Functional characterization of the interaction of Paraxial protocadherin (PAPC) and Nemo like kinase1 (Nlk1) during *Xenopus* gastrulation

DISSERTATION

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This thesis is dedicated to

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1. Summary

During the development of an organism, gastrulation is a critical step laying the foundation of organogenesis. Cells in gastrulating embryos undergo a variety of coordinated cell movements to establish the three germ layers, ectoderm, mesoderm and endoderm. In this, concerted cell movements are orchestrated by multiple signaling pathways, but most importantly by different Wnt signaling cascades, such as the non-canonical Wnt/planar cell polarity (PCP) pathway. Paraxial protocadherin (PAPC) is a central player during *Xenopus* gastrulation regulating cell adhesion, polarization and the Wnt/PCP pathway. However, the mechanism of PAPC mediated signaling is not entirely understood.

In this work I could show for the first time that PAPC interacts with Nemo like kinase 1 (Nlk1), an atypical Serine/Threonine MAP kinase and a known inhibitor of the canonical Wnt/β-catenin pathway. The interaction of Nlk1 with PAPC is mediated by the intracellular domain of PAPC, which triggers recruitment of Nlk1 to the plasma membrane. I further provide evidence that binding to each other causes a post-translational stabilization of both proteins, by inhibition of ubiquitination. Using a kinase negative mutant of Nlk1, I could show that this is independent of its kinase activity. At the same time, mutations of the putative Nlk1 phosphorylation sites in PAPC indicated that phosphorylation of these sites is required for Nlk1 mediated stabilization of PAPC.

Notably, I could demonstrate that Nlk1, probably via its interaction with PAPC, is required for the regulation of Wnt/PCP signaling and gastrulation during early *Xenopus* development, in addition to its function in Wnt/β-catenin signaling. Interestingly, I found that the kinase activity of Nlk1, while required for canonical Wnt modulation, is dispensable for its role in Wnt/PCP signaling and regulation of gastrulation.

Based on my results I propose that during *Xenopus* development Nlk1 is a versatile modulator of the Wnt/β-catenin and Wnt/PCP pathways. Moreover, I suggest that Nlk1 influences gastrulation movements by stabilizing the Wnt/PCP component PAPC.

2. Zusammenfassung

In der Embryonalentwicklung ist die Gastrulation ein entscheidender Schritt ,um die Grundlage für die Organogenese festzulegen. Eine Vielzahl koordinierter Zellbewegungen etabliert während der Gastrulation die drei Keimblätter Ektoderm, Mesoderm und Endoderm. Dabei werden die aufeinander abgestimmten Zellbewegungen von verschiedenen Signalwegen, wie z. B. dem nicht kanonischen Wnt/PCP (engl. für Planare Zellpolarität) Signalweg, organisiert. Hierbei spielt das Paraxiale Protocadherin (PAPC) eine zentrale Rolle, indem es während der *Xenopus* Gastrulation Wnt/PCP, Zelladhäsion und Polarisierung reguliert. Allerdings sind die Mechanismen der PAPC-abhängigen Signaltransmission noch unklar.

In dieser Arbeit konnte ich erstmals zeigen, dass PAPC mit der Nemo like/ähnlichen Kinase 1 (Nlk1) interagiert. Nlk1 ist eine atypische Serin/Threonin MAPKinase und ein bekannter Inhibitor des kanonischen Wnt/β-Catenin Signalwegs. Die Bindung der von Nlk1 an PAPC erfolgt über die intrazelluläre Domäne von PAPC, wodurch Nlk1 an die Plasmamembran rekrutiert wird. Weiterhin konnte ich belegen, dass die Interaktion der beiden Proteine deren Ubiquitylierung hemmt, wodurch sie sich gegenseitig post-translationale stabilisieren. Mit Hilfe einer Kinase inaktiven Mutante von Nlk1, konnte ich weiterhin zeigen, dass diese Funktion unabhängig von der Kinaseaktivität von Nlk1 ist. Dennoch lassen Mutationen von mutmaßlichen Nlk1 Phosphorylierungsstellen in PAPC vermuten, dass Phosphorylierung dieser Stellen für die Nlk1-vermittelte Stabilisation von PAPC notwendig ist.

Zudem konnte ich darlegen, dass Nlk1 nicht nur durch seine bereits bekannte Funktion im Wnt/β-Catenin Signalweg, sondern auch wegen seines Einflusses auf den Wnt/PCP Signalweg während der Gastrulation für die *Xenopus* Frühentwicklung benötigt wird. Dieser Effekt erfolgt vermutlich hauptsächlich durch die Interaktion von Nlk1 mit PAPC. Interessanterweise ist die Kinaseaktivität von Nlk1, die für die Regulation von Wnt/β-Catenin essentiell ist, für seine Funktion als Wnt/PCP- und Gastrulations-Regulator entbehrlich.

Basierend auf meinen Ergebnissen komme ich zu dem Schluss, dass Nlk1 ein vielseitiger Modulator sowohl von Wnt/β-Catenin als auch von Wnt/PCP Signalwegen ist. Außerdem beeinflusst Nlk1 Zellbewegungen während der Gastrulation durch die Stabilisierung der Wnt/PCP Komponente PAPC.

3 Introduction

3. 1 Gastrulation

Embryogenesis is one of the nature's most suave events, wherein the union of two germ cells initiates the development of a complete new organism. The process involves multiple steps including cell divisions, cell differentiation and cell fate determination, all of which are controlled by different signaling events within and between cells. As a result of highly coordinated cellular events, cells organize themselves into tissues and tissues into an organism.

Gastrulation is the central step during embryogenesis, generating the first primordial tissues. Before onset of gastrulation, the embryo is a mass of unorganized cells. During gastrulation there are changes at both cellular and molecular levels giving rise to the three germ layers: endoderm, mesoderm and ectoderm[1]. These germ layers are the foundation for future organogenesis and hence the development of a complex organism. Beside the establishment of three germ layers, gastrulation also demarcates the body axes and the rudimentary body plan [2].

The key requirements for gastrulation in any species are coordinated cell movements, proper localization of cells and proper cellular interaction. Any deviation of these processes lead to mis-regulation of gastrulation and hence defective development. So, a proper understanding of how these events unfold during gastrulation is crucial. To comprehensively understand the gastrulation process, different methods and animal models have been used by the scientific community. The African clawed frog (*Xenopus laevis*) is an animal model that was and is successfully used to elucidate the cellular signals and movements occurring during gastrulation, because the embryos are easy to obtain, manipulate and monitor. Therefore the following sections will give a detailed description of *Xenopus* gastrulation.

3. 1. 1 *Xenopus* gastrulation movements

Early *Xenopus* development is a very fast process. About 90 minutes after fertilization of the oocyte, the zygote divides for the first time, initiating a serious of rapid cell divisions. Since in this phase the divisions of the blastomeres don't involve a cell growth phase, this period is referred to as cleavage phase. Soon the embryo assumes a solid ball of cells called morula (Fig. 1A, stage 6) which, during the ongoing cell cleavages, develops a liquid filled cavity, the blastocoel. At this stage the embryo is called blastula (Fig. 1A, stage 8-9 and B, left panel). Till blastula, the embryo is just an unorganized mass of cells. At the onset of gastrulation a series of morphogenetic and coordinated cellular movements start, that shape the unorganized mass of cells into three germ layers (Fig. 1B) [2].

