the outcome of the p53 mutations was found to be conditional on the cellular context and varying in different cell and cancer types [8]. Besides, the various p53 mutants were explicitly demonstrated to differently response to oncogenic signaling [9]. Nevertheless, the detailed mechanisms underlying the tuning of p53 activity remains mainly undiscovered for both mutant and wild-type p53. Phenomenologically, this would be reasonable to expect that the functional complexity revealed for the mutant p53 variants is also characteristic for the wild-type counterpart. Although the role of p53 mutants in tumorigenesis is undoubtedly remarkable and highly attractive for studying, almost 50% of tumors retain wild-type p53. The last years yielded several publications confirming that upon oncogenic signaling, wild-type p53, in opposition to its tumour-suppressive role and similar to the mutant p53 activity, can actually contribute to cancer cells resistance to chemotherapy, survival, migration, and invasion [10], [11], [12]. In this context, further investigation of the role of wild-type p53 in oncogene expressing cells is of high interest. However, the great intricacy of the oncogene-induced alterations, multiple gene mutations, and the intensive cross-talk between various pathways make the task rather complicated. To overcome this challenge, in this work, I investigated the non-cancerous mammary epithelial MCF10A cell lines subjected to the mutant HRAS-V12 transfection in conjunction with the knockdown of the endogenous TP53. Due to the mainly intact and well-defined genetic environment, MCF10A cells serve a widely used model for mimicking the oncogenic transformation and studying the stages of tumorigenesis [13], [14]. The implementation of the system of four isogenic cell lines

differential in the statuses of the HRAS and p53 expression enabled investigation of the consequences of the oncogenic HRAS signaling, and the role of p53 activity in the regulation of its outcome.

I applied various genomic and proteomic techniques, including the nascent proteome analysis, secretome analysis, and selective isolation of the p53 on-chromatin interactors, to monitor the influence of the mutant HRAS on the protein expression, activity of signaling pathways, and functions of p53.

1.2 RAS signaling

RAS proteins are small membrane-bound GTPases playing an active role in the transmission of extracellular signals from receptor tyrosine kinases to the nucleus to modulate cellular response. By functioning as GDP-GTP-regulated switches, RAS proteins orchestrate the activity of distinct signaling pathways, such as PI3K-AKT and Raf-ERK, which, depending on signaling context and cell type, affect gene transcription and essential cellular processes including regulation of cell growth, proliferation, survival, and differentiation. The high frequency of activating point mutations of RAS genes in human cancers, and the central role of RAS proteins in proliferative signal transduction, make them an appealing therapeutic target in cancers. However, the attempts for developing direct RAS inhibitors have so far encountered biophysical limitations (GTP/GDP ratio) and did not yield a clinically applicable agent. Yet, targeting the pathways downstream to RAS is complicated by intensive cross-talk and intricate compensatory mechanisms regulating signal transduction through these cascades. Moreover, due to this high complexity, investigation of RAS activity is still challenging. In cancer cells and some of the traditional models [15], the RAS signaling is masked by additional genetic or signaling aberrations and long-term adaptations to the oncogenic signaling. Studies of RAS transformational activity in non-cancerous cell lines demonstrated that, although the cells can be successfully transfected by mutant RAS genes and develop metabolic and phenotypic changes [16], [16], those are not sufficient for actual malignant transformation without alterations in the activity of other players (e.g. c-MYC activation [17]). Along with clinical data on the RAS-driven cancer progression [2], [3], [18], these observations indicate that the RAS

contributes to tumorigenesis through cooperation with other factors. Therefore, global characterization of the effects of the aberrant RAS-signaling can help to better understand how this creates the disposition beneficial for other oncogenes and to reveal novel players of RAS-induced transformational process which could serve as prognosis markers or intervention points in RAS-driven cancers.

1.3 Cellular model

In this work, I used the spontaneously immortalized, non-malignant MCF10A cell lines derived from the human mammary gland. The cells were transfected with the HRAS harboring an activating point mutation (G12V). The obtained pair of the isogenic cell lines was subjected to the knockdown of p53 (**Fig. I**). Therefore, the cellular system of these four cell lines mimics the early stages of the oncogene-driven tumorigenesis [19] in epithelial cells and allows investigation of the role of p53 in this process.

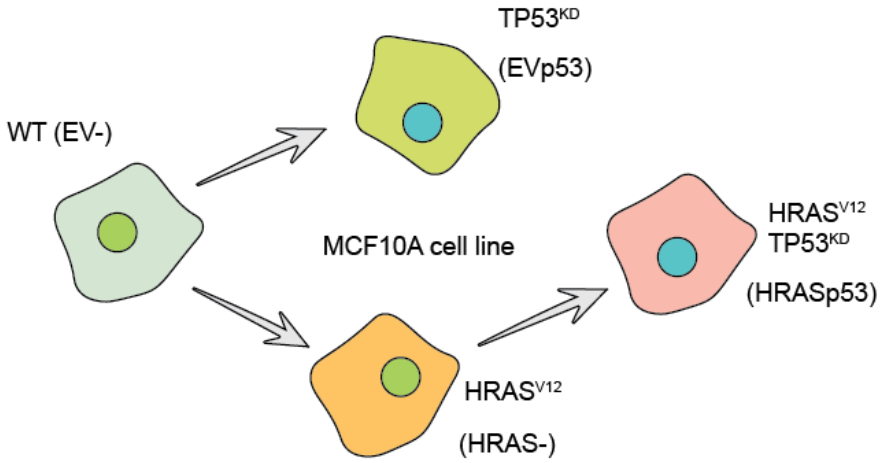


Figure I. Cell model. MCF10A, non-tumorigenic epithelial cells derived from mammary gland employed as a model system to mimic the HRAS-driven oncogenic transformation. The wild-type (WT) MCF10A cells were transfected with HRAS gene variant harbouring the activating point G12V mutation. The obtained pair of isogenic WT and HRAS MCF10A cell lines was further subjected to p53-knockdown. Thereby, the cell system includes 4 cell lines different in the status of HRAS and p53 expression and serve herein as a model for studying the effects of the aberrant RAS-signaling and of the crosstalk between the oncogene and wild-type p53 in epithelial cells.

Although mutations in RAS proteins are rather rare in human breast cancers, hyper-activation of RAS was reported for more than a half of such tumors, suggesting the supportive role of RAS signaling in the tumorigenesis [19], [20], [21]. Another malfunction typical for breast cancers is the alteration in p53 activity. The screening of clinical samples by next-generation sequencing revealed that TP53 gene mutations occur in ~30% of breast carcinomas [22]. Recent studies also demonstrated that in breast cancer, the mutant p53 serves as a mediator of survival, tumor progression, and metastasis [23], [24], showing the importance of the p53 signaling for breast cancer progression. Moreover, the role of p53 in cancer development was emphasized by revealing the correlation between abnormalities in RAS and p53 pathways in HER2 positive infiltrating ductal carcinomas [4]. The crosstalk between p53 and mutant RAS has been previously shown for colorectal and pancreatic cancers [2], [3]. In an ample number of cancer studies, p53 is considered to interfere with the transduction of signals from other players, such as MYC, NF-kappa, and YAP/TAZ [25], [26], [27]. Therefore, the mutant p53 variants can act in cancer in a cooperative manner with oncogenes. Although the wild-type p53 is conventionally considered as a guard of the genome and its functionality is traditionally assigned exclusively for tumor-suppression, including prevention of oncogene-driven aberrant proliferation, and epithelial-mesenchymal transition, a wide range of observations on pro-oncogenic properties of mutant p53 variants, and studies of its transcriptional activity in various cell types gave rise to discussions about the content-dependent nature of p53 action, and capability of oncogenes to rewire the p53 network [28], [29], reviewed in [30].

In this work, I aimed to directly evaluate how the mutant RAS signal transduction alters the impact of p53 to protein expression, and therefore, elucidate the role of normal p53 activity in the context of oncogenic signaling. To do so, I investigated the effects of mutant HRAS expression in the non-malignant MCF10A cells bearing normal p53. I also evaluated the influence of the p53 silencing in these cells transfected with the mutant HRAS.

1.4 Structure of the work

The following chapters represent the results of the characterisation of different aspects of the mutant HRAS signalling and p53 activity outcome by various methods.

Chapter 2 is focused on the quantitative analysis of alterations in gene and protein expression and on the identification of signalling pathways and cellular processes affected by the mutant HRAS activity. This chapter also includes the phenotypic characterization part.

Chapter 3 describes the results of the investigation of the p53 on-chromatin complexes, optimization of the method for the efficient detection of the p53 interactors, and the identified alterations in the p53 network composition upon the mutant HRAS expression.

Finally, in Chapter 4, the revealed effects of the mutant HRAS signalling on the protein secretion are reported.

Each chapter includes an introductory part describing respective biological and technical aspects and a discussion part summarizing the observations and conclusions reported in the chapter.

2. Analysis of gene and protein expression

2.1 Approaches to signalling pathways characterisation

From the methodological point of view, the main difficulty for studying RAS signaling lies in the high diversity of its downstream effectors. Besides the PI3K and BRAF pathways, RAS activates Rho and Ral signaling. Through these effectors, RAS modulates the wide spectrum of cellular processes associated with growth, survival, gene expression, endocytosis, and cell motility. Furthermore, current in-depth studies of RAS activity keep revealing novel targets of RAS. Particularly, RalBP1, an activator for Rac1 and CDC42 implicated in a number of cell processes, including cell migration and mitochondrial division, was shown to be directly interacting with RAS on the cellular membrane. Another relatively novel RAS interactor is RASSF5 [31]. This protein was found to be involved in the regulation of Hippo pathway through MST1/2, mammalian homologs of the drosophila Hippo kinase, and therefore, to cell cycle progression. However, the recent studies showed that RASSF5 also modulates apoptosis and RAS-driven senescence through activation of p53 and Rb1 [32], [33]. Additionally, it possesses the Hippo-independent function in cellular growth control [34]. Therefore, besides illustrating the diversity of the RAS network, this example introduces the internal complexity of distinct signaling cascades regulated by RAS. Moreover, many of these cascades are known to crosstalk with each other at different levels. Particularly, the above-mentioned RalBP1 accumulates signals from both RAS and Ral proteins and consequently serves as a node for both Rho/Ral and Ras signaling [35], whereas the Rho/Ral proteins themselves are known to be affected by RAS through TIAM1 [36]. The crosstalk between Raf-ERK and PI3K-mTOR, both of which are affected by RAS, is well studied due to the long-term efforts for targeting their distinct members with selective small molecule inhibitors for cancer therapy. It was revealed that Raf-ERK and PI3K-mTOR are interconnected by an utterly large number of feedforward and negative feedback loops regulating the signaling dynamics and, more

importantly, creating the compensatory mechanism that redirects the mitotic signal transduction through one of these pathways in case of inhibition of the other (described in details in the review – [37]. Yet, parallel inhibition of both of those pathways remains challenging because of the increased toxicity of the double-agent therapy and adverse events caused by the limited specificity of the drugs [38]. Overall, RAS proteins are interplaying with an intricate network of partners involved in distinct signaling cascades that regulate various cellular processes essential for normal cell physiology and tumorigenesis. This role of RAS as a signaling hub requires simultaneous consideration of a number of individual pathways for adequate characterization of RAS properties. As many of the pathways are regulated through successive phosphorylation of their members, monitoring of the proteins phosphorylation status, using western blotting or phosphoproteomics, might allow detection of the RAS-dependent activation of these pathways [39], [40]. However, even leaving aside technical limitations of these methods, a number of additional pathway regulatory mechanisms – such as other PTMs, protein translocation, dimerization, or degradation – would be ignored upon focusing on phosphorylation. Furthermore, the central process determining the cellular response to a stimulus is an alteration of gene expression which happens downstream of these pathways, in the nucleus, and also involves complex regulatory mechanisms [41].

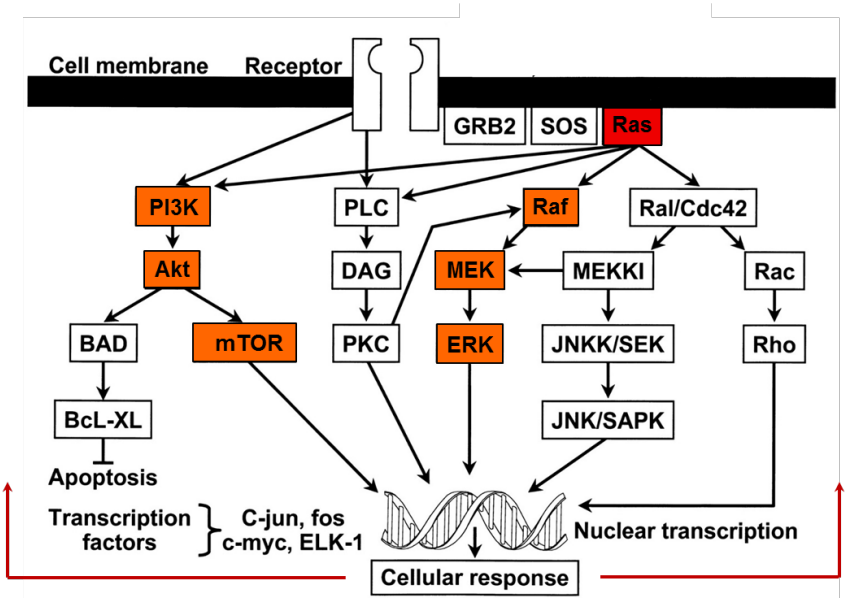


Figure II. Simplified scheme of the RAS downstream effectors (Adapted from A. Adjei *et al.* [42])

Gene expression is regulated by hundreds of transcription factors (TFs). The activity of these factors depends on the upstream stimuli transmitted through the signaling pathways [41]. Some of those are considered to be predominantly associated with certain signaling cascades. For instance, MYC is known as the terminal effector of Raf-ERK/PI3K-mTOR and thus RAS [43]; TCF/LEF TFs serve as endpoints of Wnt signaling [44]; YAP/TAZ proteins are mediators of Hippo pathway activity [45]. However, these examples rather represent a historically and clinically determined focus of biological studies. Such a TF-pathway correspondence is known for only a limited number of TFs and even for the ones mentioned above, mechanisms of their activity are not confined by interaction with respective pathways [44], [43]. In general, gene expression is determined by cumulative upstream signaling and cooperation between various transcription factors and their interactors, particularly in macromolecular complexes on chromatin [46], [47]. Therefore, transcriptional regulation by TFs represents an additional layer of the interplay between signal transduction cascades and should unavoidably be considered for a proper understanding of the signaling outcome. Unfortunately, the techniques for analysis of TF activity, such as genomics and proteomics affinity-purification-based approaches, are fundamentally restricted to targeting only a subset of TFs or genomic loci per a sample and depending on antibody accessibility [48], [49] or endogenous tagging (reviewed in [50]. The novel techniques implying enrichment of chromatin-associated proteins provide restricted sensitivity due to the large difference in abundance of TFs and do not guarantee the specificity of the enrichment [51], [52], [53].

Overall, our understanding of transcriptional alteration mechanisms modulated by upstream stimuli (e.g. RAS mutation) is still far from being complete, partially due to the lack of standard methods for direct and global evaluation of the activity of cellular TFs. This methodological impediment can be bypassed by analysis of the results of the transcriptional regulation that are represented by mRNA transcripts (transcriptomics) or expressed proteins (proteomics). Although, the changes in mRNA or protein synthesis do not provide information on the mechanisms those were caused by per se, the expanding knowledge of TF target genes, and numerous players involved into cellular processes allows interpretation of these changes and their association them with the activity of respective TFs or cellular pathways. Transcriptome analysis enables the determination of total mRNA

relative abundance in the cells that reflects the difference in the activity of certain genes [54]. The modern platforms for RNA-sequencing (RNA-seq) ensure high accuracy, reproducibility and high transcriptome coverage [55], [56], [57]. This made mRNA-seq being a widely and successfully applied tool for quantification of gene expression and consequent analysis of pathways, TFs activity, co-expression patterns, and other parameters using integrative databases combining information from RNA-seq, DNA-seq, and ChIP-seq experiments [58], [59], [60]. Nevertheless, mRNA-seq has a fundamental limitation – it does not take into account the regulatory events which happen after mRNA synthesis. The gap between a mature mRNA and the derived protein is covered by the translational process which involves the ribosomal apparatus and a diversity of other RNA-binding proteins. Although, a quantitative study of mammalian gene expression control showed that protein abundance mostly correlates with mRNA levels and, therefore, its changes can be explained by regulation of gene transcription [61], this proposition was found to be relevant mainly for variations at steady-state conditions and covering up to 84% of protein abundance variability [62]. Further implementation of mass-spectrometry along with RNA-seq in last years revealed that discordant expression changes of mRNA and proteins are characteristic for many physiological and pathological conditions. For instance, post-transcriptional regulation was shown to play an important role in cell cycle control [63] and metabolism of tissues [64], [65]. This translational control also contributes to cancer development. Particularly, the outcome of aberrant estrogen receptor activity in breast cancer was found to be partly determined by microRNAs that are capable to inhibit mRNA translation in a non-degradative manner [66], [67]. The importance of posttranslational regulation through miRNA was also reported for ovarian cancer [68]. Another recent study proposed that protein expression in colorectal cancers can be orchestrated by a set of RNA-binding proteins [69]. Indeed, previous investigations of RNA-binding proteins revealed that some of them can modulate the translation of specific mRNAs, and, therefore, characteristically alter the cellular processes and proteome content. Thereby, eIF4E was shown to drive expression of the cell cycle machinery components (e.g. CCND1, CDK2, RNR2) and factors of angiogenesis (VEGF, FGF-2), particularly, through regulation of nuclear-cytosol translocation of the respective transcripts [70], [71]. Simultaneously, RNA-binding proteins ELAV1 and AUF1 were found to mediate proliferation and survival of

cancer cells by stabilization mRNAs of various genes, including cyclins and BCL2 [72], [73]. These examples represent merely an apex of the growing pile of data about the role of post-transcriptional mechanisms in the regulation of cellular processes.

A comprehensive analysis of signaling pathways, therefore, ideally requires focusing on proteome as the resulting of the cumulative influence of all upstream regulatory events and direct determinant of phenotypic properties of the cells. Nevertheless, the proteomic analysis also meets a few limitations. First of all, despite recent technical improvements, the in-depth proteome coverage achieved by the modern mass-spectrometry methods is still typically lower than the one obtained by RNA-seq. The human proteome contains ~20000 of protein-coding genes [74]. Though the particular results depend on many factors, this might be approximated that the modern RNA-seq platforms, such as Illumina, allow quantifying >12000 gene transcripts using the standard library preparation protocol [75], [76], whereas routine methods for global proteome analysis commonly yield ~5000-6000 proteins quantified by TMT or SILAC, albeit applying of advanced chromatography and extensive sample fractionation can push this number toward 10000 [77], [78], [79]. Thereby, the proteome analysis provides additional information on the protein expression regulation in comparison to RNA-seq but loses in the depth. Besides, there are a few reasons allowing to say that the relevance of the total proteome analysis for the characterization of cellular processes might be controversial. Recently developed methods of pulse SILAC-labelling [80] gave a raise to the wide investigation of protein translation, processing, degradation, and overall turnover. This was then shown on a proteome-wide scale that the life-time and stability of the proteins often depends on the cellular state in cancer and stem cells [81], [82]. Furthermore, aging-related studies demonstrated that PTM status of proteins, their integrity, and functionality differ over their life-time [83], [84], [85]. These two observations illustrate that the same total abundances of a protein can conceal different turnover and functional state.

More importantly, proteins vary in their absolute copy numbers, often up to 6 orders of magnitude [86]. Thereby, the same fold-change alterations in amounts of high- and low-abundant proteins need unequal resources from the cell, probably, require the different intensity of regulatory events and can possess distinct physiological effects [87]. In the

context of mass-spectrometry, the protein abundance also influences quantification as upon decreasing the overall signal intensity of a peptide, the accuracy of its quantification reduces proportionally. Furthermore, due to the limited dynamic range of the modern mass-spectrometers, which typically is ~4 orders of magnitude for protein quantification and strongly depends on the sample parameters [88], the accumulation of the highly abundant proteins in the total cellular proteome diminish the potential of successful identification and accurate quantification of the ones with low abundance. Besides, the highly-abundant proteins commonly possess structural functions and accumulate in the cell over a long time, whereas the proteins with higher turnover rates are involved in more immediate cellular response and, therefore, more affected by regulation of signaling and gene expression [89]. Taking all this together, analysis of the total cellular proteome is a powerful standard tool especially relevant at the conditions when the steady-state proteome content is of interest or other technical alternatives are unavailable, however, it also has limitations that narrow down its applicability in cellular signaling studies.

Currently, besides the protein turnover studies, the pulse-labeling techniques are implemented for analysis of cellular short-term response to stress, growth signaling, and other stimuli [90], [91]. Briefly, these methods are based on the substitution of normal amino acids in the culture media to the ones labeled by isotopes (SILAC) or chemical groups (for instance, azidohomoalanin). Thereby, the proteins synthesized after the substitution are distinguishable of the “old” ones by mass-spectrometry. The labelling can be coupled with treatment and, thus, the newly-synthesized proteins reflect the cellular response to this treatment. The combination of SILAC and chemical tagging with azido-group allows improvement of the approach by adding the newly-synthesized proteins enrichment step [92], [93], [93]. In this case, the methionine in the media is substituted with its functional homolog, azidohomoalanin (AHA). The azido-group of AHA serves as a tag in the click-chemistry reaction with the alkyne-group (azide-alkyne Huisgen cycloaddition) immobilized on resin or beads. Then, the non-azido-labelled “old” proteins are eliminated from the sample by washing and the SILAC labels can be used for relevant quantification of a few nascent proteomes. Noticeably, the removal of “old” proteome accumulated over time normalizes the abundance of proteins in the sample to their translational rates. Taking into account the considerations regarding the total proteome analysis discussed above, the

AHA-based enrichment for the newly-synthesized proteome might be a method of choice for the investigation of cellular signaling. This approach allows targeting the nearly end-point of events regulating both gene and protein expression in the cell and avoids the proteome-specific complications related to protein turnover and wide abundance range.

2.2 AHA-labelling for the nascent proteome analysis

In this work, I used the click-chemistry approach developed by *Eichelbaum et al.* [92] and modified by Dr. Gertjan Kramer (unpublished data) to study the alterations in protein expression upon mutant RAS signaling and knockdown of p53 in MCF10A cells.

As introduced in the previous section, the AHA is recognized by cellular translational machinery and then incorporated into all proteins synthesized after the cultural media substitution instead of methionine. During the incubation time with AHA, cells accumulate the labeled newly-synthesized proteins proportionally to their translation rates. After harvesting and lysis of the cells, the lysates are subjected to a click-chemistry reaction with alkyne-resin. The covalent binding of the AHA-containing proteins to the resin at this step is followed by intensive washes for the removal of pre-existing cellular proteins. The combination of AHA and SILAC labelling allows processing two cellular lysates in one sample for decreasing technical variations and enables a quantitative pairwise comparison of MCF10A cells with different signaling status.

The nascent proteome analysis ranks proteins by signaling-dependent protein synthesis rate rather than abundance. Besides, due to the elimination of non-labeled, pre-existing proteins, the method contributes to the overall sensitivity of protein detection, identification, and quantification and facilitates the identification of proteins presented at a relatively low amount. Furthermore, in conditions, impairing the cellular growth, and division (e.g. EGF depletion for the growth-factor-dependent epithelial cells), the method allows exclusion of the proteins belonging to inactive, senescent, or dead cells with a low expression rate. In other words, independently of the conditions, the targeting newly-synthesized proteins nearly guarantee that the considered proteome belongs to alive, properly functioning cells,

in contrast to the total proteome analysis which can be partially post-mortal at some conditions.

For the steady-state nascent proteome analysis, the labelling time choice is limited mainly by the considerations of sufficient protein accumulation along with restriction of the protein degradation and excessive increase of the share of AHA-containing proteins, albeit the latter was shown to do not make a substantial effect on cellular processes [94]. Taking into account the reported median protein half-life (~11.6 hrs [95]) and doubling time (~22 hrs [14]), 6-hour labelling was used in this work. This was efficient to reach the depth of the quantification comparable to the total proteome. This approach was applied to characterize the effects of mutant RAS signaling on protein expression and elucidate the affected cellular processes and consequent phenotypic changes.

2.3 Results of the global proteome analysis

To estimate the sensitivity of the standard total proteome analysis for quantification of alterations in protein expression driven by a single activating G12V mutation in HRAS, proteomes of wild-type (WT-), and mutant-HRAS-transfected (HRAS-) MCF10A cells were compared using SILAC-labelling. Briefly, the cells were labelled upon passaging in SILAC media, collected, lysed, and processed down to tryptic peptides using the original SP3 method described by Hughes et al. [96]. The peptides were separated using high-pH HPLC, pooled into 12 fractions, and subsequently analysed by an Orbitrap Fusion mass spectrometer (**Fig. 1.1**).

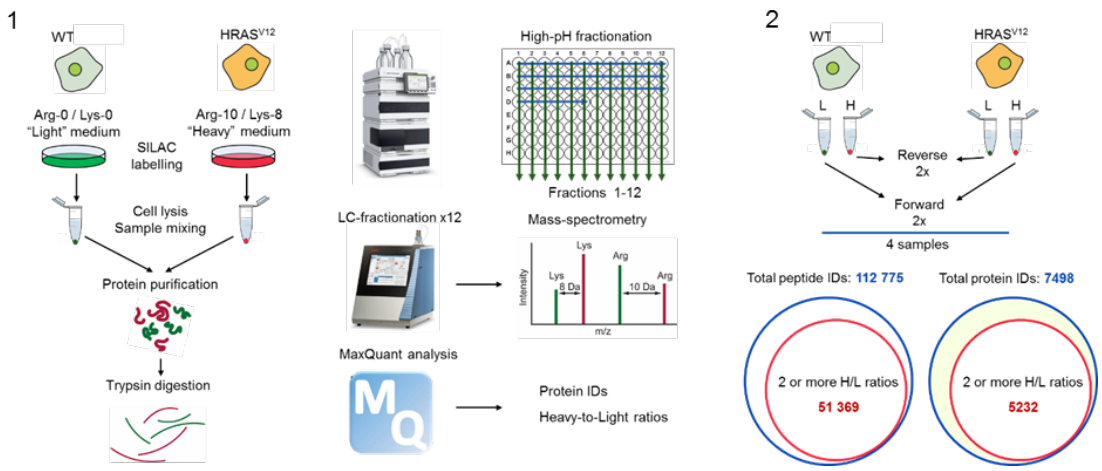


Figure 1. Global proteome analysis. (1) SILAC-labelling and shotgun proteomics approach. Whole cell lysates of were mixed in a 1:1 ratio, followed by protein isolation and trypsinization. The resulting peptides underwent fractionation by HPLC, were pooled in 12 fractions and analysed by LC-MS/MS. Mass spectra were identified and quantified using the Andromeda/MaxQuant pipeline. Briefly, peptides derived from distinct cells were distinguished by the known m/z shift corresponding to the isotope-labelled amino acids. Relative protein amounts were calculated on the basis of MS1 spectra using MaxQuant Software. (2) Sample preparation. Each cell line was twice independently labelled by both "Light" and "Heavy" amino acid. Lysates were mixed as indicated to generate 4 replicates.

Measurement of 4 biological replicates (**Fig. 1**) resulted in the identification of 7498 proteins, of which 5232 proteins were quantified in at least two replicates and used for further analysis (**Fig. 2**).