These cell movements are evolutionary conserved and are categorized as follows.

1. **Epiboly**: Epiboly is an isotropic expansion of cells that result in the thinning of the cell layer[3]. At the end of blastula stage, the cells in the animal pole of the *Xenopus* embryo start spreading in all direction. The cells from the upper layer mix with cells of the lower layers and vice versa resulting in ultimate thinning of the expanse of cells. The typical cell behavior during *Xenopus* epiboly is radial intercalation of the cells (Fig. 2B).

2. **Emboly** (Internalization): During gastrulation the mesodermal and endodermal precursor cells move inside the embryo. This movement in *Xenopus* takes place via the blastopore. Internalization starts when a group of cell near the blastopore lip elongates to form bottle cells. Both mesodermal and endodermal precursor cells then invaginate into the interior of the embryo. Later they move as a coherent layer along the blastopore lip, by a process called involution (Fig. 2D) [1].

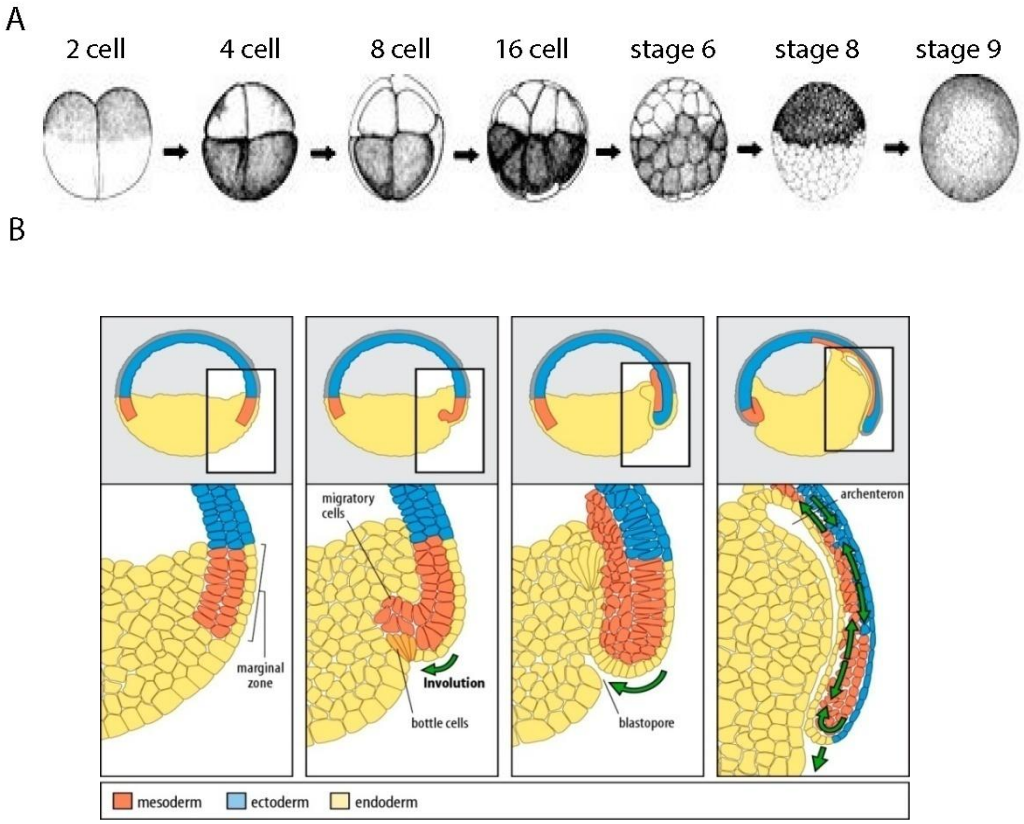

Figure 1. Schematic drawings of early *Xenopus* developmental stages. **A:** After fertilization the egg starts cleaving producing a 2-cell, then 4-cell, 8-cell and 16-cell embryo. The arising solid ball of cells from stage 6 on is called morula and develops during further cleavage to a hollow ball called blastula (stage 8-9).(Adapted from Xenbase). **B:** Shown are schematics of hemisectioned embryos and the magnified insets with the indicated color coding of the three germ layers. Dorsal is right. Gastrulation commence by the formation of bottle cells on the dorsal side of the embryo followed by the involution of mesodermal cell towards the blastocoel roof (BCR) forming archenteron (Wolpert, 2006).

3. Convergent extension movement (CE movements): As the name indicates, in this process cells collectively come together and elongate along an axis (Fig. 2). It is a highly conserved cellular movement in different species like birds, mice, ascidians, fish [4–6]. During *Xenopus* gastrulation the CE movement leads to anterior posterior elongation as well as mediolateral narrowing of the embryo. Typical cell behavior during CE movement is collective cell migration and cell intercalation (Fig. 2F) perpendicular to

the elongating axis [7]. Head mesoendodermal cell show collective migration during gastrulation, they originate from dorsal endoderm of the pre-gastrula embryo and are followed by pre-chordal mesodermal cell. These cells act as a collective sheet. Initially they are randomly polarized but undergo a bipolarization along animal vegetal axes during the CE movement. Similarly, the mesodermal progenitor cells undergo convergent movement and later chordamesodermal cell show intercalation and move towards the interior of embryo the [3]. Collectively these movements change the shape of the whole embryo and visualize the embryonic body axes.

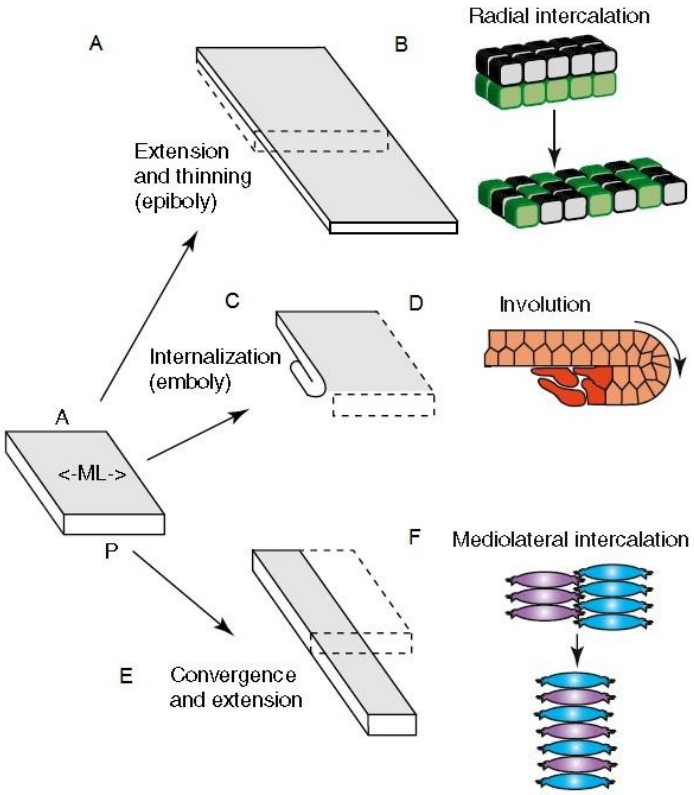

Figure 2. Schematic representation of cell behavior during gastrulation movements. A/B: show the behavior of cells during epiboly: a sheet of cells gets thinner by the radial intercalation of cells. **C/D:** Show the cell behavior during emboly, wherein the cell layer (mesoendoderm) is internalized and involutes beneath the ectodermal cell layer, creating two separate layers from one sheet. **E/F:** show the cell behavior during CE (convergent extension) movements. Here, a mass of cells collectively polarize along a given axis and converge together by mediolateral intercalation, leading to the elongation of the cell mass along the developmental axes.(Image modified from, LilianaSolnica-Krezel 2005).

Interestingly CE movements are also exhibited by excised *Xenopus* dorsal mesoderm tissue or by animal cap (ectoderm) tissue induced to become mesoderm by TGF-β treatment [8]. Using these explants the regulation of gastrulation and CE movements have been extensively studied in *Xenopus* and Zebrafish.

3. 1. 2 Tissue separation during *Xenopus* gastrulation

During gastrulation layers of cells move on or above other layers of cells without mixing. This behavior is called tissue separation and is required to form boundaries between different tissues. A failure of boundary formation leads to cell mixing and uncontrolled cross signaling between different tissues, a behavior often occurring in cancer.

During *Xenopus* gastrulation, border formation can be seen when the cells of mesodermal origin move towards the blastocoel roof (BCR) of the embryo as a separate cell layer. Cells from mesoderm and ectoderm don´t mix and remain separated by a cleft called Brachet´s cleft. Brachet's cleft is characterized by an anterior and posterior margin formed by the vegetal rotation of endoderm and mesendodermal cell movement around the blastopore (Fig. 3) [9,10]. Brachet's cleft formation is frequently used as a readout to analyze proper tissue separation during gastrulation [11].

The comprehensive molecular mechanism explaining how tissue separation is established and maintained during gastrulation is not fully understood yet. For example, it is known that a fibronectin fibril network which is present at the BCR is not dense enough to cause ectodermal and mesodermal separation alone. Furthermore, different cadherins, well known cohesive molecules, are expressed in BCR and mesoderm, and are implicated in the separation behavior [9,12,13].

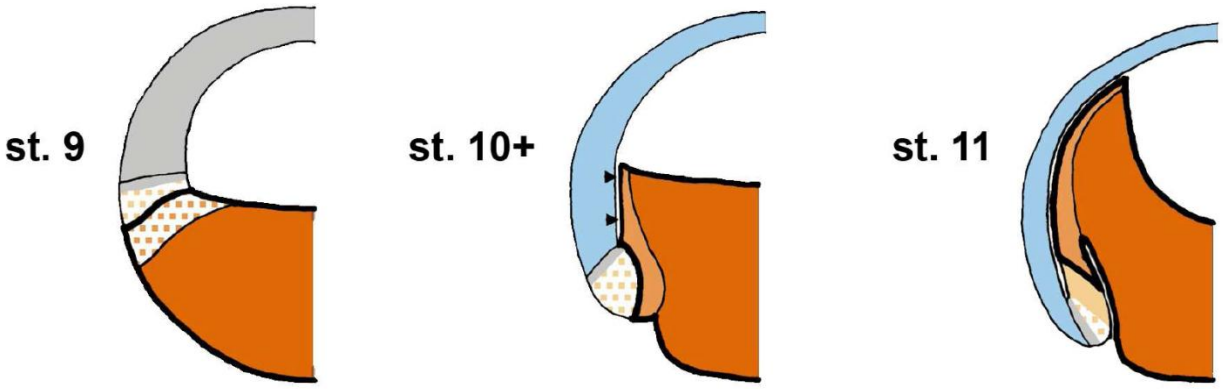

Figure 3.Tissue separation during *Xenopus* gastrulation. Dorsal side of developing embryo from stage 9,10+ and 11 are shown as cross sections. At stage 9 (left), tissue separation starts at the vegetal region (orange), and at stage 10+ (middle) spreads to anterior and posterior mesoderm (lighter orange), which does not mix with the ectoderm due to its repulsive behavior (blue). Arrowhead shows the anterior and posterior margins of Brachet's cleft. This feature gets more pronounced when gastrulation proceeds (right, stage 11). Color scheme: grey- cells showing no separation behavior, blue- cell repulsion behavior ,dotted orange- prospective region for separation, light orange-later separation, orange- early separation behavior, black arrow heads indicate the Brachet's cleft (adapted from Wacker et al. , 2000).

3. 2 Cadherins

Cadherins represent a superfamily of more than a 100 conserved transmembrane proteins. Cadherins are glycoproteins by nature and show calcium dependent cell adhesion. They have a characteristic extracellular cadherin (EC) domain, whose number may varies in different cadherin families [14]. Cadherin proteins are not only involved in cell adhesion but also mediate cell signaling. Different members of the family are reported to regulate cell polarity, cell sorting and coordinated cell movement during development [14]. Cadherins can be broadly classified into four categories, each of them differing from the others by either the EC domain number or conserved sequence motifs (Fig. 4) [15].

3. 2. 1 Protocadherin and Paraxial Protocadherins (PAPC)

Protocadherins represent the largest subfamily of the cadherin superfamily, with about 70 members already reported. Classical cadherins are considered to be evolved from protocadherins. The members of this family are similar to classical cadherins with 5 or more EC repeats, which have conserved cysteine (C) instead or tryptophan (W) motifs (Fig. 4) and they lack any β-catenin binding site on their cytoplasmic tail [15]. Protocadherins show weaker homophilic adhesion behavior than classical cadherins and the diversity in their cytoplasmic tails point towards roles other than adhesion.

Direct orthologs to vertebrate protocadherins are missing from invertebrates such as *Drosophila* or *C. elegans*. A variety of protocadherins have been reported to be involved in embryogenesis and development of *Xenopus*, such as Paraxial Protocadherin (PAPC), Axial Protocadherin (AXPC), Neural Fold protocadherin (NFPC) and Protocadherin in Neural crest and somites (PCNS)[16,17].

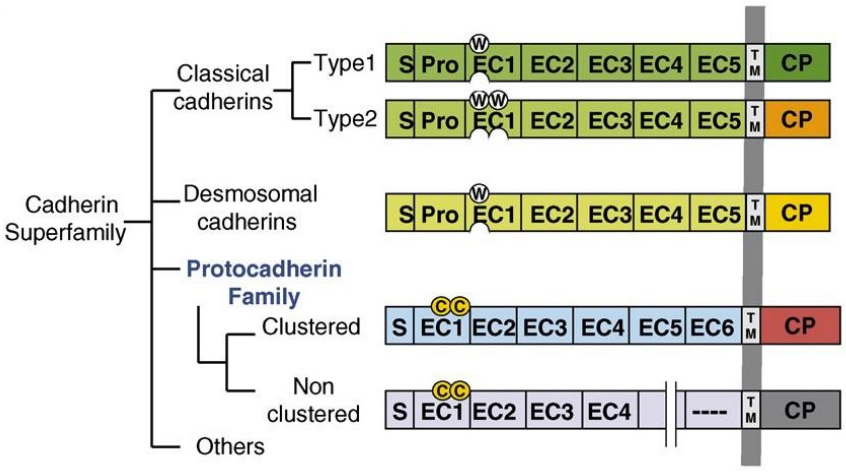

Figure 4. Cadherin superfamily categories. Classical Cadherins are characterized by conserved Tryptophans (W) in the EC1 domain, one or two hydrophobic pocket and a cytoplasmic tail which can bind Catenin. Desmosomal cadherins are similar to classical cadherin despite their different cytoplasmic tail. The Protocadherin family has 5-6 EC domains, no hydrophobic pockets and conserved Cysteine (C) instead of Tryptophans in EC1 when compared to classical Cadherins.(Modified from Morishita& Yagi, 2007).