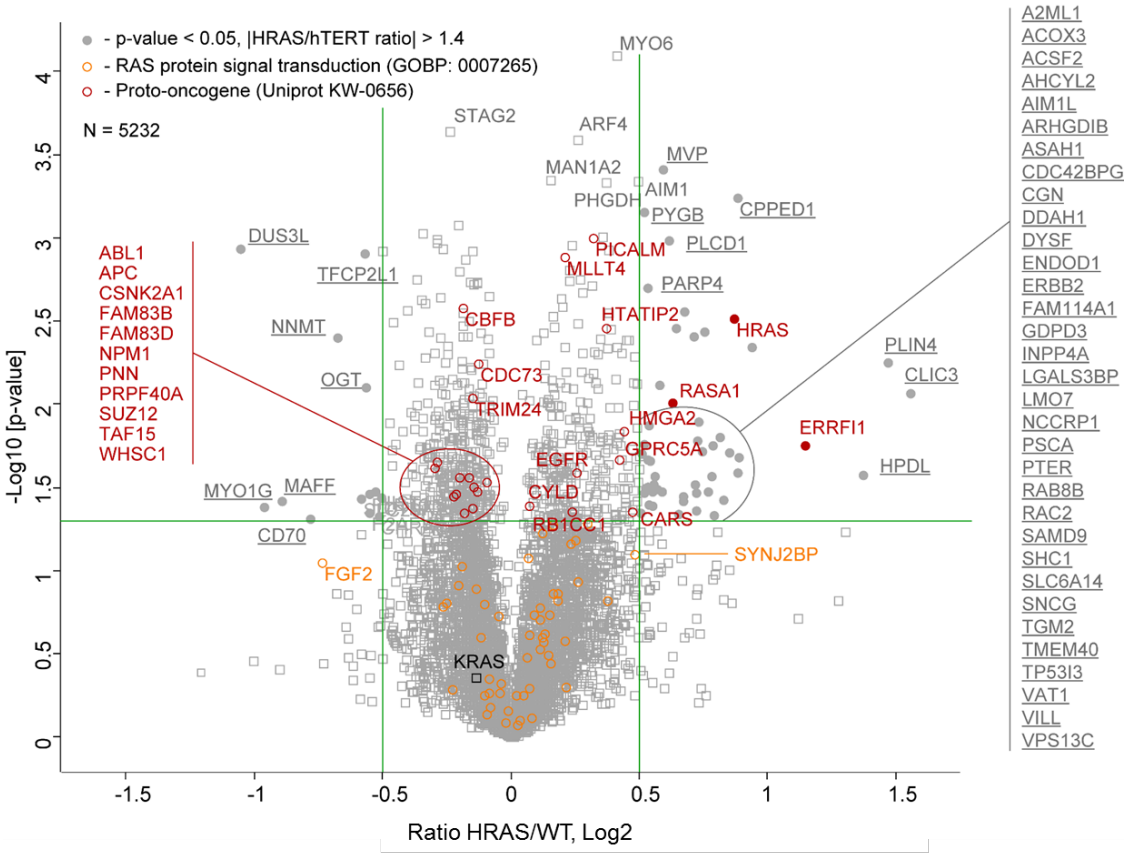


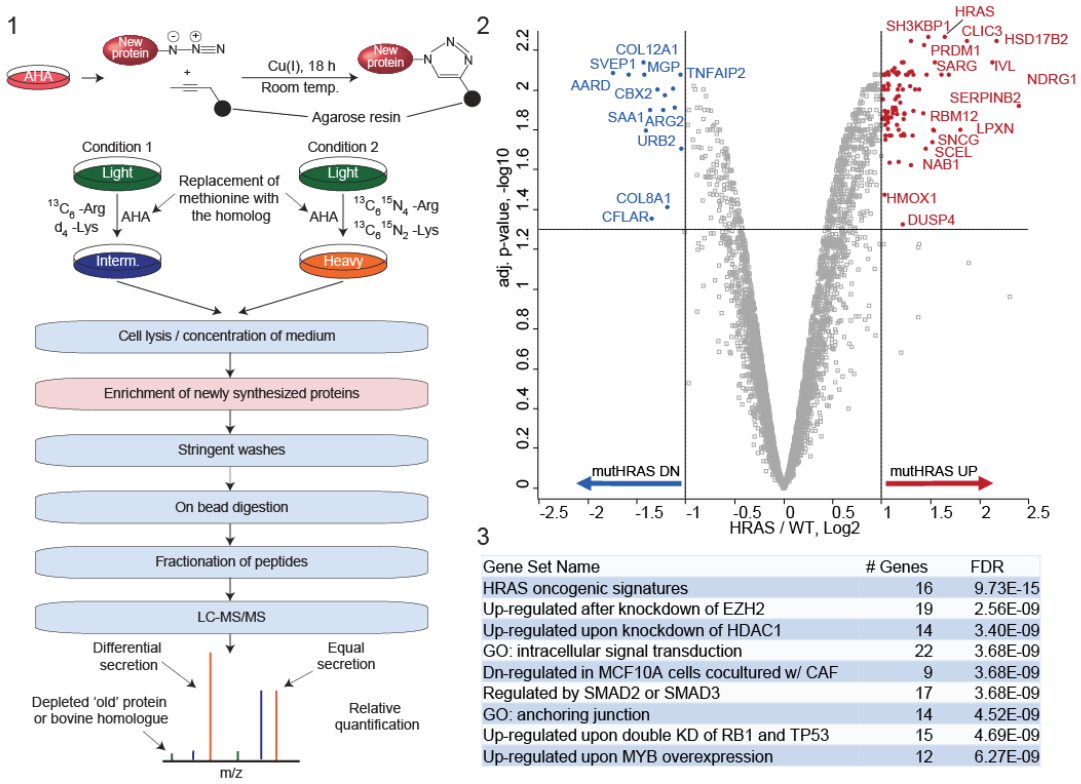
Figure 2. Volcano-plot. Proteins quantified in global proteome analysis. Log2-transformed ratios of protein abundance in HRAS and WT cells over –Log10-transformed p-values calculated by the t-test on the basis of 3 to 4 replicates. Proto-oncogenes and RAS-signaling-related proteins are highlighted in colours using gene ontology data.

Overall, this analysis demonstrates the high equality of protein abundances in the total cellular proteome of those two cell lines. Even at relaxed parameters for assignment the

differentially expressed proteins (1.4-fold changes), only 51 and 13 of 5232 ones were found to be up- or down-regulated, respectively. Although several up-regulated proteins can be associated with EGFR-like tyrosine kinase receptor signaling (CAMK2D, SHC1, and receptor protein ERBB2) or RAS activity (RAC2, RASA1) and, therefore, serve as indicators of the pathway activation, the low total number of such proteins and minor changes in their expression do not allow to make unambiguous conclusions on the status of signaling pathways in the examined cells using the global proteome analysis.

2.4 Comparison of the total and nascent proteome analysis

To estimate the capability of the nascent proteome analysis to improve the quantification of mutant RAS-driven alterations in protein expression, this method was applied to the same pairwise comparison of WT and HRAS MCF10A cells as for the global proteome analysis (**Fig. 3**). Consequently, I quantified 5117 proteins in at least two of three replicates, the number equitable to the results of the global proteome analysis. Furthermore, focusing on the nascent proteome allowed detection of more pronounced changes in protein expression. In HRAS MCF10A cells, 532 proteins were found to be significantly upregulated, 103 of which – more than 2-fold. As this might be expected, further gene ontology analysis revealed the prevalence of known mutant HRAS-effectors among these genes, particularly, RASA1, RIN1, ARFGAPs, and MAPKs that are involved in the mediation of RAS activity [97]. This provides sufficient evidence of the increased activity of RAS pathway in the mHRAS-transfected MCF10A cells. Furthermore, we observed the upregulation of a number of proteins playing a role in epithelial-mesenchymal transition (EMT), such as FN1 (fibronectin), VIM (vimentin), SCEL (sciellin), (LCP1) L-plastin [98], CLIC3, SERPINs [99].



Gene Set Name	# Genes	FDR
HRAS oncogenic signatures	16	9.73E-15
Up-regulated after knockdown of EZH2	19	2.56E-09
Up-regulated upon knockdown of HDAC1	14	3.40E-09
GO: intracellular signal transduction	22	3.68E-09
Dn-regulated in MCF10A cells cocultured w/ CAF	9	3.68E-09
Regulated by SMAD2 or SMAD3	17	3.68E-09
GO: anchoring junction	14	4.52E-09
Up-regulated upon double KD of RB1 and TP53	15	4.69E-09
Up-regulated upon MYB overexpression	12	6.27E-09

Figure 3. Nascent proteome analysis. (1) Principal scheme of of click-chemistry based purification of azidohomoalanine (homolog of methionine, AHA) containing proteins with alkyne-resin (Adapted from the work of Eichelbaum ae al. [92]). For pairwise comparison of expressed proteins different cell lines were grown in a regular medium, then the media was changed to the one containing AHA instead of methionine and SILAC versions of amino acids. Incubation time (6 hours) was chosen to accumulate a representative pool of newly-synthesized intracellular and secreted proteins and to avoid considerable influence of protein turnover rates. The incubation was followed by collection of cell pellets and growth media which were further purified accordingly to the enrichment protocol. (2) Volcano-plot. Average Log2-transformed ratios of newly-synthesized proteins in HRAS over WT MCF10A cells against p-values calculated by Limma (R-package) on the basis readings from three replicates and adjusted by Benjamini-Hochberg procedure. (3) GO enrichment analysis for the significantly up-regulated proteins.

This is in conformity with known properties of mutant RAS as the control of EMT by RAS signaling has been shown on mouse and cell models over the progression of carcinomas [100], [101]. In another recent study, it was also demonstrated that mutant G12V HRAS modulates expression of EMT markers in normal MCF10A cells *(N.B.: in this work the effect of HRAS was associated with suppression of TP63 activity through PI3K pathway*) [102]. The discovered upregulation of JAK/STAT pathway-related proteins (reactome, -log10 FDR >10) and targets of SMAD3/4 transcription factors (TGF-beta pathway, GSEA,

-log10 FDR >30) could mechanistically explain alterations in protein expression profile and to serve as evidence of cumulative action of RAS, JAK and TGF-beta signaling in our cell model as well as it was previously reported for breast cancer cell lines [103], [104]. Interestingly, I also found signature of suppression of histone modifiers, EZH2, and HDAC1 (**Fig. 3.3**). HDAC1/2 are global chromatin remodels and known promoters of tumor development [105], [106]. Particularly, upon hypoxic stress in cancer cells, HDAC1 was found to promote metastasis by downregulating transcription of the tumor-suppressor RECK through recruiting on its promoter in a complex with HIF1 [107]. On the other hand, experiments on mouse models and analysis of clinical data demonstrated that HDAC1 is required for attenuation of proliferation in carcinomas [108]. Therefore, the down-regulation of HDAC1 activity upon mutant HRAS expression can be explained both, by the HRAS-driven rearrangement of gene transcriptional program, and the cellular feedback to the aberrant pro-proliferative signaling. The same statement can be made for EZH2 as this member of the polycomb repressive complex 2 (PRC2) was found to be up-regulated in many cancers, including mutant RAS-driven ones [109], [106]. Yet, the fact that the loss of EZH2 was also reported to be a factor of amplified Akt and ERK activation in lung adenocarcinoma [110] highlights that the activity of histone modifiers is context-dependent and does not serve as a marker of tumorigenesis per se.

2.4.1 Summary

To summarize, in comparison to the standard SILAC-based global proteome analysis, the nascent proteome analysis showed a higher sensitivity for the detection of the changes in protein expression upon mutant-HRAS transfection. Yet, the depth of analysis was equitable for both methods: 5232 and 5117 proteins quantified in replicates in the global and nascent proteome analysis respectively. In contrast to the results of the global proteome analysis, which did not allow revealing alterations in the activity of signaling pathways due to the low number of proteins found to be regulated (64 in total), nascent proteome analysis showed up-regulation of 113 and down-regulation of 25 proteins. The examination of the up-regulated ones identified signatures of the RAS signaling amplification, an increase of the EMT markers expression, and alterations in the activity of histone modifiers and transcription factors. Overall, these results demonstrate the advantages of the nascent

proteome analysis for the investigation of signaling pathways even at steady-state conditions. The origins of its higher sensitivity were discussed in the methodological section.

Noteworthy, the single point mutation in a RAS protein has already been shown to cause moderate transcriptional changes in normal cells. For instance, in the recent work of Stolze *et al.*, only ~100 genes were found to be regulated upon the transfection of MCF10A cells with mutant KRAS [111]. This is in conformity with the findings in our cell system and also illustrates that detection of proteomic changes upon mutant RAS signaling is challenging, explaining the poor outcome of the total proteome analysis. It is notable as well that the consequences of the mutant HRAS expression were assessed herein in normal growth conditions for MCF10A cells, in the presence of EGF in the culture medium. The EGF receptor is upstream of RAS proteins and activates the RAS pathway upon binding by EGF [112]. Therefore, in this conditions, the HRAS pathway was naturally activated in WT MCF10A cells, and thus the alterations in the protein expression observed in the experiments were caused by the influence of the additional mutant copy of HRAS borne by the transfected cells and might represent both the specific effects of the mutant and the HRAS copy-number difference. To obtain the full picture of the mutant HRAS activity further studies were performed without EGF treatment.

2.5 Nascent proteome analysis of the mutant HRAS signalling outcome

The nascent proteome analysis was further used for pairwise comparison of protein expression in WT and HRAS MCF10A cells upon EGF depletion and at relatively low serum content (2% versus 5% conventional for culturing MCF10A cells [113]) to decrease the effect of stimuli-dependent signaling activation in WT MCF10A cells. RAS proteins harbouring activating point mutations are known to promote growth factor independent proliferation through self-sufficient stimulation of downstream pathways [111]. This approach was meant to focus on these effects of mutant HRAS and monitor the corresponding changes in protein expression.

Using the nascent proteome analysis as described in the previous section, I quantified >5000 proteins. This coverage is close to the numbers typical for total proteome and ample for analysis of individual players, and pathways responding to the oncogenic RAS signaling. In total, up-regulation of 587 and down-regulation of 438 proteins was observed in the mutant RAS expressing MCF10A cells in comparison to the WT. As expected, analysis of the up-regulated proteins revealed signatures of cell cycle progression, activation of mTOR-pathway, induction of MYC, and NF-kB-driven transcription (**Fig. 4**).

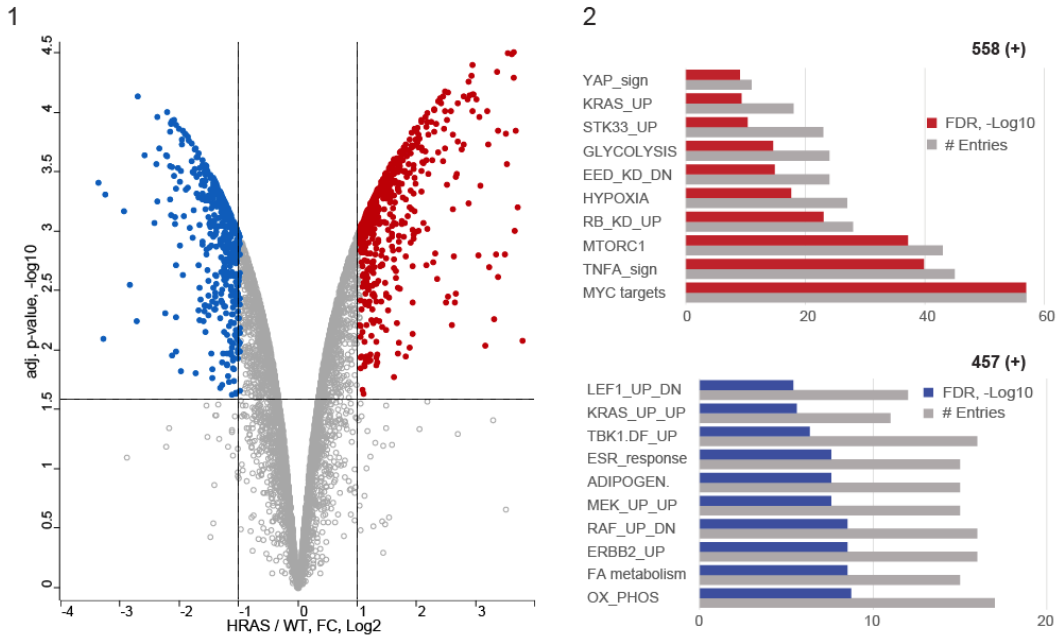


Figure 4. Nascent proteome analysis. (1) Average Log2-transformed ratios of newly-synthesized proteins in HRAS over WT cells against p-values calculated by Limma (R-package) using the 'FDR' method for calculation of the adjusted p-values. (2) The gene-set enrichment analysis (GSEA) for the significantly regulated protein functional sets, number of identified entries and the enrichment test adjusted FDR values for each set are represented by grey and blue bars, respectively.

2.5.1 Signalling pathways

The PI3K-mTOR-pathway is known to be frequently activated in breast cancers and contributes to the tumorigenesis via regulation of proliferation, and survival [114], [115]. As a downstream effector of RAS, it has also been reported to be activated in a variety of human cancer cells expressing mutant RAS [116], [117], such as breast, kidney, prostate, and liver carcinomas. Using gene set enrichment analysis at gene expression profiles for

human epithelial cell lines and patient-derived tumor samples different in the activity of mTOR (GSE47108, GSE26599), 44 hallmarks of mTOR-activation-driven gene expression were identified among proteins overexpressed in the mutant-HRAS-transfected cells. Additionally to the PI3K-mTOR, RAF-ERK pathway activation hallmarks were identified among proteins regulated upon mutant HRAS expression: 15 proteins down-regulated upon constitutively active RAF1 expression in MCF10A (GSEA, M2726) were also decreased in expression in the HRAS MCF10A cells. This demonstrates that the employed proteomic approach is sufficient for the characterization of mutant-RAS-driven alterations in the activity of signaling pathways and these alterations correspond to the expected effects of the mutant RAS.

2.5.2 Transcription regulators

MYC is a family of oncogenic transcription factors that play a pivotal role in the regulation of proliferation, cellular growth, and survival. Dysregulation of MYC through chromosomal translocations, amplification, or aberrant stabilization contributes to the development of many human cancers [118]. RAS proteins regulate the accumulation of MYC by posttranslational phosphorylation in Ser62 via RAS-dependent downstream pathways, such as mTOR or ERK [119], [120]. Thereby, oncogenic cooperation between RAS and MYC is known to be crucial for tumorigenic transformation [121], [122], [123], [124]. Among known targets of MYC, 57 ones were found to be up-regulated in the HRAS MCF10A cells (M5926, SGEA database), including proteins MCM2-7, and CDC20 reported to be involved in modulation of MYC oncogenic activity [125], [126]. This supports that the effects of the mutant RAS signaling in the MCF10A system are partially mediated by stimulation of MYC transcriptional program. Noteworthy, MYC family proteins were not regulated in the mutant-HRAS-transfected cells, suggesting that the activation of the MYC transcriptional program is modulated rather through RAS-driven stabilization than through regulation of MYC expression.

Analysis of alterations associated with other transcriptional regulators revealed signatures of the RB1 activity down-regulation (GSEA 26 proteins, GSEA, [127]. However, these proteins (MCMs, PCNA, POLD1, PRIM2, and others) are also involved in DNA replication

and thus the enrichment can be explained by the higher proliferative activity of HRAS MCF10A cells upon EGF depletion. In conformity with the results of the protein expression analysis in the presence of EGF discussed in the previous section, the disturbance of the PRC2 complex (EZH2, EED) functions were discovered by comparison with the polycomb target genes published in the study of Braket *et al.* (31 overlapping entries, -log10FDR>9, GSEA), [128]).

2.5.3 Hippo pathway

Interestingly, 11 conservative signatures of YAP activity were found among up-regulated proteins, including TK1, CDC20, DUSP1, GADD45, and SERPINE1 [129]. YAP/TAZ proteins are the final transcriptional modulators of the Hippo-pathway activity. The aberrant signaling through this pathway is a known factor of tumorigenesis as the Hippo controls cell proliferation, survival, and can promote loss of cell-cell contact inhibition of cellular growth (reviewed in [130]. As it was mentioned in the introduction section, RAS proteins are capable to regulate YAP/TAZ activity by affecting MST1/2, the kinases participating in the regulation of the YAP/TAZ phosphorylation status and translocation mediated by LATS1/2. The crosstalk between RAS and YAP plays a significant role in the development of RAS-driven cancers and resistance to inhibitors targeting the downstream RAS pathways [131], [132]. Although the RAS and Hippo signaling were shown to control the expression of overlapping sets of genes [133], [134] and YAP is known to functionally compensate the loss of RAS activity in cancer cells [132], [135], [136], the evidence regarding the relationships between the RAS status and activation of YAP are contradictive. It was previously reported in studies of various human cancer and artificially transformed cell lines that mutant RAS signaling can promote YAP/TAZ transcriptional activity, particularly through the positive feedback loop mediated by RAS-driven overexpression of AREG, an analog of EGF which can stimulate the YAP activation [137], [138]. However, other reports suggest that YAP activity is not directly regulated by mutant RAS expression [139] and the YAP-RAS connection depends on the cell line and additional genetic alterations [140], [137]. This proposition is supported by the recent studies showing that the activating effect of RAS on YAP through the downregulation of the hippo signaling is partially mediated by the downstream factors such as localization of MEK kinases, the

status of the ubiquintinase BTRC, and dimerization of MTS1/2 [141], [142], [143]. Nevertheless, the YAP signaling is a well-established significant component of RAS-driven tumorigenesis. As this will be discussed below, in the context of this study, the role of YAP in the promotion of metastasis is of especially high interest [144]. In the recent report, it was also shown that mutant RAS and YAP activities intersect in the regulation of the epithelial-mesenchymal transition (EMT) [134]. Noteworthy, it was also demonstrated in this publication that RAS and YAP converge on the transcription factor FOS, a downstream effector of RAS whose transcription targets are partially shared with YAP in MCF10A cells [145].

2.5.4 AP1 complex

Interestingly, in the MCF10A system, the FOS proteins, along with JUN, were found to be among the most dramatically upregulated ones upon mutant HRAS expression (>16-fold change for FOS). The families of transcription factors FOS and JUN regulated gene transcription in the form of mono- or heterodimers which is historically called AP1 (Activating Protein-1). AP1 is an effector of many pathways: mTOR, ERK, Wtn, NFkB, JAK, Hippo, and others. AP1 regulates the full spectrum of cellular processes associated with these pathways, including proliferation, survival, migration, and autocrine stimulation. Consequently, dysregulations of AP1 activity play a role in tumorigenesis, however, the actual oncogenic or onco-suppressive function of AP1 is defined by the combination of the upstream signaling, the genetic background of the cell, and the composition of the complex as distinct FOS/JUN family members exhibit the different regulatory properties [146], [147], [148]. AP1 regulates cell cycle through transcriptional regulation of CCND1 (cyclin D), CDKN1A (p21), and TP53 (p53) and might thus mediate the RAS pro-proliferative activity [149], [150] as RAS is particularly reported to interfere the cell cycle through Cyclin D1 [151]. Indeed, the comparison of HRAS and WT MCF10A cell revealed up-regulation of cyclin D and p21 in mut-HRAS-transfected cells along with the up-regulation of cyclin-dependent kinases CDK1-4 which participate in RAS-induced centrosome amplification in mammary epithelial cells [152].

2.5.5 Phenotypic markers

To focus on alterations in protein expression which might be effective for phenotypic characteristics of the mutant-HRAS-transformed cells, I considered the players of metabolic processes and revealed the signatures of respective changes. Particularly, the up-regulation of proteins related to glycolysis (e.g., PYGL, PFKP, GALK2, AKR1A1, GPI, and ENO1; GSEA, 24 entries, -log10FDR>14) coupled with down-regulation of ones playing a role in oxidative phosphorylation (e.g., HADHA/B, PDP1/4, ACAT1, NNT; GSEA, 17 entries, GSEA, -log10FDR>9). Taken together these observations refer us to the Warburg effect, the metabolic reconstruction typical for cancer cells that reflects in inhibition of the citric acid cycle, and the predominance of glycolysis for generating ATP [153]. Various disturbances of cellular processes are known to provoke this metabolic shift, including aberrant activation of mTOR, Myc signaling, or p53 mutations. In combination with previous studies, our findings confirm, that mutant RAS activity is potent to remodel cellular metabolism causing the Warburg effect [154] and, therefore, provide the detailed molecular profile of the RAS-driven alterations in glycolysis and oxidative phosphorylation in the perspective of changes in protein expression. Noteworthy, the observed metabolic shift can be associated with the activity of hypoxia-induced factors: the analysis revealed the up-regulation of hypoxia-induced protein (HMOX1, NOCT, AKAP12, and others). The hypoxic response is typical for cancer cells due to the fact that oxygen supply is often decreased in tumors [155]. However, the hypoxia-induced metabolic and transcriptional regulation also plays a role in the tumorigenesis itself (reviewed in [156], [157]. The hypoxic response is mainly mediated by the hypoxia-inducible factors (HIF) possessing the TF activity and modulating the expression of proteins involved in secretion, oxidative phosphorylation glycolysis, and other processes [158]. Importantly, HIF factors are known to affect the transcriptional activity of MYC, acting as a co-factor [159]. Furthermore, it cross-talks with p53 through the complex network of intermediators, such as PTEN, FOXO, TP300, and thus influences the cell fate determination [160], [161]. HIF was also reported to cooperate with mutant RAS in tumours, and cell models [162], [163]. Noteworthy, the sensitivity of the proteomic method did not allow to quantify HIF proteins, however, the results of the transcriptomics analysis, which will be discussed in the following section, revealed significant up-regulation of HIF1A transcripts upon the mutant-HRAS

transfection and down-regulation upon knockdown of p53 in HRAS MCF10A cells (~3- and 2-fold, respectively). This is in conformity with the effects of the p53 knockdown described below.

Considering other phenotypic markers, I observed the upregulation of Epithelial-Mesenchymal Transition (EMT) hallmarks, such as FN1 (fibronectin), VIM (vimentin), SCEL (sciellin), L-plastin (LCP1), CLIC3, SERPINB1/2, SERPINE1/2 that elucidates the role of RAS over-activation on malignancy development [98], [99]. Epithelial-mesenchymal transition is defined as the loss of epithelial properties and the gaining of a mesenchymal phenotype and originated from the embryology field. However, these phenotypic changes are also typical in tumorigenesis, as they promote invasion and establishment of metastatic features of cancer cells. In breast cancers, EMT is associated with mammary tumor progression and poor prognosis [164]. Previous experiments on cell lines showed that mutant RAS can contribute to the initiation of EMT [165] through NF-kB and NOTCH signaling activation [166]. And, indeed, along with, we also revealed signatures of NF-kB activation, and up-regulation of proteins involved in related to NOTCH signaling pathways in breast cancer: CCNB1, JUN JAG1, CCND1, CDK4, FOS, MMP9 (Elsevier Pathway Collection). The RAS-driven regulation of EMT proteins might also depend on the overexpression of AURKA/B proteins, mitotic serine/threonine kinases responsive to RAS signaling, and reported to contribute to EMT [167]. Indeed, according to the RNA-seq data AURKA/B are up-regulated in HRAS MCF10A cells. However, our further results from the p53-knockdown experiments described in the next section did not confirm this supposition.

2.5.6 Summary

To summarise, the discussed alterations in protein expression demonstrate that the mutant-HRAS-transfection in our cell system yields a predictable outcome, typical for oncogenic RAS activation and that the employed proteomic approach allows detailed analysis of activated pathways and distinct players of RAS-driven signaling. Besides the activation of the RAS-dependent signal transduction through mTOR, ERK, and other pathways, the mutant RAS expression in MCF10A cells causes alterations in the activity of transcriptional

regulators, such as PCR2, MYC, YAP/TAZ, AP1. These effectors are modulated by the cumulative effect of the signaling downstream RAS, and therefore, the direct relationships between those and the mutant RAS activity remain to be clarified. Nevertheless, these results show that the combination of the MCF10A cell system and the nascent proteome analysis represents a promising platform for investigation of individual players of RAS signaling (and, more broadly, of tumorigenesis) by subjecting the cells to specific perturbations such as selective inhibitor treatment or knockdown of specific targets. In this work, the MCF10A cells were further used for investigation of the functions of wild-type p53 in the context of the oncogenic signaling.