Among these protocadherins, PAPC was initially identified as a gene specifically expressed in dorsal blastopore lip during gastrulation and was consequently implicated in Brachet's cleft formation [11,18]. During *Xenopus* embryogenesis expression of PAPC starts after MBT (mid blastula transition) in anterior mesoderm, in dorsal blastopore lip and its expression expand laterally in the marginal zone (Fig. 5A-B). During neurulation the expression of PAPC is confined to paraxial mesoderm while it's missing from the notochord (Fig. 5C-D). Later in development PAPC shows a presomitic expression (Fig. 5E-F).

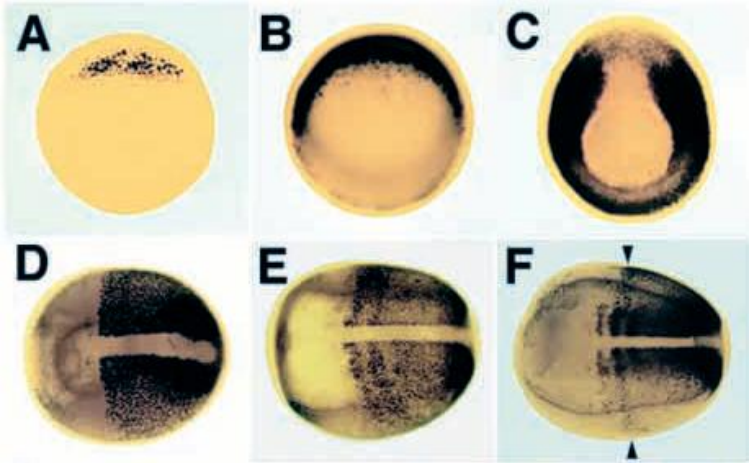

Figure 5. Expression of PAPC during *Xenopus* embryogenesis. A: PAPC expression is restricted to dorsal blastopore lip (Stage 9. 5).**B:** Expression expands 180° laterally(Stage 10. 5).**C:** Expression expands as dorsal mesoderm involutes (Stage 11. 5).**D:**PAPC expression demarcate head and trunk mesoderm (Stage 13).**E:**PAPC expression can be seen during neurula (stage14). **F:** Expression of PAPC in lateral plate mesoderm (Stage 17).(Modified from Kim et al. , 1998)

The expression of PAPC is regulated by different pathways like Nodal-related signaling, β-catenin signaling and Wnt5a/Ror2 signaling [19]. Proper PAPC membrane localization also requires ubiquitination [20]. Moreover, PAPC itself is known to regulate cell polarization, tissue separation, and gastrulation by interacting with different Wnt pathways components. The correlation of PAPC and Wnt signaling will be described in

detail (Sec. 3.4), after a thorough description of the distinct Wnt pathways and their role during gastrulation.

3. 3 Wnt signaling pathways during gastrulation

Gastrulation is a delicate process requiring a tight balance of different factors and regulators. Different signaling pathways are known to be a part of regulatory network regulating gastrulation movements or tissue separation during development. Wnt signaling has been attributed in majority of observations as a key component of gastrulation regulation in *Xenopus* [21–23].

Wnt signaling pathways depend on the presence of a lipid modified ligand molecule called, Wnt.Int1 (later termed Wnt1) was initially identified as proto-oncogene [24], encoding a protein characterize by a CRD (cysteine rich domain). Later Wingless (Wg), the *Drosophila* Wnt homolog was also discovered [25]. Subsequently, Wnt and its homolog have been identified in a myriad of cellular events ranging from early development, stem cell maintenance, differentiation, cancer etc. [26,27]. Typically Wnt pathways involve binding of a Wnt ligand to its cognate receptor and activating the downstream molecular components. There are multiple Wnt and many Frizzled receptors present in different species. Depending on which Wnt ligand and receptors are involved and more importantly the involvement of β-catenin, Wnt pathways can be broadly categorized into two type i.e. canonical β-catenin dependent Wnt pathway or non-canonical β-catenin independent Wnt pathway. Canonical β-catenin dependent Wnt pathway is the most studied among the two pathways.

3. 3. 1 β-catenin dependent Wnt pathway

Canonical β-catenin dependent Wnt pathway is activated by the binding of canonical Wnt ligands such as Wnt3ato a Frizzled (Fz) receptor in presence of co-receptors like LRP5/6 [28,29].In the absence of a Wnt ligand, β-catenin in the cytosol is bound by a multimeric protein complex, which is called destruction complex, since it leads to the destruction of β-catenin (Fig. 6A). The kinases CK-1 and GSK-3β are components of

this destruction complex that leads to β-catenin phosphorylation [30,31].This phosphorylation of β-catenin primes it for β-Trcp mediated ubiquitination and degradation[32]. Upon binding of a Wnt ligand to the Fz receptor, a cascade of events takes place leading to the recruitment of the destruction complex to the membrane where disheveled (DVL) act as a scaffolding protein causing the disassembly of the destruction complex in the cytoplasm and hence to a stabilization of β-catenin (Fig. 6B). Stabilized β-catenin can translocate into the nucleus [33]. Inside the nucleus it act as a transcriptional co-activators of transcription factors like TCF/LEF proteins (Fig. 6) [34,35]. In context of *Xenopus* development the canonical Wnt pathway plays a central role in cell fate determination and axis specification. During early development cortical rotation moves components of the Wnt pathway to the dorsal side. Studies have revealed that the cortical rotation leads to nuclear localization of β-catenin on the dorsal side of the embryo which results in the establishment of the dorsal Spemann-Mangold organizer and specify the future body axis [36,37]. Supporting the same notion, if β-catenin is ectopically expressed in the ventral side of the embryo, it leads to the formation of an additional head on the ventral side of the embryo. Beside establishing the dorsal organizer, canonical Wnt pathway is also involved in neural patterning and formation of anterior head structures [38]. However, the role of the canonical Wnt pathway in regulating gastrulation movements is poorly investigated so far.

3. 3. 2 β-catenin independent Wnt pathways

Binding of Wnt ligand doesn't always leads to a stabilization of β-catenin. Certain Wnt ligands induce different sets of intracellular signaling cascades and hence these pathways are categorized as non-canonical β-catenin independent Wnt pathways [39,40].Interestingly these pathways can regulate both transcriptional and non-transcriptional events in cell. Non canonical Wnt pathways regulate crucial cellular events like cytoskeleton rearrangement, cell polarity and cell migration, and are frequently involved in cancer metastasis [41–43]. In contrast to β-catenin dependent Wnt signaling there are several distinct β-catenin independent Wnt pathways which are organized into three major categories described below.

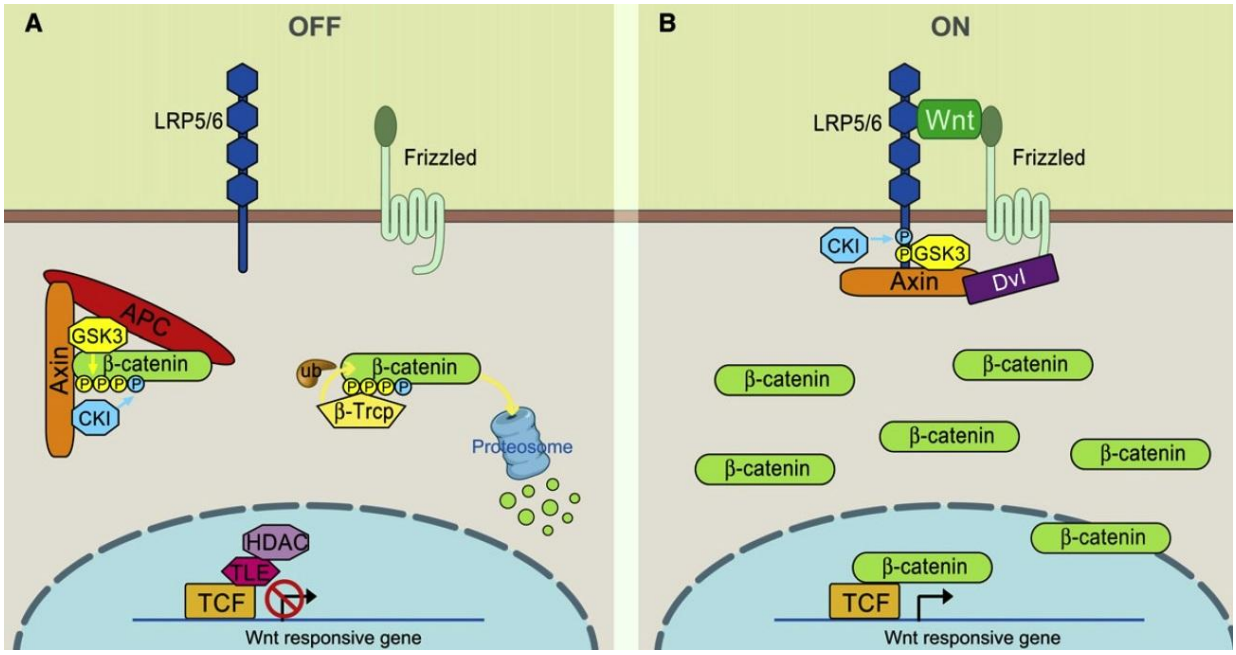

Figure 6. Overview of the canonical Wnt/β-catenin pathway. A: In the absence of a Wnt ligand β-catenin is a part of destruction complex. It is phosphorylated and destined for proteasomal degradation. **B:** Wnt ligand binding induce membrane event and disassembly of the destruction complex and hence leads to an accumulation of β-catenin. β-catenin translocates into nucleus and activates Wnt target genes.(adapted from MacDonald et. al.2009)

3. 3. 2. 1 Wnt/PCP signaling

Directed cell migration during morphogenetic events such as gastrulation requires the polarization of the cells. The Wnt/planar cell polarity (PCP) pathway is known to regulate cell polarity during tissue development. It was initially identified in *Drosophila* genetic screen where mutations in certain genes lead to a loss of cell polarity and randomize the orientation of epithelial hair cells. Similar organization for vertebrates is seen in stereocilia of inner ear epithelia, and dorsal mesoderm during gastrulation[44]. Also during *Xenopus* development Wnt/PCP is a central pathway regulating gastrulation movements such as CE movements and tissue separation [45–47]. Any alteration of Wnt/PCP signaling during gastrulation leads to severe developmental defect like axis shortening and spina bifida [48,49].

Although the PCP pathway was initially found in *Drosophila*, there is so far no Wnt ligand known to be responsible to activate this pathway in *Drosophila*. However, invertebrates Wnt4, Wnt5a and Wnt11 are well characterized Wnt/PCP ligand. Wnt/PCP signaling doesn't require LRP5/6 as a co-receptor to activate the pathway. Instead different co-receptors like Ror1/2, Ryk, PTK7 and PAPC are known to be involved, depending on the tissue and cellular context[50]. Once bound to the receptor the ligand activated small cytosolic GTPase like RAC1 and RHOA, which in turn further activates RHO kinase (ROCK) and c-Jun N-terminal Kinase (JNK) (Fig. 7, left). These cascades of events ultimately lead to activation of transcription factor like c-Jun, ATF2 or AP2 which contribute to change cell polarity or cytoskeleton rearrangement.

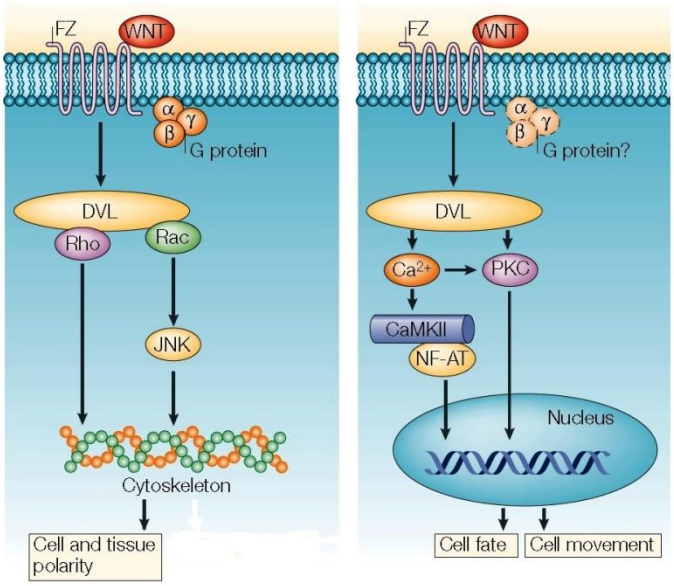

Figure 7. Schematic representation of Wnt/PCP pathway (left) and Wnt-Ca2+ pathway (right). DVL is involved in both the pathways. Detailed description is present inthe main text (adapted from Ciani & Salinas, 2005).

3. 3. 2. 2 Wnt/Ca^{2+} signaling

Binding of Wnt ligands to Fz receptors can also activate another branch of Wnt signaling, called the Wnt-Ca^{2+} pathway (Fig. 7, right) (Kohn & Moon, 2005).This pathway shares some components with the Wnt/PCP pathway, like the ligands and receptors but the key difference is that here pathway activation leads to an increase in

cytosolic Ca^{2+} level. Initial binding of receptor ligand activates PLC (phospho lipase C), a membrane bound enzyme that produces IP3 (inositol 1,4,5 triphosphate) and DAG (diacyl glycerol). IP3 in turn induces the release of $Ca^{2+}$from ER. Increase in Ca^{2+}level leads to an activation of calcium sensitive proteins like protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII). These two proteins can then interact and activate a variety of proteins like cAMP response element binding protein (CREB). Increased Ca^{2+}can also activate calcinurin (Cn), that in turn can activate nuclear factor associated with T-cells (NFAT). Activated NFAT stimulates downstream proteins that are involved cell motility and adhesion [51]. CaMKII activates the TAK-NLK cascade, which in turn inhibits the canonical Wnt pathway [53]. During *Xenopus* development Wnt11 and Wnt5a have been reported to activate PKC and CaMKII activity and in turn regulate cell sorting behavior.