2.6 The role of p53 in the context of the mutant RAS signalling

In many cancers, RAS activation combines with a loss of p53. p53 is a key regulator of the cellular stress response, including DNA damage, and aberrant mitogenic signaling. Previous studies showed that loss of p53 can lead to genomic instability, and increase the probability of spontaneous tumorigenesis in mice.

Dysregulation of p53-dependent cell cycle control can also promote oncogene-driven tumorigenesis in p53-compromised mice [168], [169]. Furthermore, oncogenesis was found to be often associated with gain-of-function (GOF) mutations in p53, leading to alterations in conformation, and DNA binding properties of the protein. The p53 mutants typically lose their oncosupressive functions and, in contrast, can benefit to tumorigenesis through cooperation with oncogenes, and regulation of a large number of genes involved in the regulation of migration, and proliferation [170], [171]. Particularly, GOF p53 was reported to positively control EMT and metastasis through modulation of ZEB1/2 activity in cancer cells [172]. In past years, the growing number of observations of the mutation-driven alterations of p53 activity yield the development of the therapeutic strategies targeting mutant p53 in various human tumors, including triple-negative breast cancer [173], [174]. Nonetheless, the demonstrated property of mutant p53 to be rewired depending on the oncogenic signaling, and genetic environment, raised the interest to the cellular context determined functions of the wild-type variant. Recent studies demonstrate that opposing the conventional knowledge of the wild-type p53 as of an exclusive tumor-suppressor, this

TF can act in a mutant-like manner stimulating survival and migration in cancer cell lines, and normal cells with aberrations in activity RAS or Hippo pathways [175], [12], [176].

MCF10A cells express normal p53 [177]. Previous studies showed that HRAS-transfection of the MCF10A cells does not cause growth arrest or apoptosis, whereas p53 depletion in mutant-HRAS-transfected MCF10A cells also does not enhance the xenograft tumor growth in vivo [178], albeit, in theory, the tumor suppressor loss might be beneficial for oncogenic activity and further HRAS-driven transformation. Although these pieces of evidence do not answer the role of p53 in the context of mutant RAS signaling in MCF10A cells, these suggest that p53 does not crucially suppress the oncogenic potential of mutant HRAS, similarly to how it happens in the wild-type-p53-expressing cancer cell lines. Thereby, investigation of the functional state of the wild-type p53 in MCF10A cells in the aspect of its role in the determination of the aberrant RAS signaling outcome could elucidate the mechanism underlying the functional interaction between p53 and oncogenes in cancers.

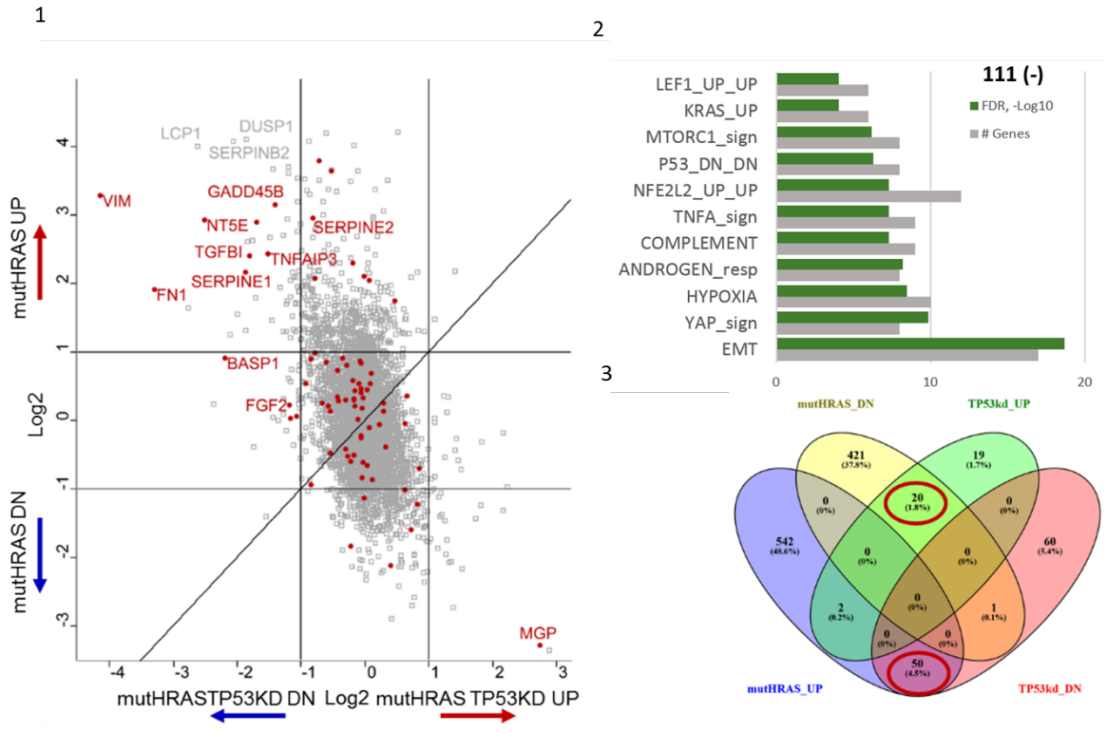


Figure 5. Effect of TP53-knockdown in HRAS-transformed cells. (1) Scatter-plot, nascent proteome. X-axis – HRAS-transformation outcome, log2-transformed ratios of HRAS over WT, Y-

axis – TP53-knockdown (TP53KD) outcome, log2-transformed ratios of HRAS after TP53kd over HRAS. Hallmarks of EMT (GSEA) are highlighted in red. (B) Gene ontology analysis of proteins down-regulated by TP53KD in HRAS-transformed cells. (C) Venn diagram overlapping sets of proteins regulated either by HRAS transformation of WT cells or p53 knockdown in the HRAS-transformed cells.

As it was discussed in the previous sections, the nascent proteome profiling is meant to provide detailed information on alterations in cellular processes caused by various molecular manipulation. I applied this method to the cellular model of the mutant-HRAS-transfected MCF10A cells subjected to TP53-knockdown to gain insights into the role of the wild-type p53 in the context of mutant HRAS signaling. The p53 knockdown in the WT MCF10A cells served as a control representing the 'canonical' p53 activity in cells with undisturbed signaling. Therefore, the difference in the consequences of the p53 knockdown in the WT and mutant-HRAS-transfected cells can be interpreted as a reflection of the functional alteration of the normal p53 activity in the context of oncogenic signaling.

2.6.1 Effects of the p53 knockdown in WT MCF10A cells

As expected for normal cells not subjected to stress and expressing p53 at a basal level, the p53 knockdown caused minor effects on protein expression [179]. Collectively, the up-regulation of 10 and down-regulation of 35 proteins was revealed upon the p53 knockdown in WT MCF10A cells. Among the down-regulated proteins, 5 were represented by direct p53 targets – TRIM22, CD82, FDXR, ZMAT3, and CYFIP2 [180]. TRIM22 is an interferon-signaling-dependent transcription factor, involved in the regulation of antiviral response. TRIM22 was also shown to regulated protein expression through interaction with translational factors eIF4E and eIF4G [181]. In normal cells, the expression of TRIM22 is controlled through the p53 axis, however, this control can be deregulated in breast cancer where TRIM22 is under-expressed in comparison to non-malignant cell lines [182]. Intriguingly, TRIM22 was also shown to promote epithelial-mesenchymal transition through regulation of AKT/GSK3β/β-catenin signaling in non-small cell lung cancer [183]. Therefore, the role TRIM22 in tumorigenesis remains unclear and likely depends on the genetic background. In MCF10A system, both TRIM22 transcription and translation remain intact upon mutant HRAS expression and down-regulation of TRIM22 is associated with p53 knockdown independently of RAS signaling. This indicates that the p53 activity

in the aspect of TRIM22 regulation is not affected by RAS signaling. FDXR, a mediator of p53-controlled apoptosis was regulated in the same mode [184].

In contrast, CYFIP2, a p53-inducible pro-apoptotic nuclear protein whose down-regulation is associated with gastric and colon cancer progression [185], [186], is also down-regulated in the mutant-HRAS-transfected MCF10A cells. Interestingly, the suppression of its homolog CYFIP1 (non-regulated in the given MCF10A model) was previously reported to induce EMT in MCF10A cell models and invasive carcinoma development in mice in a cooperative manner with an oncogenic HRAS signaling; potentially by disturbing the activity of the actin skeleton remodeling complex WAVE that includes CYFIP1/2 [187], [188]. Taken together, these observations indicate that the mutant RAS can influence the expression of distinct p53 effectors independently of p53 activity. Two other protein were found to be regulated by the p53 knockdown only in WT MCF10A cells: ZMAT3 and C82. ZMAT3 mediates p53-driven cell cycle arrest and modulates the p53 level through specific stabilization of p53 mRNA [189]. C82, a known metastasis repressor positively regulated by p53 and frequently down-regulated in the breast and prostate cancers [190], [191]. Among the up-regulated proteins, HMGB2/3 are involved into the p53 regulatory loop, can regulate p53 stability and are probably indirectly controlled by p53 [192], [193], [194].

Interestingly, HIC2, a transcription factor, was found to be dramatically (>20-fold) up-regulated upon the p53-knockdown both in WT and HRAS MCF10A cells. Furthermore, HIC2 was also up-regulated upon the mutant HRAS expression. HIC2 is a poorly studied protein (24 related PubMed publications), however, its homolog, HIC1, is a known transcription repressor homozygously deleted of hyper-methylated in many cancers [195]. Moreover, it was demonstrated that the lack of HIC1 and TP53 genes cooperatively contribute to tumor progression [196]. Therefore, the upregulation of HIC2, containing the same transcriptional repression mediating domain BTB/POZ as HIC1, might be a result of compensation of the p53 repression (in WT cell with p53 knockdown), or the insufficient p53 activity in the cells with aberrant RAS-signaling.

Overall, although the p53 silencing caused only minor effects in the WT MCF10A cells, the down-regulation of a set of p53-controlled proteins upon the p53 knockdown supports

that p53 remains active in the MCF10A cells in terms of the protein expression control, and its activity can detected by the nascent proteome analysis.

2.6.2 Effects of the p53 knockdown in HRAS MCF10A cells

Further, the effects of p53 knockdown in MCF10A cells transfected with the mutant-HRAS were estimated. I identified the up-regulation of 40 and down-regulation of 110 proteins upon p53 knockdown in mutHRAS-transfected cells. This represents the same pattern as one previously observed in wild-type cells: p53 knockdown predominantly leads to the downregulation of protein expression in the MCF10A cell system. However, the p53 knockdown driven regulation in mutHRAS-transfected cells is more pronounced (150 regulated protein versus 45 in WT cells). Particularly, among the down-regulated proteins, I identified 6 hallmarks of p53 pathways activation (CDH13, STEAP3, PROCR, HMOX1, CCND3, NDRG1 (GSEA, M5939)), and 14 known direct effectors of p53 (TRIM22, IGFBP6, LRP1, IKBIP, MMP2, CES2, STEAP3, CFLAR, GBE1, CMBL, DUSP1, GNAI1, SERPINE1, RRM2B [197]). This stronger effect of p53 knockdown in mutant HRAS expressing cells might be explained by the more active state of the p53 network induced by the oncogenic HRAS signaling. This is supported by the enrichment of the p53 pathway activation hallmarks (GSEA, 20 proteins, -Log10 (FDR) >10) among proteins up-regulated in mutant HRAS expressing cells. However, the mechanism of this expression increase might also be p53-independent as only 3 of those 20 hallmark proteins were affected by the p53 knockdown. Noteworthy, only 4 proteins were commonly downregulated by the p53 silencing in the WT and HRAS MCF10A cells (TRIM22, CES2, MCF2L2, FGF2). Therefore the p53 knockdown causes different outcomes depending on the status of RAS signaling.

Considering the normal p53 as a tumor suppressor which can partially block the action of the aberrant RAS signaling, this might be expected that the loss of p53 activity and the related downregulation of proteins controlled by p53 could facilitate the conversion of the cells into a cancer-like state by the oncogenic signaling and make the alterations in protein expression observed in the mutant-HRAS-transfected cells more pronounced. However, the analysis of the proteins down-regulated upon p53 knockdown in the HRAS MCF10A cells

revealed a more intriguing outcome. Of the 110 proteins down-regulated by the p53 knockdown, only one (CES2, a p53-induced protein) was also down-regulated by the mutant HRAS expression, whereas 50 (!) were observed to be positively regulated in the mutant-HRAS-transfected cells in comparison to the WT MCF10A (**Fig. 5.3**).

Among these down-regulated proteins, the significant enrichment for signatures of EMT (GSEA, 17 entries, -Log10 (FDR) >19), NFkB (9 entries, -Log10 (FDR) >8) and mTOR (9 entries, -Log10 (FDR) >8) signaling was revealed. Furthermore, the up-regulation of the proteins that are typically negatively regulated by the activation of RAF, a downstream effector of RAS signaling, in breast cancer cells (6 entries, -Log10 (FDR) >8, [198] was also observed upon the p53 knockdown (**Fig. 5.2**). Thus, the silencing of p53 partly abolishes the effects of the mutant HRAS signaling on the protein expression regulated by the downstream pathways.

Interestingly, the gene set enrichment analysis revealed downregulation of 8 signatures of YAP signaling (GSEA, -Log10 (FDR) >11, M2871, [129], which has previously been found to be up-regulated upon the mutant HRAS expression. In the same manner, the p53 silencing also effected the hallmarks of hypoxia, such as AKAP12, ANGPTL4, GBE1 (GSEA, -Log10 (FDR) >9, M5891) (**Fig. 5.2**). Nevertheless, the most pronouncedly the p53 knockdown influenced the expression of the EMT defining proteins. It led to a decrease in expression of 17 of those, 7 of which were previously found to be overexpressed in mutant-HRAS-transfected cells, including VIM, FN1, FBN1 (GSEA, M5930) (**Fig. 5.1**). The role of p53 in regulation of YAP signaling, hypoxia, and EMT requires further investigation. The recent study of the ovarian cancer model demonstrated that the basal wild-type p53 expression controls cell migration upon the mutant RAS signaling [12]. However, the study doesn't provide mechanistic explanations for this observation. As it is discussed herein in the phenotypic characterization section, the connection between the alterations in the expression of EMT markers and the phenotypic properties of the MCF10A cells was confirmed by the migration and invasion assays. Nevertheless, further detailed investigation of the players intermediating the influence of p53 on EMT and YAP signaling in this system remains to be done. The available data presented in this work allows the identification of pivotal players and can be used in further validation studies.

2.6.3 Knockdown of p53 and transcriptional regulators of EMT

The dysregulation of the p53 network has been demonstrated to affect the EMT and stemness in normal pancreatic cells through modulation (albeit, by an unclear mechanism as well) the expression of the transcription factors regulating EMT: SNAIL1/2, ZEB1/2 [199]. None of these low abundant TFs was quantified by the nascent proteome analysis in the MCF10A system. However, the transcriptome data indicate that ZEB1/2 were up-regulated upon the mutant HRAS signaling (~5- and >30-fold respectively) and that this effect was completely abrogated upon the p53 silencing (*N.B. the up-regulation was associated with low p-value, whereas the significance of the abrogation was confirmed statistically*). In this context, it is interesting that ZEB1 and p53 were shown on the breast cancer model to be functionally connected with HDAC1/2 and act as a bi-molecular (ZEB1-p53) or tri-molecular (ZEB1-p53-HDAC1/2) complex activating expression of FGF2/7, VEGF, and IL6, and contributing to tumour initiation, progression, and metastasis [200]. As discussed above, HDAC1 itself and the markers of its activity were found to be down-regulated upon the mutant HRAS signaling. Furthermore, the expression of IL6 and VEGFC was found to be correlating with the p53 status in the HRAS MCF10A cells (the up-regulation in the mutant HRAS expressing cells and the down-regulation upon the p53 knockdown). Besides, it was also recently reported that p53 can modulate the activity of ZEB1/2 through micro-RNA [201]. Although the microRNA analysis was not a part of my studies, an investigation of the dependency of the micro-RNA content on the HRAS and p53 status might provide additional information on the p53-ZEB1 axis activity in the context of the mutant RAS signaling. SNAI2 was also revealed to be dependent on the p53 expression in the HRAS MCF10A cells: >15-fold up-regulated and downregulated ~4 times upon the p53 silencing (transcriptomics data). Intriguingly, SOX9 that known to cooperate with SNAI proteins in the metastasis development and to be stabilized through the interaction with SNAI [202] was regulated in the opposite way (the proteomic data). However, this might be explained by the downregulation of YAP signaling as the SOX9 expression and its functional activity, in terms of regulation of EMT, depend on the Hippo pathway [203], [204]. The downregulation of SOX9 was also reported to be associated with metastasis in prostate cancer [205]. Interestingly, overexpression of a truncated mutant SOX9 specifically in colorectal carcinomas bearing mutant RAS and wild-type p53 is associated

with better overall survival [206], demonstrating that SOX9 can play a tumor-suppressive role in this genetic environment (the data of the context-dependent role of SOX9 in cancer is reviewed by M. Aguilar-Median *et al.* [207]). Noteworthy, despite the up-regulation of SOX9 upon the p53 knockdown, the analysis of TF targets (Enrichr database [208]) revealed the downregulation of SOX9 transcriptional activity (26/297 SOX9 targets identified among the 110 proteins downregulated upon p53 silencing, -Log10 (FDR) >18). This conforms with the previous findings that the nuclear localization of SOX9 is conditional on the status of p53 [209]. Overall, these transcription factors and cofactors (ZEB, SNAI, SOX9, YAP/TAZ) represent promising targets for further studies on the MCF10A model due to their functional role in EMT or cancer development and the known physical interactions between these TFs (except for SOX9) and p53 [210], [211], [212]. These interactions were also shown to be altered by mutations in p53 and, thus, are depending on its functional state which, as this could be hypothesized on the basis of the previous findings [28], [11], [12] and my results, possesses the potential to be modified by the signaling context.

2.6.4 Summary

In summary, the investigation of the effect of the p53 silencing upon the mutant HRAS signaling revealed that the p53 knockdown in the HRAS MCF10A cells partly abolishes the effects of the mutant HRAS, and conversely, the outcome of HRAS signaling in the given cell system depends on the activity of wild-type p53. This is especially relevant for RAS downstream pathways (mTOR, RAF), YAP signaling, and EMT. Therefore, the wild-type p53 cooperates with the oncogene and exhibits rather mutant-like properties. The broached difference in the consequences of the p53 knockdown in WT and HRAS MCF10A cells will be explicitly illustrated upon the discussion of the transcriptome data in the following section.

2.7 Gene transcription and activity of p53 in WT and HRAS MCF10A cells

To investigate whether the alterations in protein expression correlate with mRNA abundances, the RNA-seq analysis was performed. Conceptionally, the transcriptome data allows distinguishing which of the observed regulatory effects of the mutant HRAS signaling and p53 knockdown are intermediated by modulation of respective genes, and which of them might be modulated post-transcriptionally. Besides, the data provides a more detailed view of the mutant RAS signaling outcome and the contribution of p53, due to the lack of missing values and the higher depth of the RNA-sequencing comparing to the proteomic analysis as this has been already illustrated by the extraction of the RNA-seq expressional data on ZEB1/2 and SNAI1/2 for interpretation of the proteomics results (section "The role of p53 in the context of the mutant RAS signaling"). At last, the independent transcriptomics readout serves for additional global validation of the observations made by the nascent proteome analysis.

2.7.1 Comparison of gene and protein expression

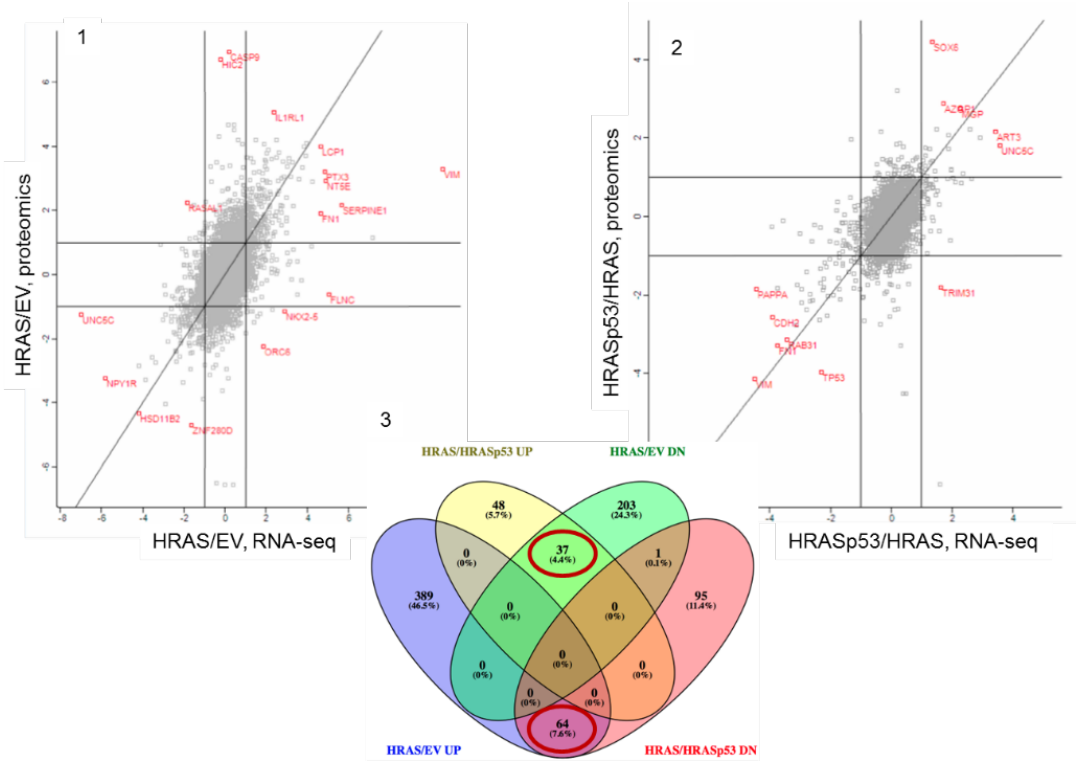


Figure 6. Comparison of proteomic and RNA-sequencing data. The log2-transformed ratios of gene/protein expression alteration upon the mutant HRAS expression (1) and the p53 knockdown in the mutant-HRAS transfected MCF10A cells (2); X-axis – transcriptomics data, Y-axis – rations obtained by the nascent proteome analysis. (3) The venn diagram overlapping sets of genes (mRNA-seq) regulated either upon the mutant HRAS signaling or the p53 knockdown in the mutant-HRAS transformed cells.

In total, the transcriptomic analysis allowed quantification of >14000 genes and anti-sense RNAs that are more than two-fold higher than the depth achieved by the proteomic analysis (>5000 proteins). Nevertheless, the mRNA-seq and proteomic data demonstrated a high correlation (Pearson correlation between ~0.5 and ~0.6, (**Fig. 6**)). Previous systematic studies of the mRNA and protein abundances dependency reported the typical conformity of 56%–84% of the cellular proteome to mRNA levels [213], [214], [87]. Therefore, the obtained results are in conformity with the expected level of correlation. Furthermore, the analysis of alteration caused by the mutant RAS signaling on the mRNA level revealed the perturbations of the same cellular pathways and processes as the elucidated by the nascent proteome analysis. Among the 453 up- and 241 down-regulated genes (≥2-fold, FDR < 0.05) the cancer hallmark analysis (Webgestalt, Hallmark50) revealed signatures of EMT, hypoxia, and signaling of RAS and NFkB (**Fig. 7**). The over-representation analysis using the datasets of the Broad Institute [215] also led to similar results as for the proteomic data, albeit identified more overlapping entries for each category and reported lower p-values. Thus, along with the up-regulation of mTOR and RAF activation, the coverage of the YAP conservative targets found among the up-regulated proteins was increased: 18 entries (-Log10 (FDR) >20) vs 11 in the proteome. Furthermore, the markers of alterations in some of the pathways that were not found to be enriched at the sufficient significance by the proteomic method were efficiently identified by mRNA-seq. Particularly, the mRNA data confirmed the increase of the inflammatory response (26 entries, -log10 FDR>18) and TGFB pathway activity (31 entries, -log10 FDR>25) markers that could be interpreted from the proteomic data only on the basis of upregulation of JAK/STAT pathway-related proteins and targets of SMAD3/4, respectively.

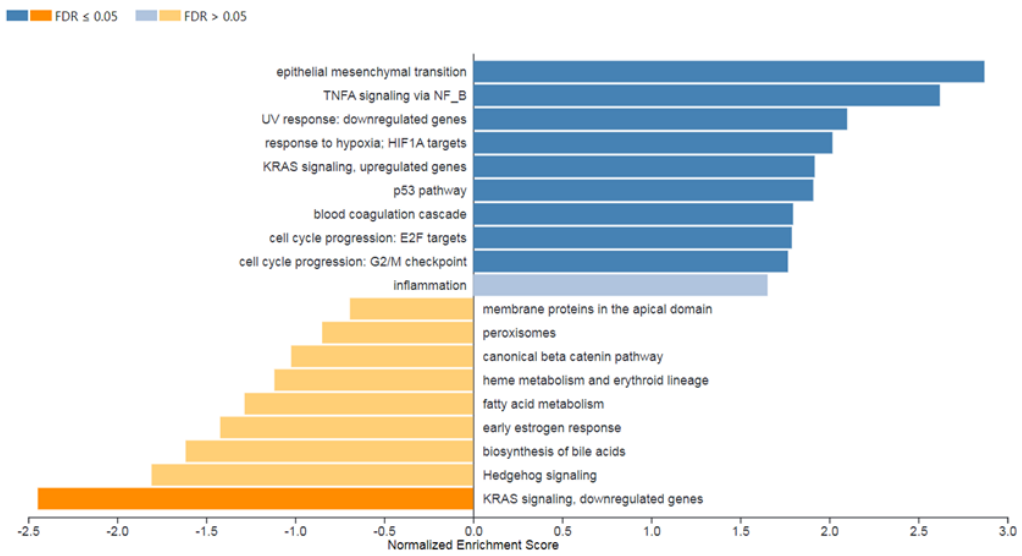


Figure 7. The pathways differentially the mutant HRAS signaling in the MCF10A cells according to the transcriptomic analysis (WebGestalt, Hallmark50).

The transcriptomic analysis revealed enrichment for the hallmarks of p53 activation (29 entries, -Log10 (FDR) >22), including the ones which were not identified in the proteome, such as GADD45A, RRAD, and TRIB3. Noteworthy, among the 17 markers which were also quantified by the nascent proteome analysis, 16 (except for RPL36) were also found to be significantly up-regulated by the mutant RAS signaling. In this context, this might interesting that RPL36 was reported to act as the tumor suppressor preventing the KRAS-driven pancreatic cancer progression in the zebrafish model [216]. Furthermore, the RPL36 expression was found to be regulated by a long non-coding RNA PLAC2 in glioma [217], and albeit this observation does not serve as direct evidence, together with the proteomic/transcriptomic results, this highlights the potential that RPL36 translation can also be regulated post-transcriptionally. Overall, the results of the RNA-seq analysis correspond to the conclusions made on the basis of the proteomic data. The higher enrichment rates for the pathway activation markers obtained from the transcriptomic data might be explained by a higher depth of the RNA-seq analysis. However, the numbers of genes/proteins found to be regulated in proteomic and transcriptomic experiments are comparable. Therefore, it seems that the higher productiveness of the RNA-seq gene ontology analysis is rather originated from the fact that the vast majority of the annotations and the consensus gene-sets are generated on the basis of transcriptomic data [218] and,

thus, are favorable for the analysis of the RNA-seq derived expressional data due to the technical (the depth of the analysis) and biological (post-transcriptional regulation events) reasons. The comparison of the expression alterations at the proteomic and transcriptomic levels in the MCF10A cell system revealed the incomplete overlap between the sets of regulated genes and proteins (**Fig. 6.1, 6.2**). This can be explained by translational regulation, and the experimental approach used in this work, indeed, provides a suitable tool to investigate the difference in transcriptional and translational regulation in this one or other systems. However, as this was not an aim of the given project, I will limit the consideration of the results to the discussion of which consequences this divergence might lead to for the characterization of the oncogenic RAS signaling.

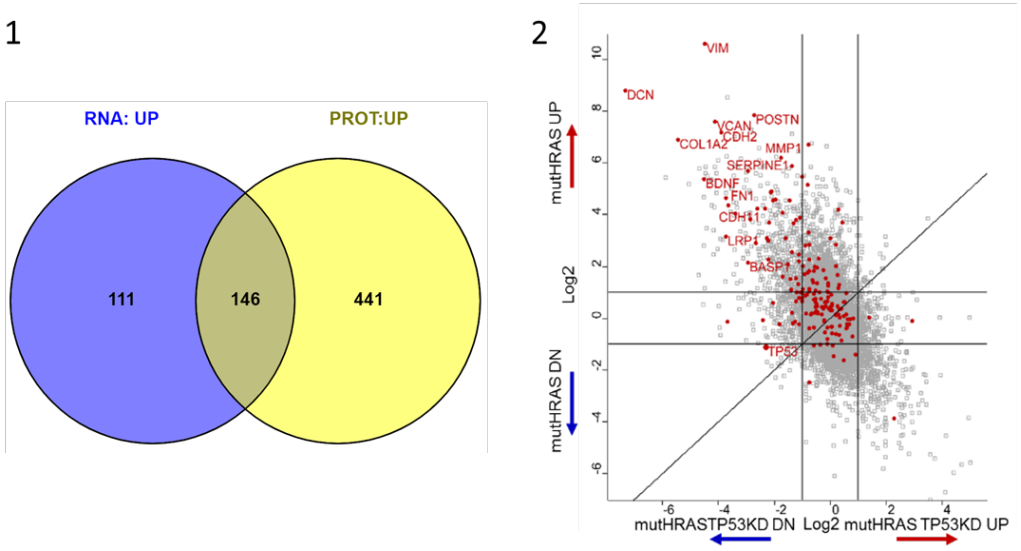


Figure 8. Comparison of proteomic and RNA-sequencing data. (1) The Venn diagram illustrating the overlap between the gene and proteins found to be up-regulated upon the mutant HRAS expression by transcriptomic and proteomic analysis; the RNA-seq-identified genes which expression was not quantified by the nascent proteome analysis were excluded. (2) Scatter-plot, mRNA. X-axis – the mutant HRAS expression outcome: log2-transformed ratios of HRAS over WT MCF10A cells, Y-axis – p53 knockdown (TP53KD) outcome in the HRAS MCF10A cells, log2-transformed ratios of the cells subjected to the tp53 knockdown over intact HRAS MCF10A cells. Markers of EMT (GSEA, M5930) are highlighted in red.

2.7.2 Transcriptional alterations

As it is illustrated by the **Fig. 8.1**, only ~60% of the genes found to be upregulated by the RNA-seq upon the mutant RAS signaling were also found to be regulated in the same

manner at the protein level (importantly, the entries that were not quantified by the proteomics analysis are excluded), translation of almost all the rest were not affected. Nevertheless, as it is discussed above, the functional relevance of the regulated genes and proteins was found to comparable. Indeed, the isolated analysis of the 146 entries regulated both transcriptionally and translationally, shows the enrichment for virtually all the GO terms found to be representative for the mutant HRAS signaling (see the nascent proteome analysis section): mTOR, RAF, NFkB signaling, markers of hypoxia, p53 activation, and others; whereas the 111 genes exclusively regulated at the mRNA level are significantly enriched only for EMT markers. Yet, the 441 up-regulated proteins are mainly represented by the MYC and E2F targets, and the hallmarks of the cell cycle progression; the latter might be also a reflection of the translational regulation of the proteins involved into the cell cycle as this control mechanism has already been reported for distinct groups of these proteins, such as cyclins [219] and at the whole proteome scale by the integration of RNA-seq and proteomic data [220]. Therefore, despite the remarkable difference between the translational and transcriptional regulation outcome at the level of individual genes and proteins, in terms of the cellular processes, this outcome reflects the same effects of the oncogenic RAS signaling in the MCF10A, independently of the type of the analysis.

2.7.3 Effect of the p53 knockdown

Although, herein I considered only the effects of mutant RAS expression, the RNA-seq, and proteomic data revealed the same alterations in the cellular processes upon the p53 knockdown. As it has been previously discussed in the nascent proteome analysis section, the markers of EMT were found to be the most pronouncedly affected by both the mutant HRAS expression (up-regulated) and p53 knockdown in the HRAS MCF10A cells (down-regulated). The same pattern remained for the mRNA (**Fig. 8.2**). Among the 64 genes significantly up-regulated upon the mutant HRAS expression and down-regulated upon the p53 knockdown in the HRAS MCF10A cells, 17 were represented by the EMT markers such as SERPINE1/2, FN1, VIM, CDH2, TGFBI, and others (in total, 38 EMT markers were down-regulated in the HRAS-p53KD cells). Furthermore, similarly to the proteomic data, the p53 knockdown was also found to affect hypoxia-induced gene expression (9 entries, -log10 FDR>10) and conserved signatures of YAP activation (6 entries, -log10

FDR>9 [129]). The search for the TF targets among the 64 differentially regulated genes revealed the enrichment for targets of TWIST1/2 (ChEA3, mean rank: ARCHS4 and GTEx databases [221], [222]), mediators of EMT cooperating with SNAI1/2 [223]. Interestingly, recent studies reported that the basal p53 expression could modulate the level of TWIST1/2 in a dual manner and either promote the mesenchymal cellular properties [224] or suppress the EMT [225], probably, depending on the genetic background.

Overall, the results of the RNA-sequencing analysis strongly correspond to the proteomic data and both of these data sets can serve for comparing the effects of the p53-knockdown in WT and HRAS-transformed cells.

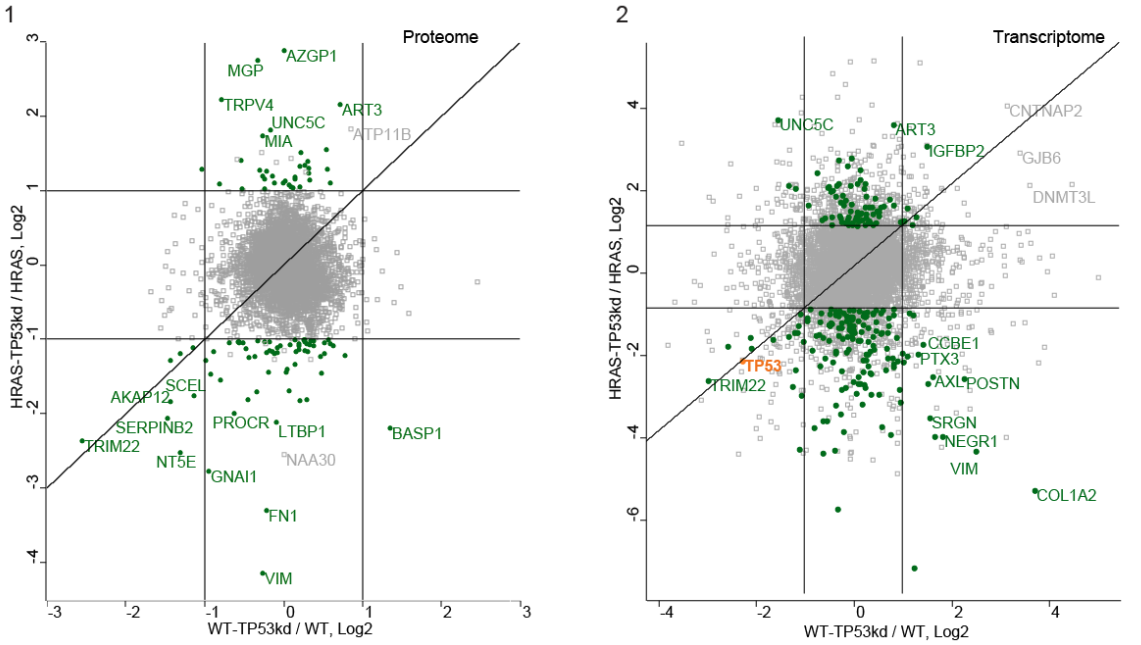


Figure 9. The differential effects of the p53 knockdown in WT and HRAS MCF10A cells. The log2-transformed ratios of protein (1) or gene (2) expression quantified by the nascent proteome and RNA-seq analysis, respectively, upon the p53 knockdown in WT (X-axes) and HRAS (Y-axes) MCF10A cells. Proteins/genes significantly regulated upon the p53-knockdown in the HRAS MCF10 cells are highlighted in green. p53 expression is indicated in orange to illustrate the strongly similar effect of the silencing on its expression in both cell lines.

As it was previously mentioned in the section discussing the role of p53 in the context of the mutant RAS signaling, the alterations in the protein expression caused by the p53

knockdown in the HRAS MCF10A were dissimilar to the ones in the WT cells with minimal correlation (**Fig. 9.1**). Nevertheless, the number of proteins regulated upon the p53 knockdown in the WT cells was relatively low (10 and 35 up- and down-regulated respectively). Yet, the transcriptome analysis revealed more pronounced changes in gene expression: 140 down-regulated and 134 up-regulated genes in WT cells, however, the comparison with the genes regulated in HRAS cells (84 up- and 160 down-regulated genes) subjected to the p53 knockdown showed the same dissimilarity (**Fig. 9.2**).

2.7.4 Effect of the p53 knockdown on the activity of transcription factors

Interestingly, the analysis of the TF targets enrichment of the regulated genes revealed the downregulation of TP63 targets in the WT cells (53 entries, ChEA3, 1 mean rank). TP63 (p63) is a member of the p53 family that shares its tumor-suppressive and pro-apoptotic functions, albeit similarly to p53, p63 is also considered as a pivotal oncogene, depending on the cellular context [226]. Besides, expression and stability of p63 is controlled by p53 through transcriptional regulation and physical interaction and can be disturbed by the mutations in p53 [227], [228], [229]. Furthermore, the knockdown of p53 was shown to decrease the expression and activity of p63 in epithelial cells [230], [102]. Therefore, although the mRNA abundance of p63 itself was found to be slightly (1.4-fold) but still statistically significantly decreased, the down-regulation of the p63 targets upon the p53 knockdown in the WT cells confirms the previous findings. Noteworthy, p63 as well as p53 [231] was found to regulate EMT in epithelial cells so that the EMT-promoting influence of p53 silencing might be mediated through the down-regulation of p63 [232], [233], [102]. Indeed, 10 EMT markers were found to be up-regulated upon the p53 knockdown in the WT MCF10A cells. As only two of them, VIM and SERPINE2, were also quantified in the proteomic experiment (only minor changes in expression of the both), there is was an opportunity to estimate whether these markers are regulated only at the mRNA or also at the protein level. However, the phenotypic characterization of the cell migration and invasion did not reveal significant changes in these properties depending on the status of the p53 expression in the WT cells, indicating that the alteration in the gene expression might be suppressed by the downstream regulatory events.

In this context, it is rather surprising that upon the p53 silencing in the HRAS MCF10A cells p63 targets were found to be up-regulated (24 entries, ChEA3, 2 mean rank). Importantly, the recent studies reveal the intensive cross-talk between p53, p63, and RAS effectors such as TGFB [229], [234], NOTCH (reviewed in [235], and PI3K [102] pathways, MYC and WNT signaling [236], [237]. Furthermore, p63 creates the regulatory network with the EMT-modulating TFs, SNAI, ZEB, and TWIST [238], [239], [233]. Besides, p63 is also considered as a maintainer of enhancers which can modulate the transcriptional activity of other TFs, including p53 [230]. Therefore, the downregulation of the EMT markers (and properties) upon the p53 knockdown in the HRAS MCF10A cells might be explained by the HRAS-mediated alteration of the p63 activity. However, this would require further validation, and within this work, only the preliminary conclusions can be made.

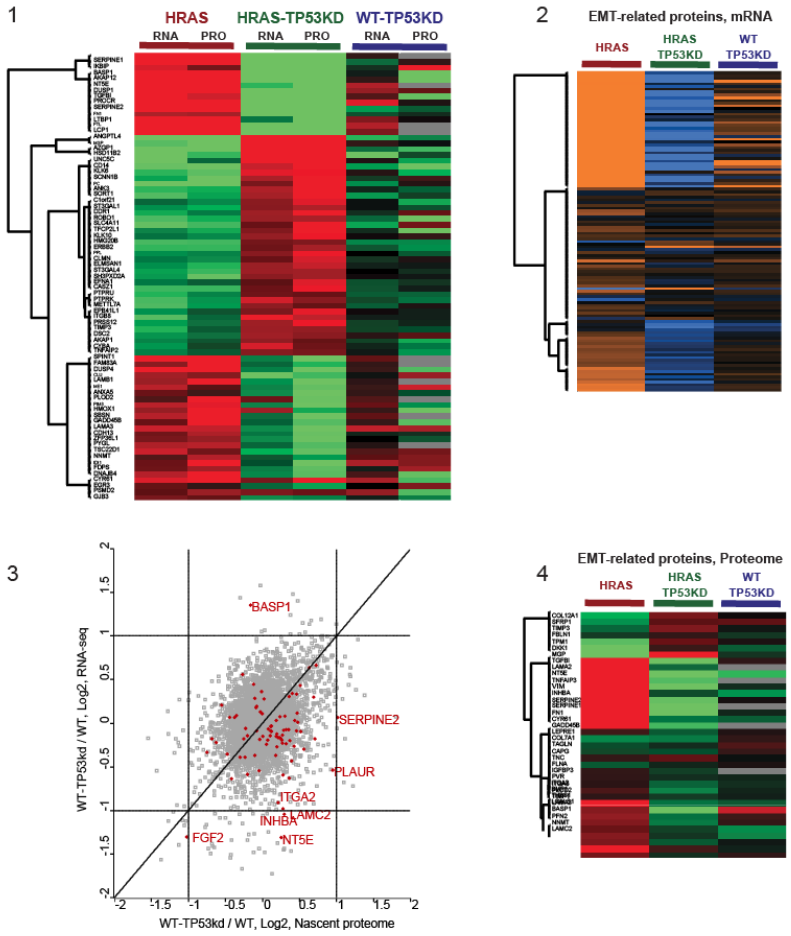


Figure 10. Comparison of the HRAS-transformation and TP53-knockdown effects at the proteome and transcriptome levels. (1) Heat map of genes/proteins that were significantly regulated (more than 2-fold, FDR<0.05) upon HRAS-transformation of TP53KD in HRAS transformed cells both in proteome and RNA-sequencing experiments. Heatmaps indicating the alterations in the expression of EMT-related proteins regulated dependently on HRAS and p53 status: RNA-seq (2) and nascent proteome analysis (4) data. (3) The scatter-plot illustrating the changes in gene transcription (Y-axis) and protein expression (X-axis) upon TP53-knockdown in the WT cell line; the EMT-related proteins are highlighted in red.

Notwithstanding the discussed anti-correlation in the activity of p63, according to the transcriptomics data, the p53 knockdown caused different effects in the WT and HRAS cells except for several p53 activity markers, such as TRIM22, CYFIP2, CLCA2, and p53 itself that were found to be down-regulated in both cell lines subjected to the p53 silencing. Noteworthy, the efficiency of the p53 knockdown was the same in WT and HRAS cells (**Fig. 9.2**). The combined analysis of the transcriptomic and proteomic data explicitly demonstrates the oppositely directed influence of HRAS signaling and p53 knockdown on the transcription and translation in the HRAS MCF10A cells (**Fig. 10.1**) whereas the p53 silencing in WT cells does not cause the similar effect and rather promote the expression of some of EMT markers (**Fig. 10.2, 10.4**). Nevertheless, the role of p53 in the regulation of EMT in WT cells was moderately pronounced at both transcriptional and translational levels of regulation (**Fig. 10.3**). It worth mentioning here that the loss of p53, particularly in normal cells has been shown to stimulate the activity of the EMT-driving TFs (TWIST, SNAI, ZEB) and mesenchymal properties in a number of studies [240], [199], [231].

2.7.5 Summary

To summarise, the data of the transcriptome analysis were found to conform with the results of the nascent proteome experiments in terms of the high correlation between the quantified changes in expression of individual genes and proteins as well as of the concurrency of the revealed alterations in particular pathways (such as mTOR, RAF, and Hippo) and biological processes (such as hypoxia and EMT) caused by both mutant HRAS signaling and p53 knockdown. Supporting the proteomic data, the RNA-sequencing also confirmed the difference in the consequences of the p53 knockdown in WT and HRAS MCF10A cells, elucidating the dual, conditional role of wild-type p53 in the regulation of the cellular processes in the normal genetic environment and upon the oncogenic signaling.

Furthermore, the RNA-sequencing allowed retrieving the information of changes in the expression and activity of TFs that were not covered by the proteomic analysis (TWIST, SNAI, ZEB, SOX9, TP63) and might be involved into the mediation of the p53-dependent alterations driven by RAS.

2.8 Phenotypic characterisation

To investigate whether the alteration is the expression of EMT markers revealed by the nascent proteome and transcriptome analysis of the MCF10A cells different in the status of HRAS and p53 expression affect the respective phenotypic features of the cells, the characterisation of migration and invasion properties of the MCF10A cells was performed. The development of the mesenchymal phenotype implies the decrease of the cellular contact along with elevation of the cellular polarization and motility [241]. These alterations can be quantified by measurement of the capacity of the cells to migrate towards an attractant through a cell-permeable membrane [242]. Besides, the role of EMT in the cancer progression includes its contribution to the capacity of the cells possessing the mesenchymal phenotype to invade the surrounding tissues and consequently to penetrate the blood vessels and develop metastases [243]. Along with the increased motility, this property is associated with the modification of the cellular secretome to excessive secretion of the extracellular matrix (ECM) remodeling proteins, particularly, metallopeptidases cleaving collagen, gelatin, fibronectin, and other components of EMT (reviewed in [244]. Thus, the invasiveness present a combination of the cellular motility and ECM degrading capability. The invasion properties, therefore, can be measured using the same membrane-based approach, despite that in this case the membrane is covered by a collagen, gelatin or the matrigel, a protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells [245], [246], to estimate how the cells can degrade and penetrate the matrix.

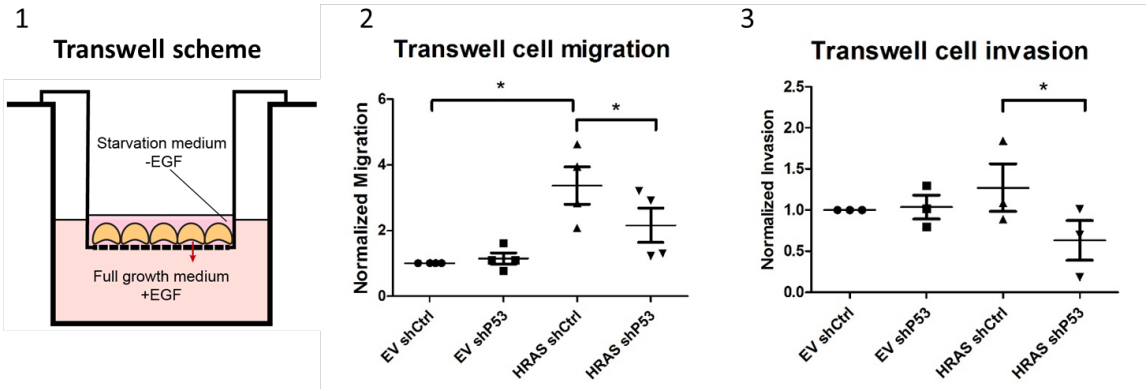


Figure 11. Phenotypic characterization: the effect of HRAS-transformation and TP53-knockdown on cell migration and invasion. (1) The experimental design. The assay assesses the capability of the cells different in the status of HRAS and TP53 to migrate though the transwell membrane towards the high serum and EGF containing media. For the invasion assay, the membrane was pre-coated with the matrigel to imitate the penetration through extracellular matrix. The cells were quantified by microscopy using the Hoechst staining. (2) Relative number of cells migrated through the membrane. (3) Relative number of cells migrated through the membrane pre-coated with the matrigel. The stars indicate the significantly ($p<0.05$) different migration/invasion.

The experiments were performed in the collaboration with the group of Prof. Stefan Wiemann (DKFZ, Heidelberg), by Dr. Cindy Körner and Angelika Wörner. Commercially available transwell chambers were used for measuring the cellular invasion through the matrigel and migration toward the medium contacting EGF that served as an attractant (**Fig. 11.1**). The analysis of cell migration revealed a significant increase in the migration of the MCF10A cells upon the oncogenic RAS signaling (**Fig. 11.1**). Likely, this effect is associated with the overexpression of EMT markers, such as VIM and FN1 that, among other functions, are responsible for regulation of the adhesion to the ECM and cellular motility [247], [248]. Indeed, mutant RAS signaling has been reported to stimulate migration in various cell types [249], [250], though in the normal MCF10A this effect typically connected with additional genetic changes [14], [178]. Interestingly, while the silencing of p53 in WT cells did not influence the migration properties, the HRAS MCF10A cells subjected to the p53 knockdown exhibited a significant reduction of migration in comparison to the HRAS MCF10A cells, albeit it was still higher than for WT cells. This corresponds to the down-regulation of EMT markers upon the p53 knockdown in the HRAS MCF10A cells that was previously revealed by the transcriptome and nascent proteome analysis.

Therefore, the analysis of the migration features of the MCF10A cells confirmed the gene/protein-expression-based assumptions on the phenotypic changes caused by the mutant HRAS expression and the p53 knockdown (see the nascent proteome analysis section). These data provide a direct evidence that p53 activity mediates the mutant-HRAS-driven stimulation of migration. Furthermore, this also strengthen the suggestion about the role of wild-type p53 as a cooperator on the oncogenic signaling. Noteworthy, although the pro-migratory activity associated with oncogenic signaling has been reported for mutant variants of p53 [251], [252], to the best of my knowledge, such a role of the wild-type p53 has been described only in ovarian cell lines [12]. Besides, Furth *et al.* previously reported that the compromised LATS expression affects functionality of p53 in MCF10A cells and promotes cell migration [176].

The invasive properties were also found to be dependent on the HRAS and p53 status, however, the oncogenic HRAS signaling did not caused statistically significant stimulation of the cellular invasion. Surprisingly, the knockdown of p53 in the mutant-HRAS-expressing cells led to the reduction of invasion in these cell lower than the basal level for the WT MCF10A. As the invasion is associated with remodeling of the ECM by the secreted proteins, these observations might point on the disadvantages of the mutant-HRAS-driven alterations in the cellular secretome for the matrigel degradation. In this case, the lower matrigel degradation capability of the HRAS MCF10A cells can be compensated by their higher motility, whereas upon the p53 knockdown and the respective reduction of the cellular migration, the contribution of the altered secretome become clear. Nevertheless, a proper examination of this hypothesis might require performing of the invasion assays using alternative coating (such as collagen or gelatin), quantification of the matrix degradation (for instance, using a fluorescein-labeled [253]), and further functional characterisation of changes in the secretome composition: although the secretome analysis (see the Chapter 2) revealed differential secretion of individual proteins, such as SERPINE1/2, KLK5-7, MGP, and other in the HRAS MCF10A cells, the role of these alterations in the aspect of the ECM remodelling remains to be further investigated.

2.9 Discussion

The usage of the normal MCF10A cells as a model of the tumorigenesis initiation allowed monitoring the effects of oncogenic RAS signaling in the intact genetic environment. For a comprehensive analysis of the mutant HRAS activity outcome in the aspect of the protein expression in these cells, two proteomic approaches were used: the conventional whole lysate (global proteome) analysis and the novel method for quantification of alteration in protein translation rates – nascent proteome analysis. The comparison of these two approaches showed that the latter has a higher sensitivity and potency to detect changes in the activity of signaling pathways and transcription factors upon the mutant HRAS signaling, yet preserving the depth of the analysis. This better performance of the nascent proteome analysis is thought to be mainly associated with the extenuation of the dynamic range that was achieved by specific enrichment for proteins synthesised in the cells within 6 hours and removal of pre-existing proteins. This enabled normalizing the protein concentration in the samples to the translational rates, to avoid the effects caused by the accumulation of long-living structural proteins, and improve the quantification. The 6-hour period was chosen to prevent the influence of protein degradation (the median protein half-life in the MCF10A cells ~11 h). The implementation of this approach allowed quantification of >5000 proteins in the WT and HRAS MCF10A cells. The further analysis of the differentially expressed proteins revealed activation of the known RAS downstream effectors: mTOR and RAF pathways, MYC, and NFkB transcription factors. Furthermore, the oncogenic RAS signaling caused overexpression of proteins involved in glycolysis and hypoxic response. Taken together with the down-regulation of oxidative phosphorylation, this can be interpreted as a Warburg effect-like metabolic shift typical for tumorigenic transformation [254]. Most pronouncedly the aberrant RAS signaling influenced the expression of EMT markers upregulating VIM, FN1, LCP1, and others. Therefore, the nascent proteome analysis allowed a detailed characterisation of the mutant RAS activity outcome in the normal cells. All of these findings were then confirmed by the transcriptomic analysis, which demonstrated high correlation between the alterations in abundances of individual mRNAs and protein (Pearson correlation >0.5) and in the activity of signaling pathways and cellular processes. The higher depth of the transcriptomic

analysis enabled recognition of the TFs that were affected by the mutant RAS activity and be involved to the mediation of its consequences. Particularly, ZEB1/2, SNAI1/2, and TWIST1/2 were found to be upregulated along with their targets mainly represented by EMT-related genes. The activity of TP63, SOX9, and YAP/TAZ was also found to be positively regulated by the mutant RAS. Noteworthy, the factors can crosstalk, and therefore participate in the joint modulation of the expressional response. The integration of the transcriptomic and proteomic data also allowed the identification of a set of genes/proteins that can be regulated post-transcriptionally that can be used for further studying of the regulatory mechanisms underlying the oncogenic signaling outcome. Both the RNA-seq and nascent proteome analysis were applied for investigation of the role of p53 in the context of the aberrant RAS activity. The MCF10A cells express the wild-type p53, and this expression was found to be untouched upon the mutant RAS transfection. Contrary to the pre-assumption of the exclusively tumor-suppressive function of wild-type p53, the silencing of p53 in the HRAS MCF10A cells did not cause further progression of the RAS-driven transformation but was shown to abolish some of effects of the mutant HRAS transfection at mRNA and protein levels, including the expression of EMT markers and activity of Hippo pathway. The outcome of the p53-knockdown was also revealed to depend on the HRAS status as in the WT MCF10A cells, the p53 silencing caused dissimilar effects. The discovered influence of RAS signaling and p53 expression on the EMT markers was validated by the phenotypic characterisation. The migration and invasion assays confirmed that the basal p53 expression is crucial for the pro-migratory activity of RAS. Taken together, these evidences indicate that in the given cellular system the wild-type p53 alters its functionality in the context of the mutant RAS signaling and cooperates with the oncogene to promote the alteration in the activity of signaling pathways and transcription factors. Overall, this demonstrates that depending on the genomic environment the wild-type p53 can exhibit the features that were previously reported for the mutant variants, such as cooperation with the mutant RAS, activation of YAP, and elevation of migration. The mechanisms underlying these features might include crosstalk and functional interactions with YAP, TP63, and other transcription factors, along with alterations of p53 PTM status and physical on-chromatin interactions. Although, the latter is partly discussed in this work, in the Chapter 3, the further detailed investigation of the

wild-type p53 activity upon the oncogenic HRAS signaling remains to be done. Taking into consideration the data described herein, this might provide valuable insights into the biological functions of p53 and its role in cancer.