3. 3. 2. 3. Wnt5a/Ror signaling

Receptor tyrosine kinase-like orphan receptors (Ror1/2) are evolutionary conserved single pass tyrosine kinase receptors. These proteins, like Wnts and Fz receptors also contain a Cysteine rich domain (CRD). Ror1/2are known to interact with Wnt5a and transduce Wnt/PCP signaling [54,55]. However, in recent years there is growing evidence that Ror1/2 can also transduce its signal though a cascade independent of RHOA and RAC1. Instead it can signal through CDC42 and MKK7 to activate JNK signaling (Fig. 8). Notably, this signaling pathway is known to activate *papc* expression during *Xenopus* development [19,55]. As mentioned earlier, PAPC itself is a key protein regulating other branches of Wnt pathways.

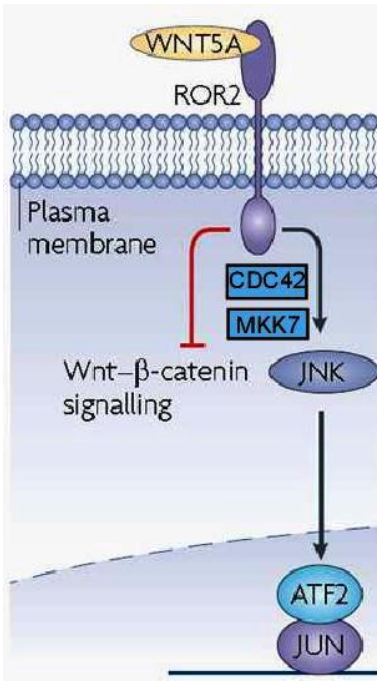

Figure 8. Simplified diagrammatic representation of Wnt5a/Ror2 signaling pathway. Binding of Wnt5a activates JNK which in turn activate JUN kinase and ultimately PAPC is induced (modified from Angers et. al 2009).

3. 4 PAPC and Wnt signaling during *Xenopus* development

PAPC was initially identified in a screen to search for gene exclusively expressed in dorsal blastopore lip. Since this area is also a Wnt signaling hub during gastrulation, PAPC emerged as an obvious candidate to be involved in Wnt signaling.

PAPC is a well-studied molecule during *Xenopus* development. The expression of PAPC is regulated by different pathways like Nodal-related signaling, β-catenin signaling and Wnt5a/Ror2 signaling [19]. Itself, PAPC is known to regulate cell sorting by regulating C-cadherin mediated cell adhesion [45]. PAPC interaction with Fz7 ectodomain can regulate the tissue separation [46,47]. Also the cell polarity and CE movement are reported to be regulated by PAPC, as PAPC can activate JNK and Rho which further governs the CE movement [19,47].

Interestingly, cell sorting, tissue separation and adhesion behavior of PAPC is primarily mediated by the membrane domain of PAPC, while the cytoplasmic domain of PAPC is

involved in intracellular signaling and reported to be interacting with multiple proteins. It interacts with Spry2(Sprouty2), which is a negative regulator of Wnt/PCP signaling, and hence positively modulate the Wnt/PCP pathway [56]. It also interact with a positive regulator of Wnt/PCP signaling, ANR5 (Ankyrin repeat domain protein 5), and modulates its activity [57]. So, PAPC possibly maintains a fine balance of Wnt pathways for a proper gastrulation to take place. In line with this, CK2β (Casein Kinase 2β) has been recently reported to interact at the same domain of PAPC as Spry2 [58]. CK2β is the regulatory subunit of the CK2 holoenzyme (comprising CK2β and CK2α) which is known to be a positive regulator of canonical Wnt β-catenin signaling [59]. Therefore, binding of CK2β to PAPC, negatively regulates the canonical Wnt pathway.

PAPC also plays an important role during development in other species. In *Zebrafish*, PAPC also regulate CE movement similar to *Xenopus* [60]. A few studies have shown that PAPC regulates gastrulation movements in mice too. Lim1, a transcription factor needed for proper axis formation and anterior structure in both *Xenopus* and mice, regulates the expression of PAPC [61]. In a nutshell, PAPC is an evolutionary conserved membrane protein that can regulate morphogenesis via its extracellular (adhesive) and intracellular (signaling) interactions with effectors of different pathways. However the molecular mechanism of PAPC's function, especially in intracellular signaling and interaction network is still obscurely established and will be examined in more detail in this work.

3. 4. 1 Yeast two hybrid screen identified Nlk1 as a potential interaction partner of PAPC

To unravel the molecular function of PAPC in intracellular signaling events, our lab performed a Yeast two hybrid (Y2H) screening using the intracellular domain of PAPC (PAPCc) as bait and *Xenopus* cDNA clones library as a prey. In this screen we identified many interesting protein as potential interaction partners of PAPCc. Two interesting candidates, Spry2 and CK2β have already been investigated and reported in detail [56,58]. However, another highly interesting candidate that came up in the study was Nemo like kinase 1 (Fig. 9) (Yingun Wang, unpublished data).

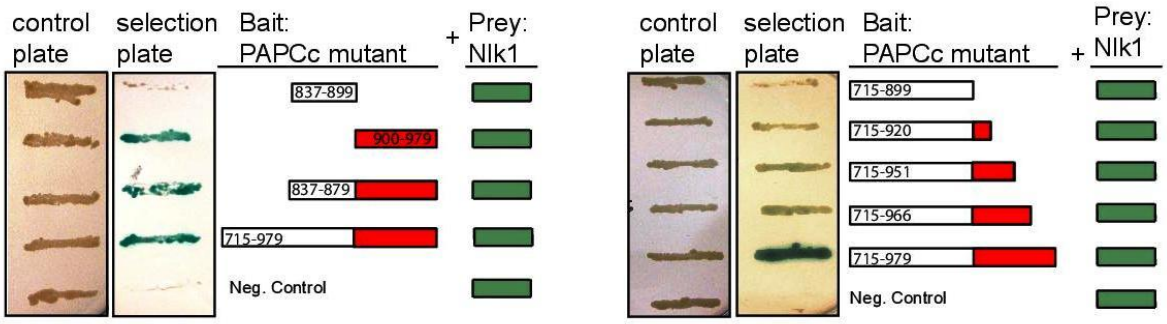

Figure 9. Panel showing Yeast two- hybrid screen. cDNAs were cloned into bait vector pNLX3 or prey vector pACT2 and co-transfected into yeast strain L40.One representative colony from each co-transformation was picked and streaked onto a Leu−Trp− plate (left panel, brown) or Leu− Trp− His− X-gal+ plate(right panel, blue) and incubated for3days at 30°C.Blue colonies are seen when bait and prey proteins interact[56]. For the present study, Nlk1 was used as prey and different deletion constructs of PAPC as bait (bars next to the panels shows the respective amino acids of PAPC used, while red color in bars shows the required 80 amino acid stretch).

3. 5 Nemo Like Kinase (Nlk) and Wnt Signaling

Nemo like kinas (Nlk) is an evolutionary conserved Ser/Thr kinase of the MAPK family. The name nemo is derived from a *Drosophila* mutant, where a mutation in a gene leads to square shaped (korean-nemo) ommatidia. During *Drosophila* development Nlk regulates head formation and PCP signaling during eye development [62,63]. Later the vertebrate homologs were identified and characterized [64]. The number of Nlk genes varies in different species. For example in mammals and chick there is only one isoform present, while in frog and fish there exist two different isoforms, Nlk1 and Nlk2[65]. The latter is a larger than Nlk1 and harbors a Histidine rich stretch at its N-terminus. In *Xenopus* both the isoforms are present; however it's still not clear if both of them act redundantly or independently.