3. On-chromatin p53 interactome

3.1 Introduction

Transcriptional regulation of gene expression is a crucial process allowing the cell to respond to internal or external stimuli and coordinate various cellular functions. This response is mediated by a massive network of signal transduction cascades whose activity depends on extra-cellular signaling and crosstalk with each other. Nevertheless, the end-point of these cascades, mainly serving as a bridge between membrane receptors and nucleus, is the activation of a respective transcription factor that interacts with DNA and modulates gene transcription. Initially, the term 'transcription factor' (TF) has been used to define any proteins involved in transcription and/or modulation of gene expression. Nowadays, the term is rather applied to proteins that bind DNA in a sequence-specific manner and are capable to regulate gene transcription. Therefore, the knowledge of a protein capability to bind DNA can serve for the prediction of its activity as a transcription factor. Deciphering of crystal structures of protein-DNA complexes started in the 1980s contributed to defining the protein sequence motifs responsible for DNA binding. Some of those such as zinc finger (ZF), basic leucine zipper (bZIP), or basic helix-loop-helix (bHLH) consequently gave names to respective families of transcription factors. By the date, there are ~100 identified types of DNA binding domains (DBDs) cataloged in special databases, such as InterPro [255]. The success in recognizing DBDs, along with the progress in the human genome sequencing and computational biology, allowed applying algorithmic approaches for discovering putative transcription factors on the basis of the gene sequences. According to the Animal Transcription Factors Database [256], there are >1600 known human TFs. However, studies of the TFs activity showed that many of those are active only at certain stages of the cell differentiation, whereas in the differentiated cells, the TF activity varies between cell and tissue types. Thereby, the numbers of experimentally detected expressed transcription factors lie in the range between 150 TFs in skin and >300

in brain cells. Yet, a concrete TF repertoire is context-dependent and might be altered at various conditions [257].

Furthermore, the DNA sequences recognized by a particular TF can also vary in a similar manner. Typically, to bind DNA, a TF recognizes a consensus motif, 5-30 nt long fragment of the DNA called a binding site [258]. Nowadays, there are more than 200 specific DNA motifs known to be recognized by various transcription factors [41]. A single TF can recognize up to hundreds of DNA binding sites along the genome. Furthermore, the binding capability of a TF can be regulated through conformational changes or binding with another TF molecule so that the resulting homo- or heterodimer has the attenuated affinity to DNA sites [259]. Generally, TFs per se are not capable to modulated gene expression and, upon DNA binding, act as recruiters for other factors such as chromatin-remodeling complexes which are responsible for the regulation of DNA accessibility by histone modification and nucleosome re-positioning followed by the initiation of mRNA synthesis by RNA polymerase II [260]. The assortment of co-factors that can be recruited by a TF depends on the local genomic context, PTM status of the TF, and the cofactor accessibility [261]. Thereby, the DNA-bound complexes of a TF factor can be of high variety according to the cell type, a stage of the cell life cycle, and the activity of signaling pathways. Summarizing, the gene transcription is orchestrated by a huge number of molecules forming the intricate and dynamic functional network based on specific DNA-protein interactions and the recruitment of protein complexes.

Due to the central role of transcriptional regulation in the control of gene expression programs and maintaining specific cell states, abnormalities in functions of transcription factors, cofactors, or chromatin regulators are associated with a broad spectrum of diseases, such as cancer, autoimmunity, neurological disorders, metabolic syndromes, and many others [262]. Indeed, statistical evaluation of reported functions of TFs identified 164 of those (~12%) being directly involved in the development of 277 diseases [41]. This is especially relevant for cancer. More than 300 TFs have reported as putative oncogenes, which represents ~20% of all known oncogene candidates [263]. This is not surprising. As demonstrated by previous studies on embryonic stem cells (ESCs), the activity of only a small number of TFs is sufficient for a comprehensive reprogramming of a broad range of

cell lines. As one of the possible examples, the ectopic expression of just three transcription factors (Ascl1, Brn2*, and* Myt1) was found to be potent for the conversion of terminally differentiated mouse hepatocytes into functional neuronal cells [264]. Another group showed that the attenuated activity of Gata4, Mef2c and Tbx5 can reprogram mouse fibroblasts into functional cardiomyocytes [265]. These pieces of evidence clearly indicate how much crucial effect any alterations in the activity of TF can cause even in mature cells. Similarly to the reprogramming, tumorigenesis represents a stepwise transformation of normal cells into cancerous ones. This is often followed by so dramatic changes in gene expression and phenotype that it might be difficult to assign the tumor with the progenitor tissue that it has been derived from. The first reported TF-activity-driven cancer was acute myeloid leukemia (AML) where fusion proteins, such as PML-RARA and AML1-ETO, resulting from the translocation of the respective genes, were shown to alter the cellular transcriptional program leading to the differentiation blocking and maintaining cells in a stem cell-like state. These alterations along with an increase of genomic instability and mutation rate are currently considered as the main factor of the AML development [266], [267], [268]. Further investigations of the role of TFs in tumorigenesis revealed that many of the specific cancer cell features, summarized under the name of hallmarks of cancer in the classical work of D. Hanahan and R. Weinberg [269], are associated with dysregulation of the TF activity. Particularly, KLF8, SNAIL2, and RUNX2 were found to drive EMT in breast and prostate cancer [270]. Stimuli-independent proliferation in RAS-driven cancers was reported to be maintained by ETS1/2 [271]. The immune invasion was connected with dysregulation of MYC and STAT1 [272], [273]. Many other TFs can also be mentioned here as, being the final effectors of multiple signaling cascades, the TFs accumulate aberrant signaling from various upstream pathways and intermediate the activity of other non-TF oncogenes. Therefore, investigation of mechanisms of transcription factor activity is of high importance for the understanding of tumorigenesis and establishing novel therapeutic approaches.

3.2 On-chromatin protein complexes

As it has been introduced above, TFs typically act as a part of multiprotein complexes and require binding of various additional factors, including those which alter the chromatin accessibility and intermediate the recruitment of RNA polymerase. Thereby the regulation of transcription strongly depends on protein-protein interactions between multiple factors and the composition of DNA-bound protein complexes. The well-known example of this is the cooperative interaction of Oct4, Sox2, and Klf4 during the reprogramming of somatic cells to pluripotency [274]. An important role in this cooperation is played by the so-called pioneer factor binding when a transcription factor with high affinity to closed chromatin regions binds the nucleosome on its own and consequently intermediates the recruitment of other factors of chromatin remodeling and the gene transcription. A recent work assessing the binding properties of various DBDs revealed that the reprogramming factors Oct4, Sox2, and Klf4 have increased affinity to the nucleosomes and, therefore, exhibit the pioneer binding functionality in vivo and in vitro [275]. The transcription factors NFAT and AP-1 have been recently shown to increase the accessibility of DNA sites which are consequently occupied by ETS1 and RUNX1 for the regulation of immunological memory in T-cells [276]. In the context of cancer, GATA3, a member of the GATA family of transcription factors with high-affinity to heterochromatin, has been reported to mediate DNA accessibility at regulatory regions involved in the ESR1-driven transcription in breast cancer cell lines [277]. TFs can also mediate binding of each other to DNA, therefore modulating the transcriptional activity of their partners. A relatively well-studied example of such a cooperation is regulation of ER1 chromatin binding through interaction with a number of other TFs, including MYC, JUN, and RUNX1 [278], [279]. Furthermore, studies of the inflammation control in hepatocytes demonstrated that the transcription factor NF-KB not only simply intermediates interaction of STAT3 with its binding sites, but alters the repertoire of the STAT3 regulated genes depending on the cytokines interleukin signaling [280], indicating the connection between the activation of the upstream signaling cascades, the composition of on-chromatin complexes, and functionality of TFs.

Overall, the discovered by to date the high variety of the interactions between various proteins modulating gene transcription demonstrates that this process is based on intensive

cross-talk and hierarchical cooperation of external factors, signaling pathways, and the intricate network of transcription factors. The mechanisms of the on-chromatin complex formation remain mainly understudied due to the high complexity and still limited knowledge of the composition of the complexes. However, the availability of new high-throughput methods allows intensifying the progress in discovering the interactions between TFs. The expanding of our knowledge about the composition of DNA-binding protein complexes is promising for the deciphering of the chromatin interactome and can eventually lead to discovering of many additional intervention points for adjustment of the various cellular processes and treatment of cancer and various other diseases.

3.3 Methods for investigation of TF interactions

Chromatin is the macromolecular complex that, besides DNA, includes nucleosomes, transcriptional and repairing machinery, and the high variety of dynamically interacting regulatory proteins. The diversity and density of the complex make studying its composition, and especially TF complexes, a technically challenging tasks due to the variety of the targets, presence of contaminants, and high dynamics and instability of DNA-protein and protein-protein interactions. Nevertheless, the understanding of TFs activity and the mechanisms underlying their effect on the cellular transcriptional program remains of high importance due to its crucial role in the regulation of the majority of cellular processes. Therefore, the development and systematic implementation of techniques targeting the DNA-binding proteins is essential for the efficient progress in the field of TF studies.

Two ‘classical’ approaches for studying the activity of transcription factors in the cells include TF silencing and chromatin immunoprecipitation (ChIP) followed by sequencing of the precipitated DNA-fragments (ChIP-seq). The TF silencing approach represents a methodological knockdown (or knockout) of transcription factors in a given cell line followed by the analysis of the transcriptome outcome (RNA-seq) allowing the identification of the genes controlled by the transcription factor and the prediction of the interaction between transcription factors that possess the same targets on DNA, using bioinformatics algorithms and database on the TF knockout outcome [281], [282], [283].

The ChIP-seq is a technique based on enrichment for the DNA fragments bound by a specific protein or nucleosome. Typically, chromatin is crosslinked by formaldehyde and sheared by nuclease treatment or sonication. The obtained DNA-protein complexes are then pulled down using an antibody specific to a protein of interest, and subjected to protein removal, and sequencing of the DNA fragments [284]. This approach enables identification of the DNA regions predominantly occupied by a transcription factor of interest and serves for direct identification of TF binding sites. Being integrated with RNA-seq, the data obtained by the ChIP-seq serve for studying the correlation between TF binding patterns and gene transcription. Detection of co-occurrence of a few TF on the same DNA region, along with results of co-immunoprecipitation experiments, also serves for the screening of interacting TFs [285]. Nevertheless, both of the approaches do not allow direct analysis of DNA-protein and protein-protein complexes. Depending on the experimental design, the quantification of the complex composition is also limited or inapplicable [286].

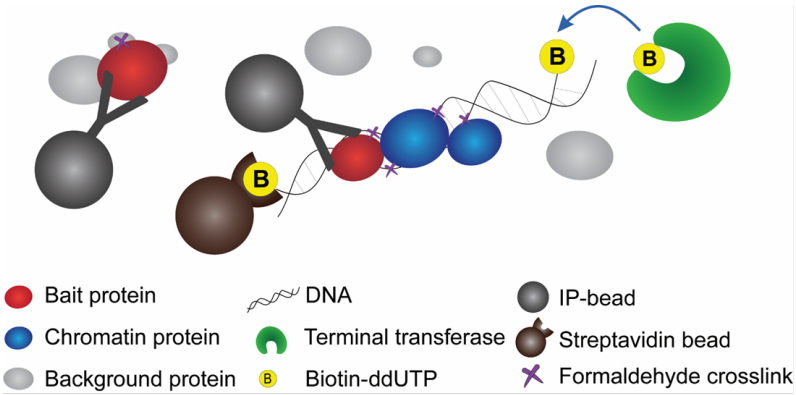
3.4 Mass spectrometry-based methods: ChIP-SICAP

The appearance of high-resolution mass spectrometry dramatically improved the technical capabilities for studying chromatin composition. Implementation of mass-spectrometry allowed the detection of proteins in DNA-bound complexes in the unbiased and quantitative manner using SICAL labelling or label-free quantification (LFQ) approaches [287]. Furthermore, the coupling of mass-spectrometry with ChIP also enabled the direct identification of proteins bound through protein-protein interaction [288]. However, the applying of mass-spectrometry for ChIP-based studies meets additional limitations specific to the protein-targeting method. The main of them is unspecific binding. As long as, ChIP-seq analysis targets DNA fragments and do not consider proteins, any of them that unspecifically bind the DNA or stick to the materials used for the antibody pulldown (such as protein G coupled resin or magnetic beads) do not affect the results. Therefore, in this case, the additional noise is created predominantly by the co-purification of unspecific proteins crosslinked to biologically unrelated DNA fragments. Those represent a small portion of contaminant proteins and the noise can be sufficiently eliminated by applying a negative control.

In contrast, by mass-spectrometry, the signal from all proteins that passed the purification step will be measured. The DNA-molecule is highly charged and provides the surface for unspecific binding of the cellular proteins during the cell lysis. Due to the difficulty of breaking these unspecific interactions and the huge difference in abundance of the highly expressed cellular proteins and TFs, these contaminants can mask the signal from the true positive targets. Furthermore, the typical lack of an appropriate control (for example, a cell line with the knockout of the protein of interest) and polycompositional character of the analysed DNA-protein complexes complicates the unambiguous interpretation of the results and efficient distinguishing of true and false positive identifications [289]. One of the approaches for overcoming this complication is the creation of databases containing results from various ChIP-MS experiments. The aggregation of data about proteins co-purified in many independent experiments allows the creation of a list of potential contaminants that are consistently identified among distinct samples. A good example of such a database is the CRAPome that represents the proteomic community-generated datasets from affinity purification coupled with mass spectrometry (AP-MS) experiments annotated by the type of experiment, cell line, tag, and method of purification [290]. This enables the generation of an experiment-specific list of the putative contaminants and, therefore, distinguishing of the true positive proteins. Another approach is increasing of the specificity of the pull-down by the introduction of additional enrichment/purification steps.

In this work, I used the ChIP-SICAP method for selective isolation of chromatin-associated proteins developed by Mahmoud-Reza Rafiee [291]. ChIP-SICAP implies tagging of DNA with biotin following the conventional chromatin-immunoprecipitation of the target TF. The biotin tag is further used for the pull-down of the DNA-bound protein complexes by the streptavidin beads (**Fig. 12**). The high affinity of biotin and streptavidin enables intensive washing by high-salt and SDS buffers serving for the removal of DNA-free protein aggregates and unspecific DNA binders. Therefore, this method allows specific enrichment for the on-chromatin TF complexes and decreasing the portion of contaminants. ChIP-SICAP was successfully applied for studying the interactors of Oct4, Sox2, and Nanog in mouse embryonic stem cells (ESCs) [291]. However, as these master TFs are relatively high abundant and active in ESCs it was remaining questionable whether this approach can be used for studying TF activity in mature cells where a TF of interests can

present in a lower amount and possess additional DNA-independent functions. To examine the applicability of the ChIP to such targets I used it for identification of the on-chromatin interactome of p53 in normal human mammary gland MCF10A cells. This application was further expanded to the mutant-HRAS MCF10A expressing cells for studying the influence of the oncogenic signaling on the composition of the p53 interactome.



Fugure 12. The ChIP-SICAP scheme. The method enables the selective isolation of a DNA-bound TFs of interest (red) along with co-localizing proteins (blue) that are then identified by mass-spectrometry. The approach includes following main steps: (1) chromatin cross-linking and shearing, (2) ChIP and DNA-biotinylation, (3) DNA isolation with streptavidin beads, (4) protein release by reversing the crosslinks, (5) tryptic digestion and MS analysis.

3.5 On-chromatin protein network of p53

The p53 pathway responds to a high variety of stress signals in the cell, including DNA damage. The level of p53 in the cells is mainly regulated through degradation of the protein by MDM2, a ubiquitin ligase mediating p53 ubiquitination and degradation of p53 in the proteasome. Upon the stress, the stability of p53 is increased by upstream mediators, typically, through p53 phosphorylation preventing its interaction with MDM2 [292]. This allows the accumulation of p53 in the nucleus and activation of its transcriptional program. This post-translational mechanism ensures a more rapid response to cellular stress than the one that might be achieved through attenuation of the p53 synthesis. In the case of DNA damage, the mediation of p53 stabilization is realized by ATM (ataxia telangiectasia-mutated) and CHK2 (checkpoint kinase 2), which are responsible both for phosphorylation

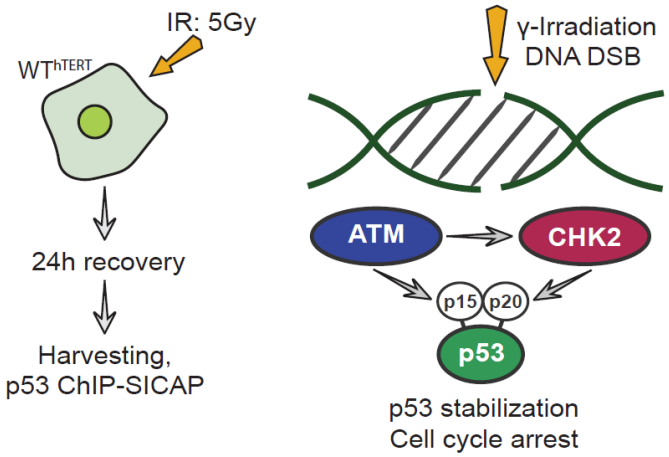
of p53 and MDM2. This blocks the ubiquitination of p53 by MDM2 and initiates the degradation of excessive MDM2. Thereby, the post-translational modification of p53 and increase of its abundance initiate the p53 transcriptional program and activation of the genes regulating DNA repair and the cell-cycle arrest, such as TRIMM19/22, DDB2, XPC, and CDKN1A [180]. However, like other transcription factors, p53 initiates transcription not per se but through the formation of a multiprotein complex. The p53 tetramer is known to recruit co-factors upon p53 binding, particularly, histone acetyltransferases (HATs) and TATA-binding protein-associated factors (TAFs) [293]. Furthermore, the protein-protein interactions involved in the regulation of p53 stability remain after binding the DNA. Therefore, these two processes, post-translational modification of p53 and its recruitment on chromatin, are co-dependent. A well-studied example of this is the interaction of p53 with p300/CBP, a histone acetyltransferase. p300/CBP is capable to acetylate p53 both in the soluble and DNA-bound form. However, the acetylation of the free, soluble, p53 is followed by dissociation of p300, whereas the acetylation of the on-chromatin p53 promotes stable DNA-bound complex formation and further activity of p300 as a chromatin remodeler [294]. The docking of p300 is also reported to affect the binding pattern of p53 and thus regulate its transcriptional program [295]. The heat-shock protein Hsp90 responsible for maintaining the stability of p53 and supporting its DNA-binding through conformational regulation was also shown to remain being associated with p53 in chromatin-bound state [296]. Interestingly, MDM2, along with its role of mediator of soluble p53 degradation, co-recruits on p53 occupied DNA sides, where it considered to participate in p53 dissociation. However, the detailed mechanisms of the MDM2 activity remain to be investigated [297].

The whole known network of p53 protein-protein interactions includes a great number of ligases, kinases, and transferases, part of which seems to be responsible for modification of soluble p53, whereas the others were found to be a part of DNA-associated complexes. The large intricacy of the p53 interactome and the reported capability of the PTMs and protein interactions to attenuate the p53 binding pattern indicate that these can play a central role in the regulation of the context-dependent p53 transcriptional activity. There are >200 directly validated p53 binding sites, and thousands identified by genomic analysis. The studies of p53 targets in various cell lines and upon different conditions demonstrated high

variability of the set of p53 regulated genes. The molecular mechanism of this binding regulation remains mainly unclear [298]. Yet, the number of reported physical interactors of p53 exceeds one thousand (BioGRID database), [299]. Despite the progress in the identification of these interactions, their connection with cellular signaling, cooperative action, and role in the p53-driven modulation of cellular processes still require further investigation. The expanding of our knowledge of the context-specific composition of the DNA-bound complexes can elucidate novel mediators of p53 activity and provide valuable mechanistic information on transcriptional regulation.

3.6 p53 on-chromatin interactome upon irradiation

Here, I aimed to adapt the ChIP-SICAP method for the identification of on-chromatin p53 interactors. To prove the applicability of ChIP-SICAP for targeting chromatin-associated proteins in the MCF10A cell system and to identify the DNA-damage-induced on-chromatin p53 complexes, the method was applied to the WT MCF10A cells subjected to ionizing radiation (**Fig. 13**).



Fugure 13. Experimental design. The WT MCF10A cell were subjected to ionizing radiation in the dose 5Gy to induce formation of double-strand DNA breaks (DSB). The cells were further incubated for 24h to allow the cell recovery. Over this time, DNA damage driven activation of checkpoint mechanisms was expected to lead to stabilization of nuclear p53 – predominantly, through ATM and CHK2 mediated phosphorylation – and accumulation of the protein followed by assembling of DNA-bound p53 complexes mediating the transcriptional response. The irradiated cells were further harvested, crosslinked, and used for the ChIP-SICAP analysis.

3.6.1 Technique

The dose of 5Gy was chosen as a non-lethal and proven to be sufficient for the induction of DSB in MCF10A cells [300]. The cells were irradiated at the confluency ~60%, further observations after the 24-hour recovery confirmed the growth arrest, though the cell death was not noticed. Upon harvesting, the cells were subjected to the crosslinking with 1.5% formaldehyde. The formaldehyde concentration lower than the 4%, which is usually applied in ChIP-seq experiments, was used to decrease the risk of over-crosslinking. The three-dimensional structure of chromatin and high concentration of macromolecules may cause the formation of high order complexes through the chain crosslinking of multiple proteins and nucleic acids. Though this issue is also relevant for the ChIP-seq applications [301], it is especially crucial for the proteomic experiments as the additional protein background may interfere further mass-spectrometry analysis and as the control of physiological relevance of the detected protein-protein interactions is complicated. The crosslinked cells were subjected to the nuclear fractionation and chromatin sonication. The chromatin was sheared down to ~300 bp fragments. This fragment size is sufficient for placing terminal deoxynucleotidyl transferase (TdT) at the fragments' ends for the biotinylation reaction and also is short enough to restrict to a reasonable degree the possibility of pulling down unrelated proteins attached to the same DNA strand.

The chromatin immunoprecipitation was performed using an anti-p53 antibody (DO-1/7) to target both fractions of p53, phosphorylated, and non-phosphorylated at the Ser20. The p53-bound antibody pulldown was performed on the protein-G-coated magnetic beads. The obtained mixture of soluble and DNA-bound p53 complexes was subjected to the biotinylation reaction. In this reaction, TdT catalyses the addition of Biotin-11-dCTPs to 3' terminus of a DNA fragment and, thereby, creates the tag which is further used for binding to streptavidin-coated magnetic beads. Elution from the protein-G beads is performed at high (10%) SDS concentration to release the bound p53. The consequent extensive washing of the streptavidin beads with SDS-containing and high-salt buffers allows breaking both hydrophobic and ionic unspecific interactions, respectively, and the efficient removal of the soluble fraction of p53 complexes and contaminants bound to the DNA. The high efficacy of the DNA-bound complex enrichment has been previously demonstrated on stem cells in

the work that established the ChIP-SICAP method [291]. The DNA-p53 complexes were further eluted from streptavidin beads by heating (90°C) in the presence of DTT that was followed by trypsinisation and mass-spectrometry analysis. The control samples, representing the chromatin immunoprecipitate from the same cells obtained using non-specific rabbit IgG, were processed in the same manner.

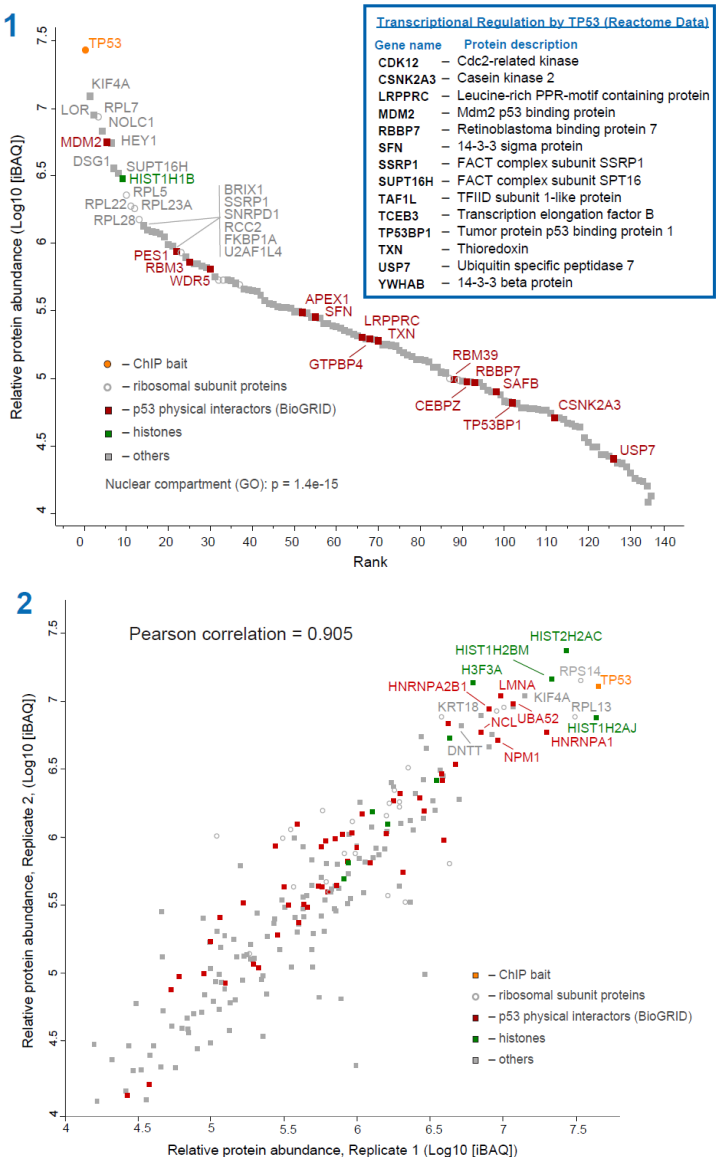


Figure 14. Analysis of the on-chromatin p53 complexes. (1) Proteins identified by MS analysis are plotted against their relative abundance, excluding identification from the negative control (unspecific IgG). p53 was identified as the most abundant protein confirming enrichment of the bait. The proteins known to directly interact with p53 (BioGRID) are highlighted in red. The table represents the players of the p53-driven transcriptional regulation (Reactome) detected in the

samples. (2) Comparison of signal intensities of proteins in both replicates indicating the high reproducibility of the method (Pearson correlation >0.9), The IDs from the negative control are also included.

3.6.2 Identified p53 interactors

In total, 135 proteins were detected exclusively in the p53 samples in replicates. The high prevalence (103/135) of proteins with nuclear localization and those involved in transcriptional regulation (73/135, Gene Ontology analysis), along with identification of p53 as the protein at highest abundance, indicates the high efficiency of the enrichment for both p53 and DNA-associated proteins (**Fig. 14**). The presence of the transcriptional initiation and elongation factors (TCEA2, TCEAB3, TAF1L) in the pulldown supports the localization of the identified p53 complexes on the enhancer regions. To confirm the specificity of the revealed interactome I referred to the BioGRID database, containing physical interactions of human p53 reported in low- and high-throughput studies. Among the proteins detected in the p53 samples, 23 have been previously recognized as direct p53 interactors. Although the BioGRID data are not assigned to a localization, condition, or biological process, this noticeable overlap supports the capability of the ChIP-SICAP to target p53 interactors. Furthermore, 14 of the identified proteins are described to be involved in transcriptional regulation by p53, according to the Reactome database (**Fig. 14**). Four of the proteins have been annotated as factors of p53-driven DNA repair. Particularly, SSRP1 was shown to intermediate the stabilizing phosphorylation of the p53 through the interaction with SUPT16 and CK2 that have been also detected in the study [302]. As the FACT complex (SSRP1- SUPT16) is involved into mRNA elongation and DNA repair, thus it is highly possible that the modification of p53 might be followed by co-recruitment of both p53 and the FACT on chromatin in the same manner as it has been previously discussed for the p53-p300 interaction. Furthermore, YWHAB, SFN (14-3-3 protein isoforms), and TP53BP1 that are capable to interact with p53 and also involved in the regulation of the cell cycle were identified in the samples. SNF (14-3-3 sigma) directly binds the C-terminus of p53 in a phosphorylation-dependent manner and regulates its transcriptional activity, presumably by prevention of MDM2-driven ubiquitination [303], [304]. TP53BP1 is a pivotal regulator of DNA double-strand break repair that also binds p53 and modulate p53-dependent transcription. Noteworthy, the DNA repair function of

TP53BP1 has been recently shown to be independent of the control of p53 activity [305]. Thus, in combination with the previous report, these data indicate the dual contribution of the TP53BP1 to the DNA damage response – as a part of the DNA repairing apparatus and as an intermediator of the p53 transcriptional program initiation. In this context, it is interesting that I also detected the ubiquitin-specific peptidase USP7. It is involved in the regulation of H2A/X ubiquitination in response to UV light treatment promoting further recruitment of BRCA1 and TP53BP1, and also is known as p53 and MDM2 deubiquitinating enzyme [306].