During *Xenopus* development Nlk is reported to regulate anterior formation and functioning downstream of P-38 MAPK [66,67]. In mice, Nlk knockout causes a pleiotropic

phenotype depending on the genetic background of the mice [68].For some strains a homozygous knockout is embryonic lethal, while in other strains it merely causes growth retardation and defects in mesenchymal stem cell development. However, there was no obvious phenotype indicating typical Wnt signaling or gastrulation defects.

The molecular function of Nlk was first revealed in *C. elegans*. The Nlk homolog in *C. elegans*, lit-1, was shown to inhibit POP1, which is a TCF homolog. Hence, this was the first report that Nlk is involved in regulating Wnt signaling pathways [69,70]. The conservation of this pathway was subsequently confirmed in higher organism. In luciferase reporter assays, as well as in *Xenopus* double axis experiments, Nlk1 was shown to act as an inhibitor of canonical Wnt signaling via the phosphorylation of TCF/LEF transcription factors (Fig. 10A) [71]. Furthermore, Nlk can associate with certain E3-ubiquitin ligases, for example NARF (Nlk associated ring finger protein) and induces the protealytic degradation of TCF/LEF and hence negatively regulates the canonical Wnt pathway[72]. Nlk was also shown to act downstream of TAK-TAB (TGFβ-activated kinase, TAK binding protein) and induce a LEF dependent negative regulation on Wnt/β-catenin pathway [73].

However, Nlk appears to be an enigmatic molecule regulating LEF/TCF, and thus Wnt/β-catenin, differently in different contexts (Fig. 10). For example, in neural progenitor cells in zebrafish midbrain development Nlk can positively regulate Wnt signaling by phosphorylating LEF1 [74]. Here, phosphorylation by Nlk1 reduces HDAC (Histone Deacetylase, global transcriptional repressor) association with LEF1 and thus activates target gene transcription (Fig. 10B). Taken together; Nlk has a dual function in the regulation of the canonical Wnt pathway.

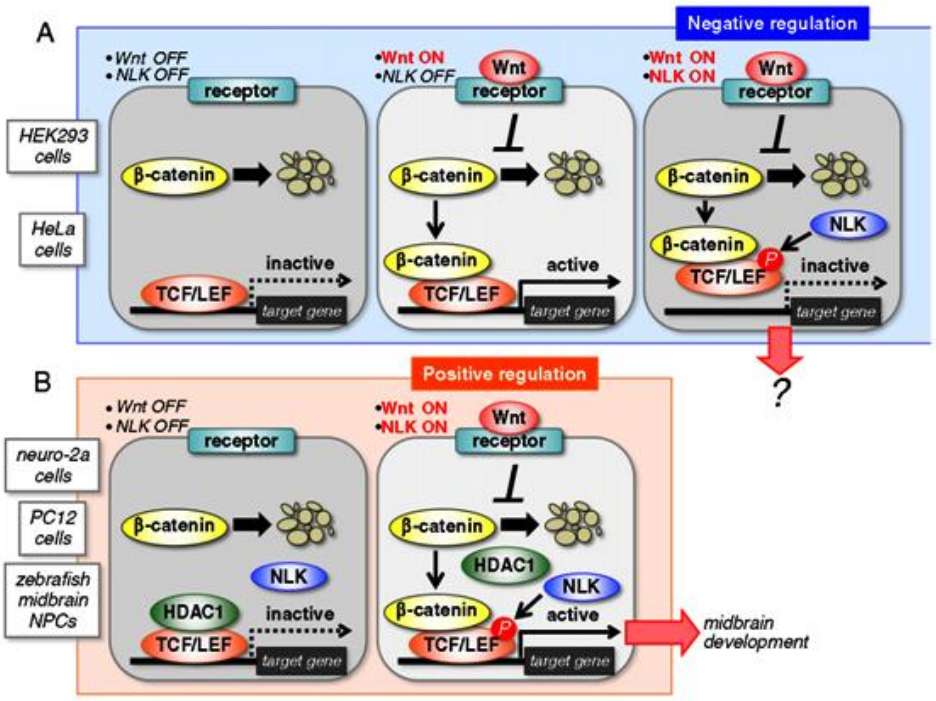

Figure 10. Schematic of the dual function of Nlk1 during β-catenin dependent Wnt signaling. A: In Hek293 or HeLa, Nlk1 causes the phosphorylation of TCF/LEF and negatively regulate Wnt/β-catenin pathway. **B:** In *Zebrafish*, PC12, neuro-2a cells, Nlk functions as a positive regulator by modulating the HDAC-TCF/LEF interaction.(Adapted from Ishitani et. al 2012)

The role of Nlk in regulating non-canonical Wnt pathways during *Xenopus* development, however, is studied much less thoroughly. A few findings suggest the involvement of Nlk in Wnt5a/Ca^{2+} signaling, Wnt5a/Ca^{2+} signaling activates Nlk mediated LEF phosphorylation and consequently down-regulates the Wnt/β-catenin pathway[53]. Furthermore, in *Drosophila* recent research suggest that Nlk is a component of the PCP pathway. It was shown that Nlk can phosphorylate β-catenin and connects E-cadherin to core PCP components [62]. So far, a function of Nlk in β-catenin independent pathways has not been investigated in *Xenopus*, where these pathways are excellently characterized.

Understanding the broad spectrum of Nlk function in cellular signaling events is also important for human disease. A number of studies in the past decade have revealed the association of Nlk with different types of cancer. In most types of cancer Nlk acts as a

tumor suppressor. For example, in colon carcinoma cells Nlk can boost apoptosis by phosphorylating and hence regulating c-Myb and its transcriptional activity [75]. Apoptosis induced by the cancer drug Taxol is increased by Nlk in laryngeal cancer [76]. Negative regulation of canonical Wnt signaling by Nlk is also associated with inhibition of non-small cell lung cancer (NSCLC) progression [77].

At the same time, Nlk1 promotes cancer progression in certain cases. For example, in the case of hepatocellular carcinoma, simultaneous inhibition of Nlk1 along with CDK2 and the Wnt target gene Cyclin D1 inhibits tumor cell growth [78]. Furthermore, it was shown that while the induction of cancer is repressed by Nlk, development of cancer metastasis is stimulated by this kinase (Jansson, Arbman, Zhang, & Sun, 2003; Tan et al. , 2012). Cancer metastases are majorly attributed to inappropriate Wnt/PCP pathway activation leading to increased cell motility [41]. An involvement of Nlk in cancer metastasis supports a role for Nlk in Wnt/PCP signaling. Therefore, enlightening the role of Nlk1 in Wnt/PCP pathway regulation would help to understand cancer and especially metastasis development. The observation, that Nlk1 might be a novel interaction partner of the crucial PCP pathway component PAPC, could help to understand the complexity of embryonic, as well as cancer development.