3.6.3 DNA damage responders

Although SICAP method does not allow distinguishing between direct protein-protein interaction and co-localisation on the same DNA fragment or nucleosome, the results demonstrate the putative involvement of the DNA damage responders into the initiation of the p53-driven transcription. Overall, the Gene Ontology analysis of the whole set of the pulled-down proteins and individual examination of the proteins with reported functions supports that the method was sufficient for the detection of known p53 interactors and the resulting interactome mainly corresponds to the expected effect of p53 activation by DNA damage. This allows extrapolating the knowledge about the functional relevance of the dataset on the identified proteins whose biological role remains incompletely characterised. An example of this might be WDR43, a poorly studied WD-repeat (WDR) containing protein. The WDR domain serves as an interface coordinating multiprotein complex assemblies [307]. The wide diversity of the WDR proteins is assigned to various cellular processes. Many of them are known to play a role in DNA damage repair and chromatin remodeling [308]. One of those is WDR5 that also was detected in this experiment. This protein has been reported to control p53 ubiquitination and regulate p53 signaling [309] and, recently, it has been also confirmed that WDR5 and p53 interact and can be co-recruited on chromatin where WDR5 coordinates gene transcription through histone methylation. Noteworthy, in the DNA damage response WDR5 cooperates with BRCA1, therefore the co-occurrence of WDR5, BRCA1, and TP53BP1 could be evidence of their joint recruitment on the p53 occupied DNA sites. The discussed functions of WDR5 enable

arise the suggestion that WDR43 might be also involved in the respective regulatory processes.

Another protein which function can be elucidated on the basis of the p53 interactome data is CCDC50. A previous study investigated the effects of the CCDC50 silencing in HEK 293 cells [310]. It has been shown that the CCDC50 knockdown causes a predominant reduction in expression of genes involved in p53 signaling. This allowed suggesting that CCDC50 might mediate the p53 activity in those cells. In another work, in HeLa cells, CCDC50 (YMER) was identified as a potential interactor of RNF126, a ubiquitin-protein ligase [311]. The further independent studies of the RNF126 activity showed that it ubiquitinates a p53 downstream effector p21 and regulates the ubiquitination of histones mediating the recruitment of TP53BP1 and BRCA1 on chromatin upon DNA damage [312], [313]. Taking into account the role of ubiquitination in the tuning of the p53 pathway, these facts enable to suggest that CCDC50 might be involved in the regulation of the interactions between RNF126 (or another yet undefined ubiquitin ligase) and p53 itself or other members of the p53 interactome (for example, MDM2 or TP53BP1). Alternatively, the CCDC50 might play a role in the recruitment of the ligase for chromatin ubiquitination. Noteworthy, RNF126 was not detected in the pulldown. This impels to make an attempt to suggest another ubiquitin ligases in the identified p53 interactome. Besides MDM2, TRIM41 was identified in the samples. This protein is of interest as it is known as a regulator of a TF activity by the example of ZSCAN21 in neural cells [314]. TRIM41 is localized in the nucleus and according to the IntAct database interacts with a number of chromatin-binding proteins such as ZFP1 and MORF4L1. Interestingly, TRIM41 has been reported and validated to interact with subunits of CK2 [315] which, among other functions, phosphorylates p53 at Ser392 and controls its site-specific DNA-binding upon DNA damage and at other conditions [316]. Indeed, these presumable functional connections require a proper investigation and validation that was not a part of my studies. Nevertheless, my speculation was meant to illustrate that the data obtained by the SICAP method are sufficient for suggesting novel transcriptional regulators by extrapolation of our knowledge about protein functions and interactions.

3.6.4 Indirect interactors

Another putative regulator of the transcriptional activity identified in my study is YTHDC1, a regulator of the sequence-specific pre-mRNA processing. YTHDC1 binds the newly-synthesized transcripts and intermediates their consequent splicing. It has been reported to play a role in cancer progression through interfering maturation of various gene transcripts, including SOX2 and c-MYC [317]. Thus the identification of the YTHDC1 in the pulldown might allude to its involvement in the regulation of p53 targets' expression. This observation also indicates the limitations in the resolution of the formaldehyde-crosslinking-based method. The formaldehyde fixation serves for linking the chemical groups that are ~2 Å apart and, therefore, predominantly targets the physically interacting molecules [318]. However, the chromatin consists of a high variety of interconnected dynamic complexes of proteins, DNA, and RNA. Due to this high complexity and unspecific nature of the formaldehyde action, the successive covalent binding of the interactors unavoidably leads to the formation of the high-order aggregates. Thereby, the chromatin structures obtained upon the pulldown combine spatially-close protein-DNA complexes co-crosslinked through intermediating molecules. Thus, the resulting interactome cannot be unambiguously interpreted as a set of the direct binders of the target TF or members of the same complex. However, the example of the YTHDC1 identification highlights that this feature can be also beneficial for discovering new putative regulators of the transcriptional outcome at distant layers of its control.

3.6.5 Phosphorylation sites

As the stability and activity of p53 are modulated through post-translational modifications, I also examined the sensitivity of the SICAP method for the detection of phosphorylation sites, using the mass spectra data. In the samples, p53 was identified with the sequence coverage of 41% by 22 peptides, including three phosphorylated sites on p53 typical for the stress response: Ser15, Ser315, and Ser392. The Ser15 is a known target for ATM-mediated phosphorylation preventing its interaction with MDM2 and, therefore, increasing the life-time of p53 [319]. P314/315 are two sites of phosphorylation by CHK1/2 in response to the DNA damage [320]. The Ser392 has been reported to be phosphorylated

upon the treatment of the cells with UV and various genotoxic stress. The phosphorylation is mediated by the recruitment of the kinase CK2 to the p53-FACT complex [316]. Noteworthy, all members of the complex were detected in the interactome, including SK2, SPT16, and SSRP1. Thereby, ChIP-SICAP enables analysis of the PTM on the targeted TFs. This is especially important for p53 as the protein contains >20 PTM sites which determine its stability, localization, DNA-binding activity, and interaction with other partners [321].

3.6.6 Summary

Overall, the composition of on-chromatin p53 interactome upon irradiation was successfully identified. The vast majority of the identified interactors have previously been reported as members of the p53 network in the context of DNA damage. This demonstrating the conformity of the new data with previous studies. Besides, the implementation of ChIP-SICAP allowed the detection of putative p53 partners which haven't been described to interact with p53 on chromatin (WDR43, TRIM41, and CCDC50). A further investigation of those might contribute to the expansion of the knowledge about the p53-mediated stress response. To summarize, these results support the applicability of ChIP-SICAP for the identification of TF interactors in non-stem cell lines. The method was further used for the identification of differential interactors of p53 in unstimulated MCF10A cells expressing mutant HRAS.

3.7 Optimisation of the ChIP-SICAP for analysis of p53 interactors

The transcriptional activity of p53 is typically associated with a stress response followed by its stabilisation. However, p53 is also present in the cell at the steady-state, albeit in relatively low amounts. There is an increasing number of publications considering the role of the basal level of p53 in the regulation of cellular processes, such as cell cycle, antiviral immune response, maintaining cell potency, and others [224], [322], [179]. In this work, I have already demonstrated that, in our MCF10A cell model, the oncogenic RAS-signaling doesn't lead to an increase in the p53 abundance (see Chapter 1). Despite this, the analysis of protein expression in the mutant HRAS-transfected cells subjected to the p53 knockdown

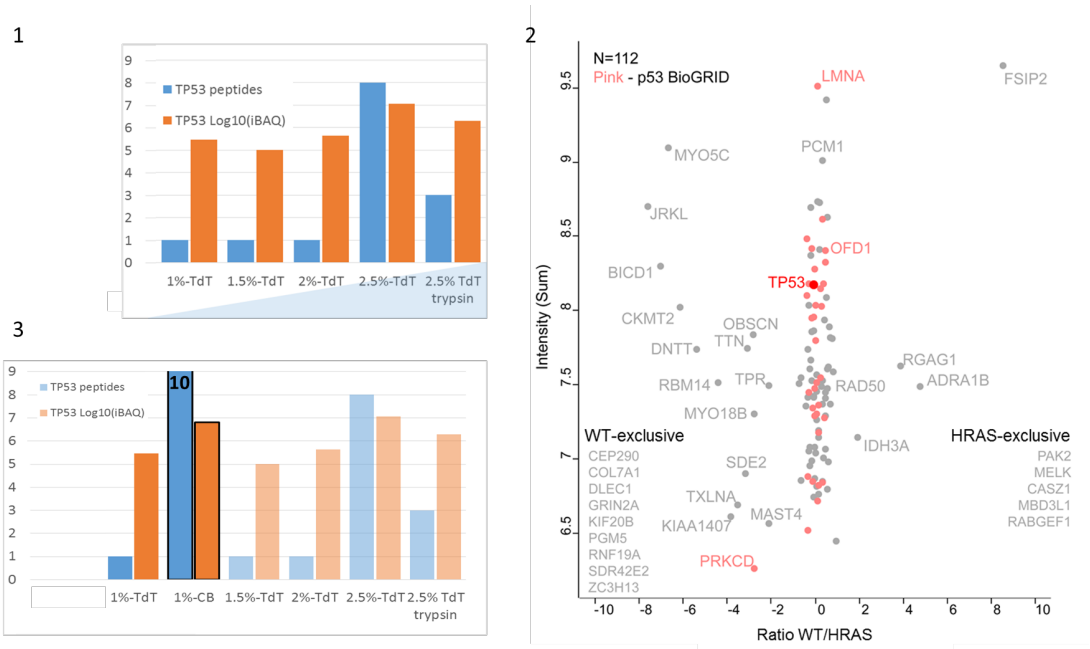
showed that the HRAS-driven alterations in the expression are partially dependent on p53. Therefore, p53 is involved in the mediation of the effects of the mutant HRAS. This makes it of high interest to investigate the activity of p53 in the context of the mutant HRAS signaling. The ChIP-SICAP provides a propitious opportunity for such investigation by the analysis of the p53 interactome. As discussed in the previous chapter, the ChIP-SICAP is capable to recognize the functionally-relevant proteins co-localized with p53 on chromatin and also detect novel putative members of the p53 network. The implementation of the method for the identification of the steady-state on-chromatin p53 complexes in the wild-type and mutant-HRAS-transfected MCF10A cells might elucidate the mechanisms of the cooperation between the oncogene and p53. Unfortunately, the basal total and on-chromatin abundances of p53 in normal cells are typically low to the extent that it complicates even the ChIP-seq analysis [323]. This explains why the rise of interest in the role of the unstimulated p53 in the cellular regulation in last years is paralleled with the improvements in the sensitivity of analytical techniques. In contrast to DNA molecules, proteins cannot be amplified during sample preparation but can be rather lost as the ChIP-SICAP targets only the on-chromatin fraction of a TF and implies multiple enrichment steps with a non-absolute efficiency. In the work of *Rafiee et al.,* the ChIP-SICAP was applied for identification of the network of Oct4, Sox2, and Nanog which are of high abundance in the embryonic stem cells [291]. Similarly, the analysis of the p53 interactors in the irradiated MCF10A cells targeted the stabilized transcription factor, which abundance was substantially higher than the basal level in MCF10A cells. These considerations highlight the challenge of the basal p53 interactome analysis. The multiple attempts to pulldown the p53 complexes brought me to the implementation of a few modifications of the protocol that has been used for the analysis of the DNA-damage-induced p53 network.

(1) The increase of the starting material. In contrast to ~30M cells applied for in the DNA-damage experiment, the amount of starting material for the basal-p53 pulldown was increased up to ~100-200M per a sample to compensate the decrease in the bait abundance. This corresponds to the typical ChIP-seq protocols [324]. The further increase of the cell number might be beneficial for the detection but still meets a few hindrances. First of all, the culturing of adherent cells on a large scale remains a laborious task and requires extra lab space. Furthermore, the increase of the starting material amount complicates the

preparation of the samples due to the limitations in the solubility of the proteins and the volumes of the samples suitable for the 'one-short' processing. Furthermore, the increase of the protein concentration in the samples can interfere with the biochemical TdT reaction. These conclusions were made empirically, on the basis of a row of successful and failed pulldowns. The systematic evaluation of the conditions for the ChIP-SICAP protocol remains of high need and might lead to considerable improvements in the efficiency and reproducibility of the method, especially for moderately and low abundant TFs.

(2) The adjustment of the cross-linking. As previously discussed in this chapter, although the intensive cross-linking increases the probability of the successful fixation of the protein/DNA complexes, the co-fixation of spatially distant molecules might cause the pulldown of unrelated proteins along with the true interactors. Therefore, it is important to properly select the optimal formaldehyde concentration, allowing both efficient enrichment for the chromatin-bound complexes of interest and decreasing the number of incidental binders. Due to the lack of unambiguous criteria for distinguishing true and false positive interactors, ideally, the ChIP-SICAP should be performed at different concentrations of formaldehyde and followed by a comparative analysis of the IDs. However, due to the requirements for the amount of the starting material and the high cost of the reagents, this might be feasible only for a limited number of highly abundant TFs. Nevertheless, here I will give an example of how the formaldehyde concentration might affect the identified interactome and the efficiency of the pulldown. In contrast to the typical ChIP-seq approaches, the original ChIP-SICAP methodology implies the in-solution crosslinking following cell dissociation by trypsin or accutase. Upon the up-scaling of the cell number for the experiment and to prevent the possible trypsin-induced short-term cellular response, I changed it to the on-plate cross-linking. To compensate the decrease in the formaldehyde diffusion, the concentration of the agent was increased up to 2.5%. This enabled the successful pulldown of p53 and its known on-chromatin interactors (BioGRID, 41 of 112) (**Fig. 15.2**). However, it was also noticed that the obtained interactome consists of a suspiciously large portion of centrosome proteins. Particularly, OFD1, a component of the centrioles, was found among the most abundant proteins, along with the identification of its centrosomal network, including CEP72, TTBK2, FOPNL, and others. The centrosomal localization of p53 upon mitosis is known from previous studies [325] and thus it was

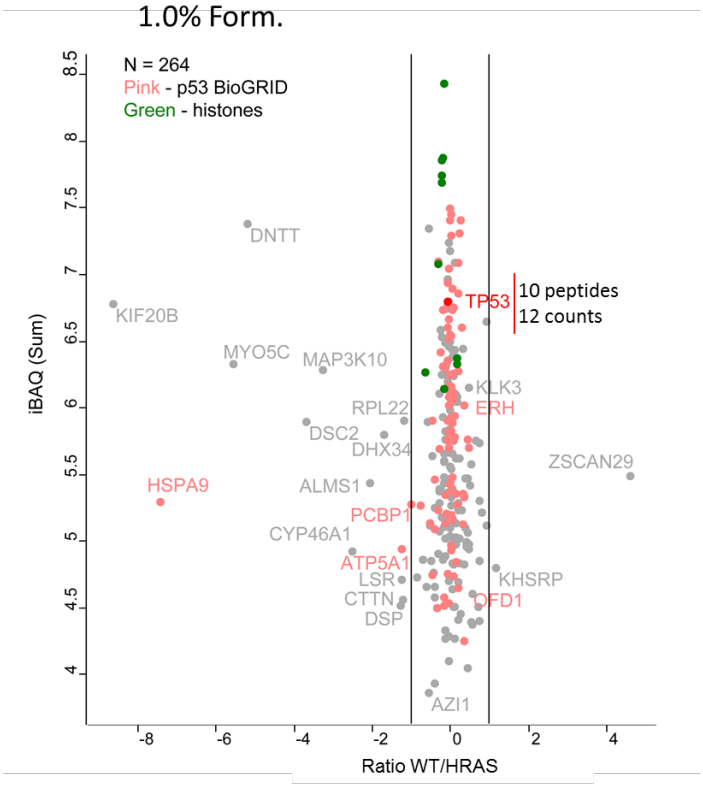
presumed that the prevalence of the centrosome proteins might have been caused by a co-crosslinking of p53, chromatin, and centrosome intermediated by the microtubules. The further testing of lower formaldehyde concentrations revealed that the lowest one still ensuring rather stable p53 detection is 1% (**Fig. 15.1**). The 1%-formaldehyde crosslinking allowed detection of 133 proteins, 54 of those have been reported as p53 physical interactors in the previous studies (BioGRID). Noteworthy, the increase in the proportion of the reported interactors should not be considered as an ultimate criterion for the estimation of the results as the affinity-capture studies underlying the core BioGRID data-set do not consider the on-chromatin protein localization. Particularly, the physical interaction with OFD1 has been reported for p53 in the centrosome-cilium interface study [326]. In the ChIP-SICAP experiments, the reduction of the crosslinking intensity resulted in the dramatic decrease of the OFD1 relative abundance and loss of the majority of the related chromosomal proteins, indicating that, in the context of the DNA-bound complexes investigation, OFD1 represents a cross-linking artifact. This emphasizes that the enrichment for the DNA-crosslinked complexes *per se* does not guarantee to the target of the true DNA-associated proteins and depends on the crosslinking. This should be also taken into account for the selection of experimental conditions and data interpretation. It is noticeable, that, along with the artifacts elimination, the applying of the milder fixation resulted in the undesirable drop in the number of identified p53 peptides (**Fig. 15.1**). This appealed to additional attempts on the optimization of the enrichment efficiency. It has been previously observed in various ChIP-SICAP experiments (e.g, TP53, SUZ12, SOX2) that the flow-through solutions after the streptavidin pulldown contain the majority of the proteins identified in the DNA-bound fraction upon enrichment. Therefore, the biotinylation of the DNA by TdT is barely complete, and an improvement in its efficiency might be beneficial for the general performance of the ChIP-SICAP analysis. For these reasons, I also tested various TdT buffers used for biotinylation.



Fugure 15. Optimisation of the ChIP-SICAP method. (1) The efficiency, the number of detected peptides and the intensity, of p53 identification in the ChIP-SICAP pull-down depending on the formaldehyde concentration. (2) The comparison of the abundances of the p53 interactors in the WT and HRAS MCF10A cells: axis X – log2-transfromed ratios, axis Y – the total intensity, the known p53 physical interactors are shown in pink, the high abundance of OFD1 is an artifact of crosslinking. (3) The improvement of the p53 detection achieved by the usage of the in-house cacodylate buffer (CB) instead of the commercial one (TdT) at 1% crosslinking.

(3) The substitution of the commercial TdT buffers. The terminal deoxynucleotidyl transferase (TdT) is used in the method for the incorporation of the biotinylated nucleotides to the ends of the DNA fragments. The TdT activity requires pH>6.8, lack of chaotropic anions, and the presence of metal co-factors, Mg, Co, or Zn [327]. Due to these reasons, the TdT buffers are based on cacodylate (e.g. Thermo Scientific) or acetate (New England Biolabs) buffering agents that are capable to ensure the pH ~7.5. It was observed in the experiments on biotinylation of the whole cellular chromatin that the TdT buffers from both Thermo Scientific and New England Biolabs cause partial precipitation of the sheared chromatin preparations. This might be explained by the high concentration of the macromolecules in the sample, at the same time, the serial dilutions of the samples showed that the visible precipitations remain even upon the decrease of the concentration. Noteworthy, the precipitation was not reproduced by the in-house cacodylate and acetate buffers alone and in combination with other declared components of the commercial buffers

(Tris, Mg and Co salts). Therefore, the particular agent causing the precipitation remained unrevealed. Nevertheless, an obvious assumption from these observations is that upon biotinylation on the protein G beads, the TdT buffer potentially causes the precipitation of the chromatin and this might reduce the efficiency of the DNA-tagging. As the in-house cacodylate buffers (CB) did not promote the precipitation, they were used for comparison to the commercial ones in the p53 ChIP-SICAP. The **Fig. 15.3** illustrates the improvement in the intensity and coverage of p53 that were used as a fair benchmark, taking into account that the consistent presence/detection of any on-chromatin p53 interactor cannot be guaranteed. The biotinylation in the CB buffer allowed to increase the number of the identified p53 peptides to 10 in comparison with 1-3 peptides typically identified in the samples biotinylated in the TdT buffer (Thermo) upon 1% formaldehyde cross-linking. The results obtained by applying the CB buffer was further used for the analysis of the p53 interactome.



Fugure 16. Analysis of the on-chromatin p53 complex composition in the WT and HRAS MCF10A cells at the optimized ChIP-SICAP conditions. Axis X – log2-transfromed ratios, axis Y – the total IBAQ intensity, the known p53 physical interactors are shown in pink.

3.8 Comparison of p53 interactors in WT and HRAS MCF10A cells

The comparative p53 interactome analysis was performed using SILAC labelled cell lines of WT and mutant HRAS-transfected MCF10A cells. The labelling allowed combining the cells directly after harvesting so that the lysis, chromatin sonication, and ChIP were performed together for both cell lines to avoid technical variations. This approach showed so high conformity of the p53 pulldown efficiency that the resulting SILAC ratios did not require normalization in the vast majority of experiments. This confirms the equivalence of the p53 expression observed in the nascent proteome analysis and suggests the on-chromatin recruitment also remains equal in both cell lines. Moreover, the composition of the identified p53-containing on-chromatin complexes was revealed to be nearly completely the same. Particularly, in the representative experiment (**Fig.16**), of 264 proteins, only 15 were >2-fold more abundant in one of the cell lines. Interestingly, the majority of the differentially represented proteins were found in the mutant-HRAS-transfected cell line. The main identity of the p53 complexes should be expected as the cell lines are different only in terms of a single mutation and the core interactome is essential for the TF functionality. As has been mentioned above, ~40% of the identified proteins were represented by reported physical interactors of p53. Furthermore, I identified a number of functional p53 interactors. Namely, a set of p53-related chaperones, including HSPA9 [328], HSPB1 [329], and HSP90 known as part of p53 transcriptional complex [330]. The interactome also included PTEN, a regulator of p53 stability and mediator of the p53-driven regulation of upstream signaling pathways [331]; SFN, another stabilizer of p53 [303]; RAD50, CCAR2, XRCC6 and DDX5, mediators of the DNA-damage control by p53 [332], [333], [334]. Similarly to the results of the DNA-damage experiment (chapter), the detected p53 partners included WDR5, several 14-3-3 proteins, and a row of zinc finger TFs (ZNF138/329/598 and others). Taking together, these pieces of evidence confirm the identification of the characteristic p53 interactome that indeed could serve a criterion of the experimental success.

Interestingly, NUP155 and FTSJ3, a paralog of FTSJ1, were identified in the pulldown. The recent publication of our colleagues from ZMBH, Heidelberg showed the influence of the wild-type p53 on the expression of the nucleoporin NUP155 and methyltransferase

FTSJ1, suggesting a regulatory loop between the nucleoporin and transcriptional effectors of p53 [335]. It has been also reported that NUP155 can modulate TF's activity by the example of its physical interaction with HDAC4 [336]. Thereby, detection of NUP155 as a part of p53 interactome could be a sign of direct regulation of the p53 transcriptional activity by NUP155 via changes in chromatin–nucleoporin association.

Although these pieces of evidence emphasise that the detailed analysis of the steady-state p53 on-chromatin network per se might be a source of information of the putative p53 transcriptional partners, I will rather describe the proteins revealed to be over-represented in the p53 complexes in mutant-HRAS-transfected cells in comparison to the WT MCF10A. This could enable suggesting the mediators of the observed alterations in p53 activity in response to the aberrant RAS signaling. One of those is MAP3K10, a downstream effector of the RAS-activated PI3K pathway. MAP3K10 involved in activating phosphorylation of JUN, the AP1 complex subunit [337]. As it has been discussed in Chapter 1, the up-regulation of JUN and another AP1 subunit, FOS, was revealed in the mutant-HRAS-transfected cells, along with the up-regulation of AP1 transcriptional targets (GSEA). Noteworthy, the p53-knockdown did not affect the JUN/FOS expression. MAP3K was also shown to modulate the activity of a transcription factor TCF3 (E47) by phosphorylation. The regulation of the p53 by MAPKs was confirmed for stress-response-mediating p38MAPK [338]. My finding suggests that MAP3K10 can play a role in the regulation of the p53 transcriptional activity. Another kinase found to be differentially represented in multiple replicates is PTK6. PTK6 activates the STAT3 pathway through phosphorylation of the TF. The recent study reported the connection between PTK6 and p53 in normal epithelial and colon cancer cell lines with wild-type p53: PTK6 expression is stimulated by p53, whereas PTK6 knockdown decreases the p53-driven expression of p21 upon DNA-damage [339]. The mechanisms underlying the functional connection was not suggested. However, it was noticed that the pro-apoptotic activity of PTK6 in normal cells switches to the pro-survival in the cancer ones. Along with other genomic alterations, the colon cancer line HCT116 used in the study expresses the mutant KRAS (G13D). Taking these observations together with the identification of PTK6 in the p53 interactome of the MCF10A cells, a possible interpretation would be that, upon the modulation by mutant RAS signaling, PTK6 alters p53 transcriptional activity by the

direct TF phosphorylation or through crosstalk with p53 interactors, for example, PTEN [340]. Thereby, in both cases, the interaction between PTK6 and its effector could remain on-chromatin and be detected by the ChIP-SICAP. Other proteins that were found to be overrepresented in the p53 interactome of the mutant-HRAS-expressing cells with different reproducibility and that might be of interest to mention are ATP5A1, KIF20B, SH3RF3.

Among the others, two junction proteins, JUP (plakoglobin) and DSP (desmoplakin) were found to be predominantly recruited to the p53 complexes upon the oncogenic HRAS signaling. Both of these proteins were recently reported to have nuclear localisation and exhibit chromatin-related functions. Using the advantages of mass-spectrometry and the proximity-dependent labeling tool BioID, DSP was found to be involved in telomere maintaining though interaction with the shelterin complex [341]. JUP was recently discovered to be a physical interactor of p53 (by co-IP) and a regulator of its transcriptional activity in MCF7 cells [342] Furthermore, in last years, JUP has been already reported as a co-factor of several other TFs, such as SOX4, LEF1 (Wnt pathway), and YAP1 (Hippo pathway), demonstrating that its role in transcriptional regulation is not confined by the interaction with p53 and that JUP might intermediate signal transduction from many pathways (Aktary *et al*. published a valuable review on the topic [343]). JUP was also shown to regulate MYC expression through cooperation with LEF1 [344]. As it was discussed in the nascent proteome section (Chapter 1), the mutant HRAS expression activates TNFA signaling, expression of YAP signatures, and MYC targets. Besides, the p53 knockdown in the mutant-HRAS-transfected cells led to partial abolishing the effects of oncogenic HRAS signaling. Furthermore, the analysis of the regulated genes showed that 19 of 115 are represented by targets of LEF1. Overall, the revealed differential on-chromatin interaction of JUP and p53 in the context of the oncogenic RAS signaling supports the previous findings on the role of JUP in transcriptional regulation. The mutant RAS driven JUP recruitment on chromatin might also explain the observed alterations in the activity of p53 in the MCF10A cells expressing mutant HRAS. A specific investigation of the crosstalk of JUP with LEF1, MYC, AP1, and p53 might be beneficial for revealing the role of both JUP and wild-type p53 in oncogeneses.

To summarise, the ChIP-SICAP method allowed the identification of the p53 on-chromatin interactome in unstimulated MCF10A cells. The interactome was found to be remaining mainly the same independently of the HRAS status, however, several proteins were overrepresented in the p53 complexes of WT or HRAS MCF10A cells. The detailed analysis of those showed that some of them might represent novel putative partners of p53. As the transcriptional functions of p53 were shown to be altered by the oncogenic HRAS signaling, these new interactors could be involved in the intermediation of the specific p53 activity. The further validation and investigation of these partners in the context of their cooperation with p53 and downstream effectors of RAS might give mechanistic insights into the crosstalk between wild-type p53 and the oncogene. Besides, the ChIP-seq of p53 in the WT and HRAS MCF10A cells might be especially informative to correlate the interactome of p53 with its binding sites and transcriptional activity outcome.

3.9 Discussion

The understanding of how TFs crosstalk with the downstream signaling pathways and interact with other proteins on-chromatin to regulate the gene transcription is important to investigate the mechanistic aspects of cancer development. The mass-spectrometry-based approaches provide a powerful tool for studying the TF interactomes as these allow direct detection of protein complexes. In this work, I applied the method for selective enrichment of chromatin-associated proteins, ChIP-SICAP [291] for identification of the DNA-bound p53 interactors in the context of DNA-damage and the mutant HRAS signaling in MCF10A cells. The ChIP-SICAP implies successive enrichment for p53 complexes and DNA-bound proteins, using the conventional ChIP followed by DNA biotinylation and pulldown with streptavidin beads. The extensive purification enables efficient removal of contaminants and selective isolation of on-chromatin complexes of the TF. The identification of p53 interactome in the irradiated MCF10A cells confirmed the high selectivity of the ChIP-SICAP as a large number of know p53 interactors and mediators of the DNA damage response, including TP53BP1, SFN, USP7, and others, was successfully detected, along with the efficient enrichment of p53 itself. Furthermore, the analysis of the p53 interactome revealed several putative interactors of p53 that might play a role in the mediation of the DNA-damage response. Particularly, WDR43, TRIM41, and CCDC50, which might

participate in chromatin remodeling and regulation of p53 ubiquitination. Furthermore, the analysis was sensitive enough to detect the p53 phosphorylation sites (Ser15, Ser315, and Ser392) typical for CHK/FACT-complex-mediated stabilization of p53 upon the DNA damage.

This approach was further expanded to study alterations in the composition of the p53 complexes in unstimulated MCF10A cells expressing the mutant HRAS. Due to the relatively low basal concentration of p53 in these cells, the methodology required considerable optimization. The in-house TdT buffer was constituted on the basis of sodium cacodylate to avoid the risk of precipitation caused by additional components of the commercial buffers at the high protein concentration in the sample. The amount of starting material was increased up to 100 million cells to compensate the low abundance of the bait. The sample preparation procedures were adjusted accordingly. Finally, the milder crosslinking conditions (1% formaldehyde vs 2.5% used in the original work) were employed to reduce the presence of artifacts of the chain crosslinking. Together, these measurements enabled the identification of the steady-state p53 interactome both in WT and HRAS MCF10A cells, using SILAC labelling. The p53 network included a wide range of known physical interactors, such as RAD50, PTEN, XRCC6, representing in total ~40% of the identified proteins. The majority of the proteins (>90%, depending on the replicate) were found to be equally abundant in the p53 complexes in WT and HRAS MCF10A cells. This supports the assumption that the core repertoire of a functioning TF partners remains the same independently of the signaling or the cell type as it is demonstrated in the previous studies of TF complexes [299]. Thus, this result also confirms the accuracy of the ChIP-SICAP quantification. Yet, several individual p53 interactors identified by the ChIP-SICAP were found to be differentially represented in the MCF10A cell lines, predominantly – over-represented in the mutant-HRAS-expressing cells, some of which have been reported to be associated with the p53 activity and might, therefore, be putative interactors of p53. Those included PTK6, NUP155, KIF20B, JUP, DSP, and others. The investigation of the effects of the p53 silencing (Chapter 1) showed that the outcome of the p53 activity differs upon the aberrant RAS signaling. Thus the divergence in the composition of the p53 interactome might reflect these alterations in the p53 functionality that could be driven by the on-chromatin interactions with other proteins, similar to the changes in the interactome

reported for the mutant variants of p53 [345]. Undoubtedly, these findings require validation, and the further improvement of the sensitivity and reproducibility of the ChIP-SICAP methodology also remain of high need for a more detailed investigation of the p53 interactome. Nevertheless, in this work, I demonstrated the applicability of the ChIP-SICAP for the identification of on-chromatin partners of a low-abundant TF in differentiated cells. Being used for subsequent studies, the obtained data can help to elucidate the mechanistic aspects of the oncogenic-signaling-dependent alterations in the activity of p53, give insights into the p53 biology and reveal potential therapeutic targets for the treatment of cancers expressing the wild-type p53.

4. Secretome analysis

4.1 Introduction

The term ‘secretome’ refers to the proteins released, or secreted, by cells into the extracellular space. It was originally introduced almost 20 years ago in the studies of the proteome of *Bacillus subtilis* [346], [347]. The share of the secreted protein in the human genome is commonly estimated to be 10% of the protein-encoding genes. These proteins are mainly represented by biologically active molecules such as growth factors, cytokines, proteases, and angiogenic factors. Thereby, they intermediate the communication between cells, and also define the properties of the extracellular matrix. Expectedly, these proteins are involved in various physiological processes such as cell differentiation, tissue organization, angiogenesis, and immune response. The classical mechanism of protein secretion implies the translocation of newly synthesized proteins into the lumen of the endoplasmic reticulum, transportation through the Golgi complex, and release into the extracellular space via vesicles (**Fig. 17**). The messenger RNAs of the proteins secreted in this way include the sequence that encodes an N-terminal signal peptide that is recognized by the cellular machinery which transports a protein into the endoplasmic reticulum. In cell biology, the signal sequence is used for the prediction of the secreted proteins from genomic data [348].

Alternatively, proteins can be secreted by unconventional mechanisms that imply direct transit through the plasma membrane bypassing the Golgi and not requiring the signal peptide. For instance, FGF2 is known to be secreted by this mechanism [349] (**Fig. 17**). The unconventional secretion is usually associated with stress and has been reported to be typical for cancer cells. This phenomenon also expands the complexity of the cellular secretome as the commonly intracellular proteins secreted through this unconventional mechanism can exhibit uncharacteristic activities in the extracellular space [350].

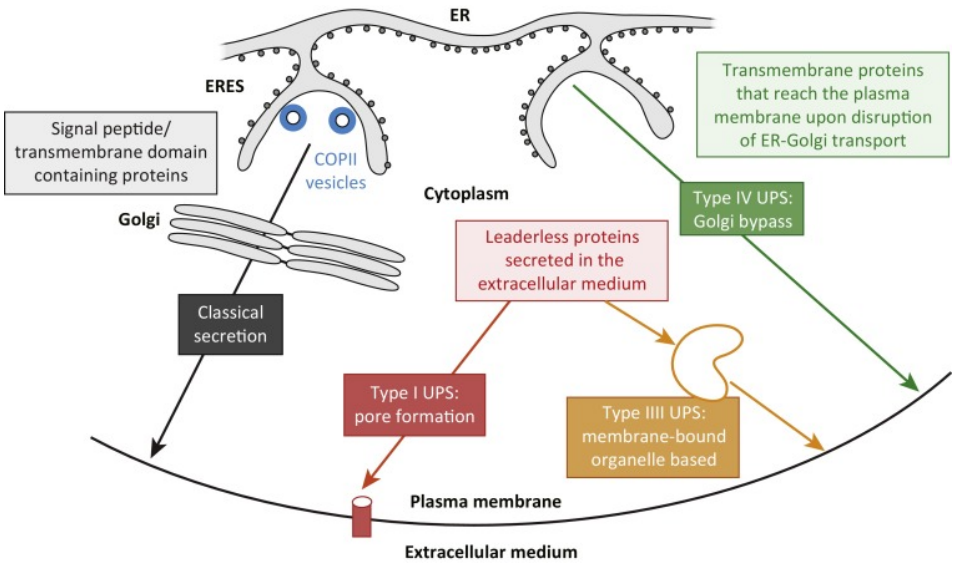


Figure 17. Mechanisms of protein secretion. Adopted from the publication of C. Rabouille *et al.* [351]

Like other proteins, the secreted ones are regulated depending on the cellular status (for the secreted protein this regulation involves not only the expression but also the release), and alterations in the cellular secretome are found to be associated with many pathophysiological conditions, including cancer, metabolic, inflammatory, and neurodegenerative diseases [352]. Thereby, the secreted proteins often serve as clinical biomarkers derived from body liquids.

4.1.1 Role of the secretome in diseases

In cancer, alterations in the secretome are tightly connected with oncogenic transformation and contribute to tumor progression. The secreted proteins are able to intermediate cell-to-cell communication as many of them can be bound by respective cellular receptors and play a role in the regulation of cellular processes involved in growth, proliferation, metabolism, and survival. Thus, the secretion allows the cancer cells to influence the other nearby ones and alter their state (paracrine stimulation). In heterogeneous tumors, cells of different types can adjust the behavior of each other through the exchange of the secreted factors and, therefore, act as an organic whole [353]. Furthermore, the tumor microenvironment includes other non-cancer cells, such as fibroblasts, immune, and endothelial cells that can be effected through the secretion or, conversely, provide the cancer cells with secreted

factors. A well-characterized example of this is cancer-associated fibroblasts (CAFs), cancer reprogrammed fibroblasts found in the stroma of human tumors. The CAFs produce the extracellular matrix proteins, proteases, and pro-tumorigenic factors supporting the cancer cells for further growth and proliferation [354]. As well as proteins secreted by cancer cells, the CAFs-produced factors can contribute to angiogenesis, drug resistance, and development of the invasive phenotypes through activation of the respective signaling pathway or remodeling of the extracellular matrix [355].

Another way how secretome can promote tumor development is the autocrine stimulation. The cancer cells are known to often require fewer growth factors than normal cells for optimal growth and proliferation [356]. One of the mechanisms supporting this feature is the aberrant endogenous production of growth factors that subsequently stimulate external receptors of the cell which produced them. The growth factors acting via an autocrine mechanism in cancer cells include TGF-a, PDGF, AREG, and many others, including GHRH that has been recently reported to stimulate RAS downstream pathways in a dose-dependent manner by the autocrine mechanism in breast cancer cells [357].

4.1.2 Secretome and oncogenic signaling

Tumorigenesis is characterized by a progressive sequence of premalignant changes, altering various cellular processes including the secretome composition. This fact, along with the progress in analytical methods, made the analysis of the cancer-cell-secreted proteins to be a promising tool for discovering biomarkers, proteins that are differentially expressed by cancer cells, and are capable to enter body fluids. Indeed, the recent studies applying mass-spectrometry for the secretome analysis managed to identify a set of putative secreted biomarkers for various cancer types [358]. For instance, the investigation of proteome composition of normal and cancer breast cell lines (MCF-10A, BT474, and MDA-MB-468) revealed the cancer-specific secretion of PI3, KLK6, and ALCAM that was further validated on clinical samples [359]. However, since the protein secretion is strongly connected to cellular signal transduction and the status of various pathways, the secretome can also be a source of valuable information on the mechanisms underlying tumorigenesis. Besides, the knowledge of how various oncogenic mutations affect the composition of

secreted proteins can open opportunities for the development of therapeutic approaches targeting undesirable effects intermediated by secreted proteins such as invasiveness, inflammation, and neoangiogenesis. Thus, the recent studies demonstrated that in thyroid cancer, the more invasive phenotype associated with resistance to dasatinib is modulated through FAK kinase driven secretion of metalloprotease MMP-9, and therefore suggested an approach for combined therapy [360]. In the other study in this field, it was shown that pro-metastatic functions of mutant p53 are associated with increased secretion of SPARC, MMP-2, and, ADAM-10, [361]. Overall, the secretome analysis is a powerful tool for studying peculiarities of oncogene-associated signal transduction in cells.

4.2 Methods of the secretome analysis

The discussed role of secreted proteins in cancer development makes them a promising object to study. Analysis of the cancer cell secretome can provide valuable information on mechanisms of the cellular transformation, intercellular communication, and dynamics of tumor progression. This can also help to reveal novel cancer type-specific biomarkers of therapeutic targets. However, for many years, the progress in the field was impeded by technical issues associated with the relatively high complexity of the cellular secretome, the difficulty of their identification and quantification, and the presence of various contaminants.

The approaches for secretome analysis can be categorized into the ones that use genomic information, and those that directly target the secreted proteins. The genomic approaches are based on the analysis of expression of the genes associated with secreted proteins by measuring the mRNA levels using various approaches, including RNA-sequencing and DNA-microarray [362]. These methods allow overcoming the complexity of clinical and cell culture samples to a certain extent as they focused on the abundance of intracellular nucleic acid and do not depend on the presence of contaminant proteins. In the modern state, they also allow the simultaneous processing of multiple individual samples [363]. However, these methods have several limitations from a biological point of view. First of

all, the analysis of the gene transcripts ignores the post-transcriptional regulation of the protein expression, and thus the measured mRNA abundance does not always reflect the actual abundance of a protein. This is especially important for secreted proteins as the levels of expression and secretion of those might also differ [87]. Furthermore, these methods do not allow to directly determine whether a given protein is secreted. The composition of secretome can differ among various cell or tissue types, and can also be condition-dependent. Therefore, ideally, the genomic approaches are applicable only for cell/tissues with pre-defined secretory profile, when the only quantification is of interest. At the same time, this limitation can be now overcome using publicly available databases of experimentally identified secreted proteins and bioinformatics tools for the prediction of secretion such as VerSeDa [364] and SignalP [365]. As it has been introduced above, classically secreted proteins contain a 15-30 amino acids long N-terminal signal peptide exhibiting a distinguishable pattern of positively charged and hydrophobic residues distribution. The modern bioinformatics algorithms allow the genome-wide identification of genes encoding such peptides, and hence, prediction of secreted proteins. The proteins secreted by unconventional mechanisms which do not bear a signal peptide can be predicted on the basis of amino acid sequence similarity with the protein that has been experimentally identified to be secreted. The prediction algorithms strongly depend on the quality of annotations and are limited in the capability to detect proteins outlying of the known patterns [366].

The methods focusing on direct protein analysis can be sub-divided into the antibody-based (protein arrays) and the proteomic ones. The samples analyzed by these methods are typically represented by body liquids such as serum, plasma or urine, and media from cultured cells. Both of these sources contain highly abundant secretome unrelated proteins (e.g. albumin) that can constitute up to 80% of the total proteins [367], making it difficult to detect low abundant proteins. This determines the requirements for both high selectivity and sensitivity of the methods since, in contrast to the mRNA, proteins cannot be amplified after the sample collection. The selectivity can be achieved by applying additional purification steps before the analysis to remove the unspecific proteins or by selective methods of identification. These methods include antibody binding or endogenous labeling of the secreted proteins of interest; the latter is unfortunately mainly inapplicable to clinical

samples. The sensitivity varies depending on the method of identification. Particularly, modern ELISA-based techniques have a detection range between 0.1 to 1 fmole [368]. The sensitivity of the mass-spectrometry analysis strongly depends on the complexity of the sample, methods of the sample preparation, chromatography separation, the type of the ion detector, and the acquisition method. The accuracy down to 0.5 ppm has been recently reported for the detection of low abundant residual host cell proteins in biopharmaceutical preparations [369]. For both methods, the range for accurate quantification can differ depending on the ranges of detection.

The protein array approaches are based on binding the secreted proteins with specific antibodies. A set of antibodies against various secreted factors can be immobilized on plastic, nitrocellulose, or glass surfaces allowing detection of the proteins in multiplexed and high-throughput formats [370]. These methods are especially sensitive in terms of the detection of low abundant proteins, though they also provide high specificity. Nevertheless, as well the genomic methods, the protein arrays require a certain prior knowledge of the secretome as the panels detecting antibody are not meant to cover the whole proteome due to reasons of cost-efficiency and limited availability of antibody with high specificity for some antigens [371].

4.3 Mass spectrometry-based methods of the secretome analysis

In contrast, mass-spectrometry-based analysis of secretomes allows direct and unbiased detection of secreted proteins using the whole range of the protein-coding genes as a reference. Modern mass-spectrometers provide high sensitivity and are capable to detect even low abundant proteins. However, in practice, the sensitivity is limited by the presence of secretome-unspecific highly abundant serum proteins from blood or animal serums in cell culture media. The typical concentration of the supplementary bovine or horse serum in the media used for growing cells is 5-10%. This gives the concentration of the serum proteins within the range of several mg/mL, which is 1000-fold higher the usual concentration of secreted proteins (ng/mL) whereas the typical range of mass-spectrometers

lies within 4 orders of magnitude [372]. Therefore, the detection of low abundant proteins in the presence of a high and complex background is a severe challenge. One way to overcome this complication is omitting of serum. In this case, before the analysis, the cells grown in the regular culture medium are washed to remove the serum proteins and then conditioned in the serum-free medium for the accumulation of the secreted proteins. This enables decreasing the amount of contaminating proteins with respective benefits for the analysis. However, the later reports [373], [374] showed that serum starvation can significantly alter cellular signaling and, consequently, protein expression and secretion. Therefore, the secretome analysis in serum-free media provides results biased by the lack of a broad spectrum of serum supplements.

Alternatively, various strategies for endogenous protein labelling can be used to distinguish cellular and serum proteins by mass-spectrometry or facilitate removal of the background by purification steps. First of all, the stable isotope labeling by amino acids in cell culture (SILAC) can be used for the incorporation of the labelled amino acid into proteins synthesized by the cells. This then allows us to process the samples according to the standard procedures and detect the peptides containing the labelled amino acids already during the mass-spectrometry analysis. This approach facilitates distinguishing of the secreted protein from the serum background, however, it does not exclude the analytical issues caused by the complexity of the samples and the broad range of protein concentrations [375]. Another alternative is endogenous tagging of secreted proteins by reactive groups that can be further used in the purification step for the secretome enrichment. These approaches include labelling of sugars [376] or amino acids [377] with the azide-group which intermediates covalent binding of the protein incorporated into an alkyne-functionalized agarose resin. In contrast to the sugar-based labelling that only partially covers the spectrum of secreted proteins as not all of those are glycosylated, applying of azide-bearing analogs of amino acids such as azidohomoalanine (AHA, a functional homolog of methionine) allows to label >90% of proteins expressed by mammalian cells. Yet, those do not include ~1% of methionine-free proteins, and ~5% of proteins containing only the N-term methionine residue that can be removed post-translationally [378]. Therefore, the azide-labelling-based proteomic approach has high sensitivity due to the efficient removal of serum proteins. It also allows annotation-

independent identification of the secreted proteins – considering the whole proteome – and the precise quantification by SILAC. Besides, the method does not require additional genetic manipulations such as endogenous tagging and can be used to any cells. Due to these advantages, the method was chosen as a tool for investigation of the secretomes of the MCF10A cells in my project.

4.4 AHA-labelling for quantification of secreted proteins

For the analysis of the secreted proteins, I used the protocol that was initially established by Katrin Eichelbaum [92] and further optimized by Gertjan Kramer to investigate the changes in protein secretion driven by mutant RAS signaling in non-cancerous human mammary gland cells. The protocol combines AHA incorporation with SILAC labelling and thus enables distinguishing the secreted proteins from residual co-purified serum proteins and to reliably quantify in one sample the proteins secreted from two different cell lines.

In my work, the MCF10 cell lines of human breast epithelial cells transfected with mutant RAS serve as a model for studying the consequence of the mutant RAS signaling on protein expression. Due to its capability to reprogram the cell behavior through modulation of downstream signaling pathways, mutant RAS variants are expected to alter protein secretion as well. Indeed, several studies, which were focused on the detection of the RAS activity related to secreted factors, revealed that RAS can contribute to the regulation of various cellular processes via secretion. Particularly, a mutant RAS was found to modulate angiogenesis and to be beneficial for tumor growth through induction of IL6/8 secretion [379]. An increase of the basal autophagy levels upon the oncogenic RAS activation was revealed to be connected to the secretion of pro-invasive factors, MMP2 and WNT5A [380]. Another study reported that the mutant-HRAS-associated metastatic potential of breast epithelial cells depends on the secretion of S100A8 and S100A9 [381]. The oncogenic RAS proteins were also shown to affect cellular secretion through the modulation of the transcriptional co-activator YAP1. Pancreatic ductal cells transformed

by a mutant KRAS showed the elevated expression of pro-proliferative and pro-inflammation secretory factors [382]. Thereby, the autocrine/paracrine regulation seems to play an important role in modulating effects of the mutant RAS signalling. However, as the previous studies were mostly focused on particular processes and applying low-throughput techniques, they provided only limited information about the RAS-driven secretion. Our mass spectrometry-based method was meant to allow more comprehensive monitoring of the effects of RAS on the cellular secretome.

4.5 Alterations in secretome upon the aberrant RAS signalling

To investigate the effect of mutant RAS expression on the cellular secretome, I performed the pairwise comparison of the protein content of culture media from wild-type MCF10A cells and the cells transfected with mutant HRAS harboring an activating point mutation (G12V).

As described above, I used the methodology that was initially established by Katrin Eichelbaum [92] and further optimized by Gertjan Kramer for selective labeling of newly-synthesized proteins with an azido-group. Being coupled with pulse SILAC labelling and consequent click-chemistry-based purification on alkyne resin, this method allows removal of background signal arising from the horse serum proteins, a conventional supplement of MCF10A cell growth media. The SILAC labelling in this context serves for distinguishing between proteins derived from different cell lines – as the samples for the pairwise comparison are combined at the stage of the medium collection – and quantification of the protein abundances in these two secretomes.

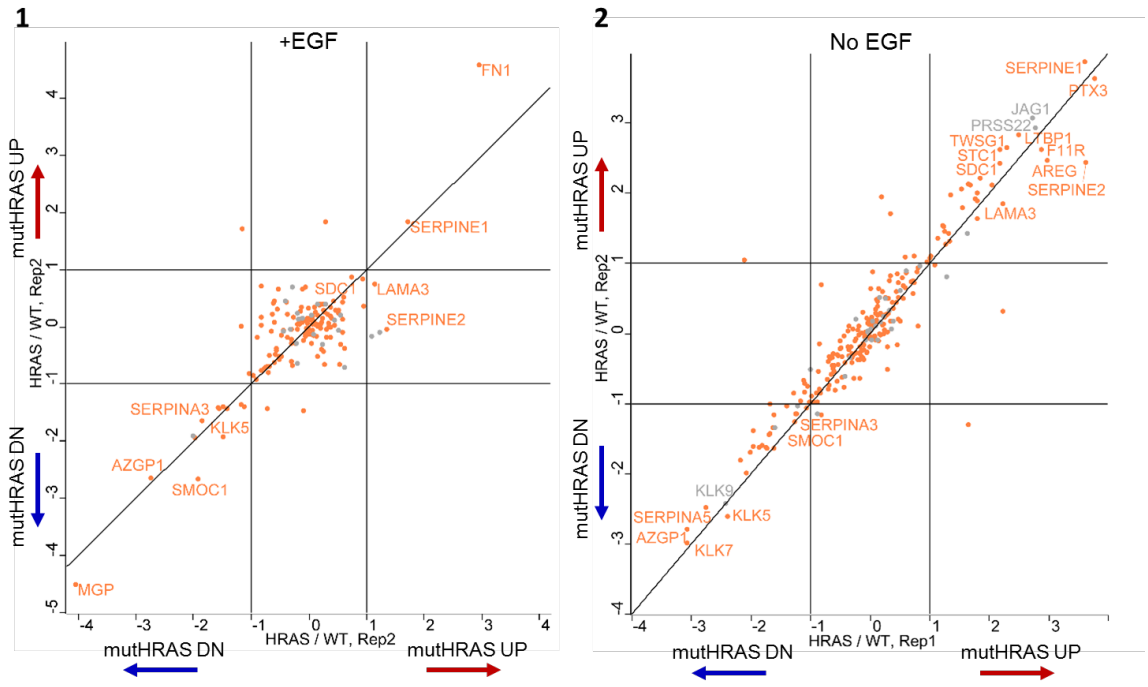


Figure 18. Proteins secreted by MCF10A cells depending on the status of RAS signaling and the presence of EGF. Labelling and sample collection was performed in the same way as for the nascent proteome analysis (Chapter 1) except that concentrated growth media was used as starting material for the click-chemistry based enrichment. The labeling time – 6 hours. The scatter plots demonstrate the correlation of Log2-transformed ratios of the protein abundance (the mutant HRAS-expressing cells over the wild-type) between replicates. (A) Secretome analysis in the presence of EGF. (B) Secretome analysis upon EGF depletion. Orange dots – extracellular proteins (AmiGO annotation).

This approach enabled quantitative identification of ~300 proteins secreted by the WT and the HRAS MCF10A cells. According to the gene ontology analysis (AmiGO annotation database), >70% proteins identified in replicates in different samples were represented by ones known to be secreted (**Fig. 18**). To reveal how the secretome composition depends on the natural activation of the EGF receptor (EGFR), an EGF-binding-dependent upstream modulator of RAS signaling, the analysis was performed in two conditions: at the presence of EGF, when the RAS signaling is active in both wild-type and the mutant HRAS-expressing cells, and upon EGF depletion, when the activation of RAS nearly exclusively depends on the activating point mutation. As expected, I observed the more pronounced mutant-HRAS-driven regulation of secretion upon the EGF depletion (39 up-regulated, and 37 down-regulated proteins) (**Fig. 19**) in comparison to the analysis in presence of EGF (only 3 up-regulated proteins, and 14 down-regulated ones). This shows that the secretion

is predominantly controlled through EGF-independent signaling mediated by the mutant RAS, and might be driven by mechanisms compensating the lack of EGF. Particularly, AREG was found to be up-regulated upon HRAS transformation at the EGF-free conditions. AREG serves as a ligand of the EGF-receptors and a promoter of cell growth. It was found to be overexpressed in various breast cancers [383] and act as an autocrine stimulator of tumor growth [384]. Interestingly, the AREG upregulation is common in the estrogen receptor (ERα)-positive tumors and its expression is known to be controlled by the ERα transcriptional activity [385]. In triple-negative breast cancers (the MCF10A cells serve as a model of this phenotype), the increase of AREG was shown to be mediated through PTEN-deficiency-driven activation of the NFkB pathway. Furthermore, EGFR activation by AREG might cause a cellular response different from the canonical EGF-mediated activation and, therefore, lead to overexpression of BIRC5 and FN1 and development of an invasive phenotype [386]. Hereby, the MCF10A secretome analysis shows that aberrant activation of RAS is sufficient for stimulation of AREG secretion in absence of EGF, whereas, wild-type MCF10A cells are not capable to initiate this EGF depletion compensating mechanism. As follows from the analysis of protein expression in the mutant HRAS-transfected cells, the mutant RAS activity leads to activation of NFkB and mTOR signaling. Therefore, the AREG expression can be induced through the same downstream mechanisms, similarly to how it was previously reported for the EGFR-inhibition resistant triple-negative breast cancer cell models [386].

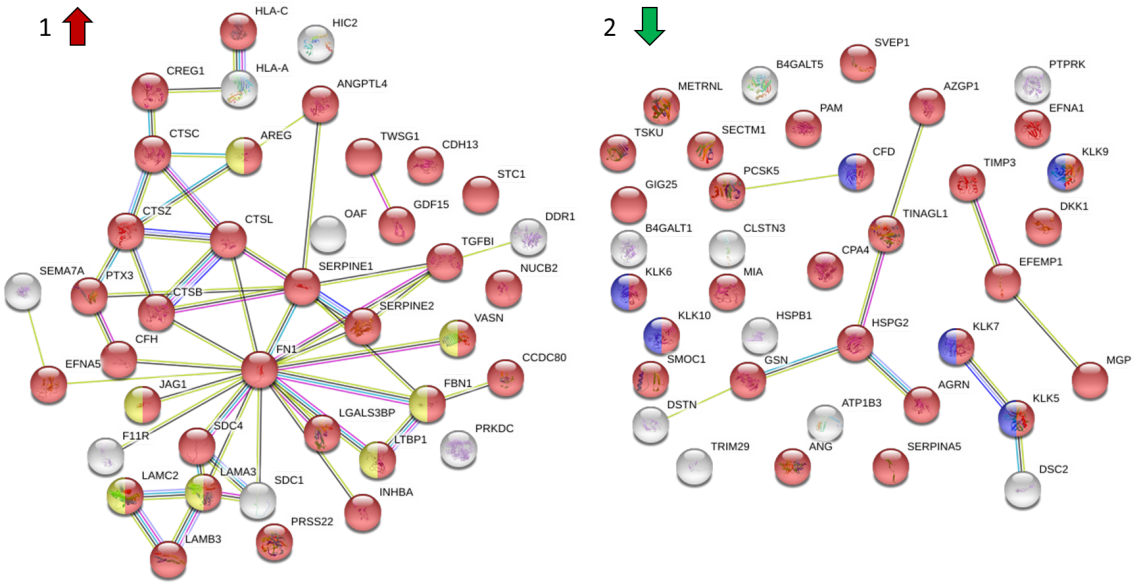


Figure 19. Secreted proteins regulated upon the mutant RAS expression. Those annotated as extracellular (AmiGO annotation, GO:0005576) are highlighted in red. The connections reflect the protein-protein associations identified by String [387]. (1) The up-regulated proteins (39); the proteins containing the EGF-like domains (InterPro, IPR000742) are highlighted in yellow. (2) The down-regulated proteins (37), the proteins exhibiting a peptidase activity (Pfam, PF00089) are highlighted in blue.

The overexpression of FN1, as well as of SERPINE1, in the context of the MCF10A cell model, seems to be induced in an AREG-independent manner as these proteins were also upregulated upon the EGF treatment. However, at the EGF-free conditions, 9 other EMT-related (according to the experimental annotation database of the Broad Institute, M5930) proteins were found to be upregulated, including SERPINE2, INHBA, FBN1, and TGFBI. Thereby, the increase in expression of EMT-related proteins observed by the nascent proteome analysis (Chapter 1) as well as the elevation of the cell migration and invasion properties (the chapter about phenotypic characterization) upon the mutant HRAS transfection, are reflected in alterations in the cellular secretome. Additionally, I observed an increase in the secretion of several proteins which were recently reported to play a role in the invasive phenotype development. Particularly, SEMA7A, a promoter of invasion in breast cancer cell lines through β1-integrin receptors [388]. CREG1, as a factor of cell proliferation and cell migration in Non-small Cell Lung Cancer Cells, that expression was shown to be strongly associated with the oncogenic RAS signaling and poor prognosis [389]. VASN (vasorin), a protein, which overexpression was reported to increase the invasive abilities of glioma cells [390].

Interestingly, among the proteins that secretion was reduced upon the mutant HRAS-transfection, we identified 6 markers typical for inactivation of p53 (EFEMP1, RNASE4, DKK1, KLK10, DSC2, AZGP1; the experimental annotation database of the Broad Institute, M2698). Thus the changes in the secretion pattern might be a result of the mutant RAS driven alterations in the p53 activity. I also observed the down-regulation of MGP, a protein that is capable to remodel ECM and augment cell migration through binding fibronectin (FN1) [391]. Noteworthy, the contribution of MGP overexpression in migration and drug resistance has been previously reported for ovarian cancer [392]. However, in ER-positive breast cancers, the negative regulation of MGP through the promoter hyper-

methylation was revealed to be associated with worse survival outcomes [393]. Thus, MGP might play an ambivalent role in tumorigenesis depending on the cellular context.

SERPINA3, another protein known to regulate EMT in melanomas and prostate cancer [394], was also found to have a decreased secretion rate in the mutant HRAS-transfected cells. This protein was also reported to be overexpressed in breast and colon cancers and associated with regulation of tumor proliferation and progression [395]. Though the downregulation of SERPINE3 might be not expected given the observed pro-migration phenotype of the cells expressing the mutant HRAS, this should be taken into account that, in the recent studies, the SEPRINE3 expression was proven to be regulated by STAT3, a member of the JAK/STAT signaling pathway. The JAK/STAT activity is modulated through both RAS and TGFB signaling. Therefore, the observed effect might represent the result of crosstalk between these pathways [396].

Noteworthy, I also observed that all detected KLK proteins (KLK5-7 and KLK9/10) were downregulated. The regulation of KLKs is largely understudied. They have been reported to be regulated through hormone signaling, androgen and estrogen receptors transcriptional activity [397], some of them such as KLK5-7 are known to be co-occurring with aberrations in RAS signaling, however, the mechanistic aspects underlying these observations remain unclear. The KLKs were mainly not detected in the intracellular proteome analysis, though the transcriptomics data confirmed that some of the family members are downregulated at the transcriptional level both upon the mutant HRAS expression and p53 knockdown in wild-type cells. Yet, the knockdown in the mutant HRAS expressing cells did not cause significant changes in the expression of KLKs. These results demonstrate that KLKs expression in MCF10A cells could be mechanistically connected with RAS-driven regulation of signaling pathways. This allows hypothesizing the role of p53-mediated transcriptional activity in this regulation.

4.6 Discussion

The implementation of the azido-tagging based technique for selective enrichment of secreted proteins allowed direct identification and quantification of the proteins secreted by the MCF10A cells upon the expression of the mutant HRAS. The tolerance of the method to the presence of serum enabled obtaining the data directly comparable to the results of the proteomic and transcriptomic characterisation of the MCF10A cells. The analysis showed that alterations in the secretome profile depend on the EGF treatment. The most pronounced changes in the secretome composition of WT and HRAS MCF10A cells were detected upon the EGF depletion. This might reflect the activation of HRAS-driven mechanisms compensating the lack of growth factor. Particularly, ligands of EGF (AREG), TGFB (VASN), and NOTCH (JAG1) receptors were found to be over-secreted by the HRAS MCF10A cells. This is consistent with the results of the nascent proteome analysis (Chapter 1) that revealed the activation of the respective signalling pathways in the HRAS MCF10A cells. Thereby, the aberrant RAS signalling can contribute to cell growth and proliferation not only through the direct activation of its downstream effectors but also through the modulation of the autocrine stimulation.

Expectedly, the aberrant RAS signalling was shown to cause an increase in the secretion of EMT markers, such as SERPINE2, FN1, INHBA, FBN1, TGFBI, and SEMA7A. The influence of the mutant HRAS on the expression of the EMT-related proteins has also been demonstrated by the nascent proteome analysis (Chapter 1). Furthermore, the pro-migration and pro-invasion effect of the mutant HRAS was confirmed by the phenotypic characterisation (Chapter 1). Therefore, the secretome analysis conforms to the findings made by the other methods in this work and allows further determination of the role of individual secreted proteins in promoting EMT in the MCF10A cells.

The secreted proteins down-regulated upon the mutant HRAS expression included the extracellular matrix (ECM) remodelling proteins. Particularly MGP, and KLK-family members, that are known to be associated with metastatic cancer progression. This corresponds to the observations made by the invasion assay (Chapter 1), demonstrating that the capability of the HRAS MCF10A cells to penetrate the ECM is relatively low in

comparison to the mutant HRAS-driven improvement in migration. Therefore, the down-regulation of the ECM remodelling proteins might be one of the factors why the mutant HRAS expression in normal epithelial cells is typically not sufficient for the development of the tumorigenic phenotype in mouse models. However, the mechanisms of the downregulation cannot be concluded on the basis of this study. Besides, the hypothesis about the decreased capability of the HRAS MCF10 cells to degrade the ECM matrix requires further examination, for example, by a quantitative analysis of the ECM destruction by the HRAS and WT cells.

The successful implementation of the secretome analysis and the observations made in this work also creates a basis for following studies of the role of the secreted proteins in mediating the effects of the mutant RAS signalling in epithelial cells. Particularly, the role of the autocrine stimulation could be assessed by treating the WT cells with a culture media from the mutant-HRAS expressing cells and by the consequent analysis of the resulting alterations in gene expression. Furthermore, the functions of individual ligands or ECM remodelers, which were found to be differentially secreted, might be elucidated by their selective removal from the culture media, for instance, by a specific antibody. The investigation of the relations between the p53 activity and secretion in the HRAS MCF10A cells might also be informative for understanding the role of p53 in the mutant HRAS signalling, as p53 was shown to affect both the expression of the EMT-related proteins and migration/invasion features of the HRAS MCF10A cells.

5. Concluding remarks

In this work, I characterized different aspects of the aberrant RAS signalling in non-cancerous human epithelial MCF10A cells and provided evidence of the oncogene-cooperative alterations in the wild-type p53 activity upon the mutant HRAS expression.

First of all, I implemented a proteomic approach, the nascent proteome analysis, to investigate the effects of the mutant HRAS signalling on the protein expression. I also compared the conventional analysis of the whole cell lysate and the nascent proteome analysis and showed that the latter ensures a more accurate quantification of changes in protein expression. More than 5000 proteins were successfully quantified in WT and HRAS MCF10A cells. In the mutant HRAS-transfected cells, I revealed the activation of RAS downstream effectors (mTOR and RAF pathways, MYC, and NFkB transcription factors), the metabolic shift towards glycolysis, and the hypoxia-like cellular response. Most pronouncedly the effect of the mutant HRAS expression reflected in the up-regulation of EMT markers and the activation of YAP signalling.

Further, I applied RNA sequencing to monitor the mutant HRAS-driven changes in gene transcription. The analysis confirmed the proteomic results and allowed revealing the increase of expression and activity of several transcription factors involved in the regulation of EMT (SNAI, ZEB, and TWIST). The comparison of the alterations in the expression of individual genes and proteins also showed a high correlation between those (the Person correlation coefficient >0.5). The non-correlating fraction of the genes/proteins could be further investigated in the context of the contribution of the post-transcriptional regulation to the mutant RAS signalling outcome.

Both the transcriptomic and proteomic methods revealed the differential effects of the p53 knockdown in the WT and HRAS MCF10A cells. In the WT cells, the silencing of p53 mostly caused a moderate downregulation of p53 targets. Yet, in the HRAS cells, the p53 knockdown was found to partially abrogate the effects of the mutant HRAS signalling. Particularly, the expression of EMT markers was severely reduced along with a decrease

in the activity of YAP. This elucidates the connection between the p53, Hippo pathway, and oncogenic signalling in the MCF10A cell system. These results were further confirmed by the phenotypic characterisation of the MCF10A cells deferential in the status of HRAS and p53. It was found that the mutant HRAS, indeed, enhances the migration and invasion properties of the cells, whereas the p53 knockdown attenuates them exclusively in the HRAS cells. Therefore, the wild-type p53 was shown to cooperate with the oncogene and partially determine the outcome of the mutant RAS signalling.

Furthermore, I investigated the composition of the p53 on-chromatin interactome. I used the methodology for selective isolation of chromatin-associated proteins (ChIP-SICAP) and optimised it to apply to the MCF10A cells. The applicability of the method was validated by the identification of the p53 network upon non-lethal irradiation. This analysis revealed a quantity of known p53 physical interactors and mediators of the p53-driven DNA-damage response, such as TP53BP1, SFN, and USP7. Besides, I identified several putative p53 interactors (WDR43, TRIM41, and CCDC50). Subsequently, this approach was used to compare the composition of p53 partners in unstimulated WT and HRAS MCF10A cells. The majority of interactors were found to be equally represented in the interactomes of both the cell lines. Yet, a number of proteins, such as PTK6, NUP155, DSP, and JUP, were found to be more abundant in the p53 DNA-binding complexes of the HRAS MCF10A cells. Further validation of the identified on-chromatin interactors of p53 might expand our understanding of the p53 activity and give mechanistic insights into the cooperation between p53 and HRAS.

Finally, I analysed the mutant HRAS-driven alterations in the composition of proteins secreted by MCF10A cells. The applied AHA-labelling-based method allowed detection of the secreted proteins in unbiased conditions, in the presence of serum. I identified an increase in the secretion of several receptor ligands (AREG, VASN, and JAG1) upon the mutant HRAS expression. Therefore, the outcome of the aberrant RAS signalling can be partially determined by the autocrine stimulation. Furthermore, the analysis confirmed the upregulation of EMT markers in the HRAS MCF10A cells. Together with the revealed decrease in the secretion of extracellular matrix remodelling proteins (MGP and KLKs),

this might be a mechanistic basis for the observed alterations in the migration and invasive properties of the mutant HRAS-expressing MCF10A cells.

This analysis revealed the upregulation of the secretion of several receptor ligands (AREG, VASN, and JAG1). Therefore, the outcome of the mutant HRAS expression can be partially determined by the autocrine stimulation. The analysis also confirmed the up-regulation of EMT markers (SERPINE2, FN1, INHBA, and FBN1) in the extracellular space and showed the down-regulation of ECM remodelling proteins (MGP and KLKs). The latter can serve as a potential mechanistic explanation of the less pronounced effect of HRAS on the cell invasion in comparison to migration.

Overall, I applied various proteomic techniques to perform the multifaceted analysis of the effects of the mutant HRAS expression in non-cancerous epithelial cells. My study allowed elucidating the mediators of the aberrant RAS activity and to make novel observations on the role of wild-type p53 in the oncogenic signalling. The results of this work could also be used for subsequent studies of individual interactors of p53 and players of the mutant RAS-driven tumorigenesis.

6. Materials and Methods

6.1 Cell culture

The MCF10A cells were cultured in DMEM/F12 medium (Thermo Fisher Scientific) supplemented with 5% heat-inactivated horse serum (R&D Systems), 10 µg/mL insulin (Sigma-Aldrich), 100 ng/mL cholera toxin (Sigma-Aldrich), 20 ng/mL EGF (Sigma-Aldrich), 0.5 µg/mL hydrocortisone (Sigma-Aldrich), 1% glutamine (Thermo Fisher Scientific), 1% Penstrep (Thermo Fisher Scientific). For the SILAC labelling, the cells were cultivated in DMEM/F-12 for SILAC (Thermo Fisher Scientific) additionally supplemented with 146 mg/L isotope labelled lysine (Sigma-Aldrich), 84 mg/L isotope labelled arginine (Sigma-Aldrich), and dialyzed horse serum (Dundee Cell Products). For the azidohomoalanine labelling, the methionine-free DMEM/F12 (non-GMP, Gibco) supplemented with 18.1 mg/L L-azidohomoalanine (Jena Bioscience). The cells were maintained at 37ºC with 5% CO2.

6.2 The nascent proteome analysis

The cells for the nascent proteome were collected by scraping, pooled into the samples for pairwise comparison, and washed with PBS. The cell pellets were resuspended in ~900 µL of the Urea buffer (300 mM Hepes pH 8, 0.75 M NaCl, 6.2% CHAPS, 8M Urea) containing the protease inhibitor (Roche). The lysate was subjected to sonication by the probe sonicator (Branson 450 Digital Sonifier): ~4, duty cycle 50%, ~5 cycles on melting ice. To remove the cellular debrises, lysates were transferred into new tubes after the centrifugation for 30 min at 15000g, 4ºC. The alkyne-agarose resin (Jena Bioscience), 100 µL per sample, was prepared by washing with the MQ water. The click-chemistry reaction mix was prepared according to the protocol for the Click-kit (Thermo Fisher Scientific). The cleared lysates were combined with the mix, and the alkyne-agarose beads and incubated at 40ºC

for 2 hours on a shaking incubator (2000 rpm. Following the incubation, the resin was pelleted by centrifugation for 1 min at 2000g and the supernatant was removed. The resin was resuspended in the MQ water, pelleted again, mixed with the 1-step reduction/alkylation solution (10 mM Tris(2-carboxyethyl)phosphine, 40 mM 2-Chloroacetamide in the SDS washing buffer (100 mM Tris-HCl pH 8.0, 1% SDS, 250 mM NaCl, 5 mM EDTA)), and incubated for 15 minutes at 70º C. on a shaking incubator (2000 rpm) and further 15 minutes at room temperature. The resin was transferred into spin columns (BioRad) allowing the continuous removal of the buffer flowing through the resin. The resin was consecutively washed five times with 1-mL portions of the SDS-wash buffer, guanidine-wash buffer (100 mM Tris-HCl pH 8.0, 6M Guanidine-HCl), and acetonitrile-wash buffer (20% Acetonitrile, ULCMS in Water ULCMS). After the washing, the resin was resuspended in ~300 µl of the digestion buffer (100 mM Tris-HCl pH 8, 2 mM CaCl2, 5% Acetonitrile) supplemented with ~0.2 ug of the bovine trypsin (Promega). Protein digestion was performed overnight (16-18 hours) at 37ºC. The resin was pelleted by centrifugation for 1 min at 2000g, the supernatant was transferred into the 96-well Oasis plate (Waters) and purified for further fractionation and/or MS analysis according to the Oasis plate protocol. The samples in this way were either used for direct analysis by the LC-MS system or subjected to the intermediate high-pH fractionation.

6.3 The secretome analysis

For analysis of the secreted proteins, the culture media were collected after the incubation with AHA, mixed in the equal proportions, concentrated using Amicon Ultra-15 Centrifugal Filter Units (Merck) down to ~300 µl, mixed with ~900 µL of the Urea buffer and identically to the protocol for the nascent proteome analysis, excluding the sonication step.

6.4 Transcriptome analysis

RNA was isolated from MCF10A cells using the NucleoSpin kit RNeasy Mini kit (Qiagen). For the RNA sequencing, 500 ng of total RNA (measured by the Nanodrop device) from each MCF10A cell line (in three replicates) were processed TruSeq RNA Sample Preparation Kit v2 protocol (Illumina). The integrity of the obtained libraries was evaluated by the TapeStation platform. The samples were further sequenced by the Illumina HiSeq 2500 V4 instrument. The reads were mapped and quantified using the STAR [398] and the RefSeq annotation. The detection of the differentially expressed genes and count normalization analysis were performed by DESeq2 [399] integrated into the UTAP platform of the Weizmann Institute of Science [400]. The p-values were adjusted using the Benjamini and Hochberg method.

6.5 Chromatin immunoprecipitation and biotinylation

The cells were fixed on a plate or following the dissociation with formaldehyde (0.5-2.5%, depending on an experiment) for 15 min. The crosslinking was stopped by adding 125 mM glycine (in PBS). The cells were washed with the PBS and pelleted. The different cell lines were combined into samples immediately after crosslinking. For the lysis and the nuclear extraction, the cell pellets were dissolved in the LB1 buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40) and incubated on ice for 20 mins. To remove the cell debrises and the cytoplasmic proteins, the nuclei were collected by the centrifugation at 1000g for 5 mins followed by the supernatant removal. The pellets were resuspended in the LB2 buffer (10 mM Tris-HCL pH 8.0, 200 mM NaCl, 1.5 mM EDTA). The nuclei were collected once again by the centrifugation at 1000g for 5 mins and the supernatant removal. For the lysis of the nuclei, the pellets were dissolved in the LB3 buffer (10 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, and 0.5% N-lauroylsarcosine) and incubated for 30 mins. The obtained mixture was transferred into 1.5 ml sonication tubes (Diagenode), 300 µL per tube, and subjected to sonication by the sonifier Bioruptor Pico, 15 cycles (30 sec ON, 30 sec OFF)

to shear the chromatin. The efficiency of the sonication was assessed by the agarose gel electrophoresis: the sonication protocol results in the main portion of the DNA fragments to be ~150 bp long. The sonicated samples were further span down for 15 min at 15000 g to remove residual debrises and large DNA fragments. The supernatant was used for the immunoprecipitation: 5 µg of the anti-p53 antibody mix (1:1 DO1 and DO7 (Santa Cruz Biotechnology)) or 5 µg of unspecific mouse IgG (for the control) were added to each sample. The samples were further incubated overnight (~16h) upon rotation at 4ºC. The next day, 30 µL of the Protein G beads (Dynabeads, Thermo Fisher Scientific) was added to each sample for binding the antibodies. Following the further incubation of the samples for ~2h upon rotation at 4ºC, the beads containing the antibody-p53 complexes were collected on a magnet stand, whereas the supernatant was removed. The beads were further washed 2 times with 1mL of the IP buffer (Triton 1%, NP40 0.5%, Tris.Cl pH= 7.5-8 50mM, EDTA 5mM) and once – with the Tris buffer (10mM Tris-Cl pH 7.5). The beads were then washed with 100 µL of the 1x TdT buffer (Terminal Deoxynucleotidyl Transferase buffer, Thermo Fisher Scientific) and reconstituted in 100 µL of the TdT reaction mix (60 U of TdT (Thermo Fisher Scientific) and 50µM Biotin-11-dATP (Jena Biosciences) in the 1x TdT buffer). Following the incubation at 37º C on a shaking incubator (700 rpm) for 1h, the beads were collected on a magnetic stand and washed 5 times with the IP buffer to remove residual biotinylated nucleotides that were not incorporated into the DNA fragments. The elution of the proteins from the beads was performed by adding 20 µL of 10% SDS with 100mM DTT and incubation at 37º C on a shaking incubator (1000 rpm) for 30 minutes. The beads were then collected on a magnetic stand. The supernatant was transferred into separated tubes, diluted with 1mL of the IP buffer, and used for the isolation of the chromatin-bound complexes on the streptavidin beads (SICAP).

6.6 SICAP technique

The complexes of p53 bound to the biotinylated DNA fragments obtained by the immunoprecipitation and DNA biotinylation were separated from the DNA-free complexes

by the pulldown with the streptavidin beads. 100 uL of the magnetic streptavidin-coated beads (New England Biolabs) was added to each sample to bind the biotinylated DNA. The samples were incubated for 2h at room temperature upon rotation. The beads were collected on a magnetic stand. The supernatant was removed (a portion of this supernatant can be further purified and used for the analysis of the proteins that were not enriched by the SICAP). The beads were washed consecutively 3 times with the SDS wash buffer (10mM Tris-Cl pH 8.0, 1mM EDTA, 1% SDS, 200mM NaCl), 2 times with the BW2x buffer (10mM Tris.Cl pH 8.0, 1mM EDTA, 0.1% TritoneX100, 2000mM NaCl), 2 times with the isopropanol wash buffer (2-propanol 20% in the MQ water), and 2 times with the acetonitrile wash buffer (acetonitrile 40% in the MQ water). This extensive washing is meant for the removal of non-biotinylated protein complexes and the proteins non-covalently bound to the DNA. Following the complete removal of the acetonitrile buffer, the beads were resuspended in 14µl 50mM of Ambic with 0.1% SDS and 100mM DTT and incubated at 95º C for 20 min to elute the proteins from the streptavidin beads. Upon cooling down, the beads were subjected to alkylation by adding 1 µL of 200 mM iodoacetamide and incubation for 30 min at room temperature. Then the beads were collected at a magnetic stand and the supernatant was transferred into new tubes. The supernatant was further subjected to the protein digestion by adding 200 ng of the bovine trypsin (Promega) and incubation overnight (16-18 hours) at 37ºC. The digested peptides were purified by the SP3 protocol.

6.7 SP3 peptide clean-up

The peptide clean-up was performed using the original protocol developed by C. Hughes, S. Foehr et al. [96]. Briefly, the peptide-containing supernatant was collected upon the overnight protein digestion. The paramagnetic beads (Sera-Mag Speed Beads, Thermo Fisher Scientific) in the amount equivalent to 5 µL of the original stock were washed with water and mixed with the supernatant to make a homogeneous solution in a PCR tube. LC-MS grade acetonitrile (~220 µL) was added to the solution to achieve the >95% final acetonitrile concentration. Upon vortexing, the tube was incubated on a bench for 10 min

to allow the peptides to the beads. Then, the beads were collected on a magnet stand and the supernatant was removed. The beads were further washed 2 times with pure acetonitrile to remove residual detergents. Following complete removal of acetonitrile, the beads were mixed with 20 µL 0.1% formic acid. The mixture was sonicated in a water-bath sonicator for 5 min. The supernatant collected after the removal of the beads on a magnetic stand was directly used for the LC-MS analysis.

6.8 High-pH fractionation

For the fractionation the samples were processed by the 1200 Infinity HPLC system (Agilent), using a Gemini C18 column (Phenomenex). 33 fractions were collected upon elution with the 60-minute linear gradient of ACN in 20 mM pH10 ammonium formate (0 to 35%) at the 100 µl/min flow rate. The 33 fractions were orthogonally pooled in 8 fractions that were used for the analysis by the LC-MS system.

6.9 LC-MS analysis

The samples were measured on an Orbitrap-Fusion mass spectrometer (Thermo Fisher Scientific) coupled with an EASY-nLC chromatography system (Thermo Fisher Scientific). The chromatography separation was performed on an Acclaim Pepmap RSLC (a 75 µm x 50 cm) analytical column (Thermo Fisher Scientific) using a 105-minute gradient from 3% to 50% (at 86 min) of the solvent B at the 300 nl/min flow rate. Solvent A – 0.1% formic acid in water; solvent B – 0.1% formic acid in 80% acetonitrile and 19.9% water (ULCMS grade, Biosolve). The eluate was ionized in the Nanospray-Flex ion source (Thermo Fisher Scientific), using a 10 µm Picotip coated fused silica emitter (New Objective), and injected to the mass spectrometer. The MS1 analysis was conducted by the Orbitrap detector, in the positive mode, at the 60000 FWHM resolution, AGC target was 1E6, and the maximal injection time 50 ms. The acquisition was performed in the data-dependent mode (DDA). The intensity threshold for the selection of ions for fractionation

was set at 5x103. The HCD-fragmentation in nitrogen was performed at the 33% collision energy. The MS2 occurred in a Linear IonTrap set to the rapid scan rate at TOP20-mode with an isolation window of 1.6 Da, AGC target 1E4, and maximal injection time of 50 ms.

6.10 Data analysis

The analysis of the generated MS raw-files was performed using the MaxQuant [401]. The reviewed human and horse UniProt proteome databases were used for the search. The default Andromeda list of contaminants was applied for the contaminant subtraction. Statistical analysis was performed by the limma R-package [402], the obtained p-values were adjusted by Benjamini and Hochberg method for multiple testing. The MaxQuant output and RNA-sequencing data were further processed with the Perseus software [403].

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Abbreviations

Abbreviation	Description
AHA	L-azidohomoalaine
BP	Biological Process
CC	Cellular Compartment
ChIP	Chromatin Immuno-Precipitation
ChIP-MS	Chromatin Immuno-Precipitation with Mass Spectrometry
EMT	Epithelial–Mesenchymal Transition
GO	Gene Ontology
HPLC	High Performance Liquid Chromatography
iBAQ	Intensity Based Absolute Quantification
LC	Liquid Chromatography
LFQ	Label-Free Quantification
MS	Mass Spectromerty
SILAC	Stable Isotope Labeling with Amino acids in Cell culture
SP3	Single-Pot Solid-Phase-enhanced Sample Preparation
TdT	Terminal deoxynucleotidyl Transferase
TF	Transcription Factors

List of publications

Dewari PS, Southgate B, Mccarten K, Monogarov G, O'Duibhir E, Quinn N, Tyrer A, Leitner MC, Plumb C, Kalantzaki M, Blin C, Finch R, Bressan RB, Morrison G, Jacobi AM, Behlke MA, von Kriegsheim A, Tomlinson S, Krijgsveld J, Pollard SM. **An efficient and scalable pipeline for epitope tagging in mammalian stem cells using Cas9 ribonucleoprotein**. Elife. 2018 Apr 11;7:e35069.

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