Aim of the Thesis

PAPC protein is known to regulate Wnt pathways and gastrulation during *Xenopus* development. The primary focus of this thesis is gaining insight into the molecular mechanism of PAPC functioning and regulation. We identified Nlk1 as a potential interaction partner of PAPC. As PAPC is a primary player in the regulation of Wnt/PCP pathway, the focus of major part of the study is Wnt/PCP regulation. I have examined the following questions in this thesis:

1. Confirming the interaction of Nlk1 with PAPC

2. Role of Nlk1 during *Xenopus* gastrulation movement

3. Molecular function and impact of Nlk1 and PAPC interaction during gastrulation

4 Results

4. 1 PAPC and Nlk1 interact

4. 1. 1 Nlk1 binds to PAPC

In our lab we are interested in understanding the molecular mechanism of Wnt signaling involvement during gastrulation. As mentioned in the introduction, PAPC is an important protein during gastrulation and in regulating Wnt signaling (Sec. 3.4). In an Y2H screen searching for novel interaction partners of PAPC, we identified Nlk1 as a promising candidate (Fig. 9).

To confirm the interaction of Nlk1 and PAPC I performed co-immunoprecipitation (Co-IP) experiments of HEK293 cell lysate overexpressing different Myc-tagged PAPC constructs (Fig. 11A) and Flag-tagged Nlk1. As expected, immunoprecipitated full length PAPC-Myc was able to pull down Flag-Nlk1 (Fig. 11B left lane). Also the cytoplasmic C-terminal domain of PAPC (PAPCc) co-precipitated Nlk1 (Fig. 11B right lane). When the cytoplasmic domain was missing, however, Nlk1 could not be detected in the Co-IP (Fig. 11B middle lane), indicating that Nlk1 binds to the C-terminus of PAPC (Fig. 11A).

The results of the Y2H data suggested that the interaction of PAPC with Nlk1 is mediated by the most C-terminal 80 amino acid of PAPC. To confirm the requirement of this domain for the Nlk1-PAPC interaction I again performed a Co-IP using a Flag tagged deletion construct of PAPCc (only the cytoplasmic domain) that lacks last 80 amino acids (PAPCcΔ899-Flag), which according to the Y2H data should not be able to pull down Nlk1 (Fig. 11C). Indeed, when compared to the positive control PAPCc-Flag, Nlk1-Myc is scarcely co-precipitating with PAPCcΔ899-Flag (Fig. 11D). This experiment confirms that Nlk1 require last 80 amino acid of PAPC for proper interaction.

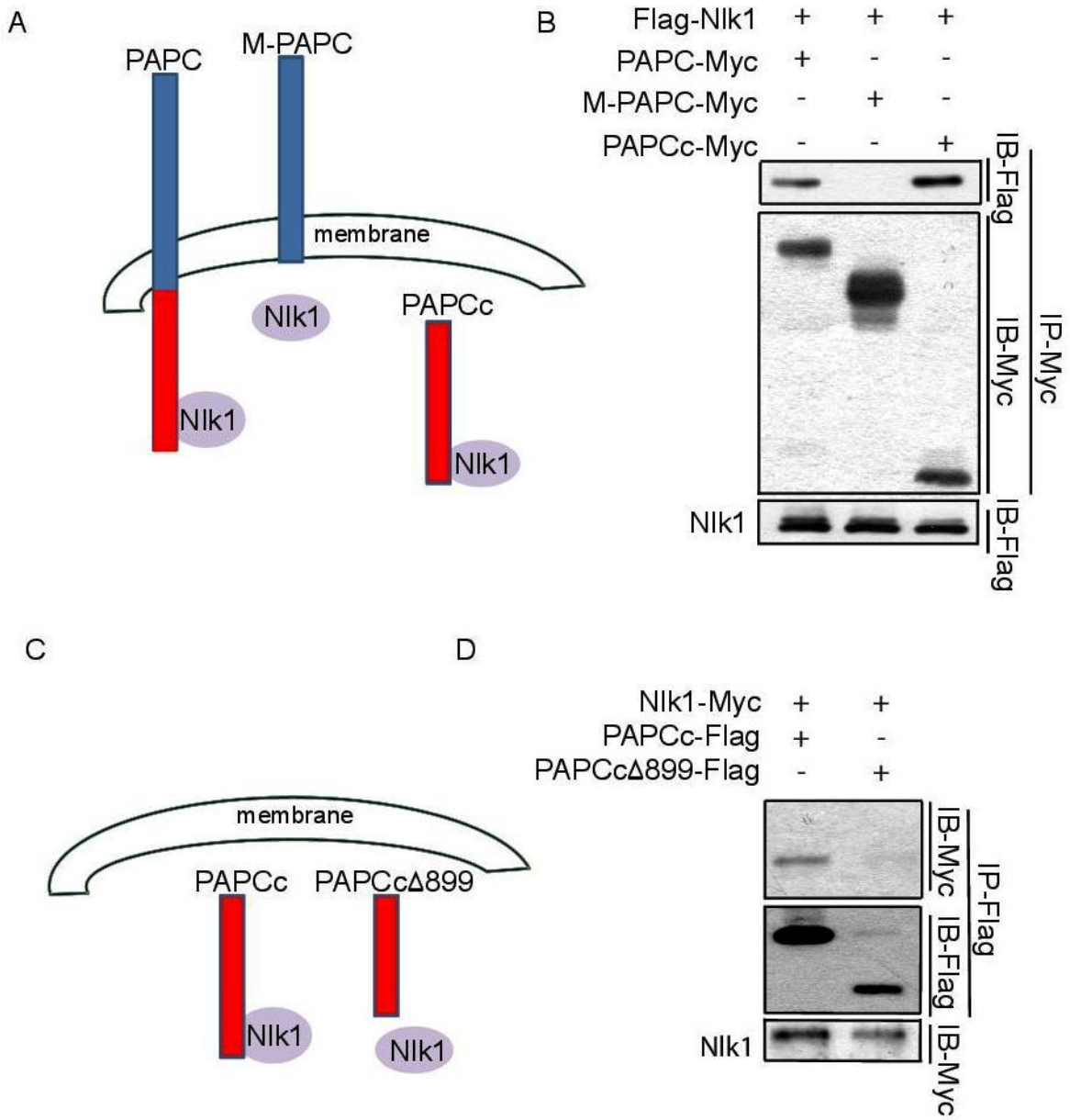

Figure 11. Interaction of PAPC and Nlk1 require the C-terminal domain and last 80 amino acids for interaction. A: Schematic overview of PAPC constructs used and result in the Co-IP experiments in B. **B:** Immunoblot analyses of Co-IP of HEK293 cell lysates overexpressing the indicated Nlk1 and PAPC constructs. The Myc-tagged PAPC constructs were immunoprecipitated (middle) and probed for Nlk1 (top). Input (bottom) shows equal expression of Nlk1 in all the samples. **C:** Schematic overview of PAPC constructs used and result of the Co-IP experiments in D. **D:** Western Blot analyses of Co-IP of HEK293 cell lysates overexpressing the Nlk1-Myc and indicated PAPCc-Flag or PAPCcΔ899-Flag. The Flag-tagged PAPC deletion constructs were immunoprecipitated (middle) and probed for Nlk1 (top). Input (bottom) shows expression of Nlk1 IB: immunoblot detecting the indicated tag, IP: immunoprecipitation.

4. 1. 2 Expression of PAPC and Nlk1 overlaps during *Xenopus* gastrulation

To confirm that the interaction of PAPC and Nlk1 is functionally relevant for *Xenopus* embryonic development the expression of the two genes was compared at gastrula stages. Comparison of *nlk1* and *papc* whole mount *in situ* hybridization (ISH) on hemi-sectioned gastrula (stage 10. 5) embryos revealed that the expression of the two genes overlap in the dorsal marginal zone (DMZ) and the involuted mesoderm (Fig. 12).

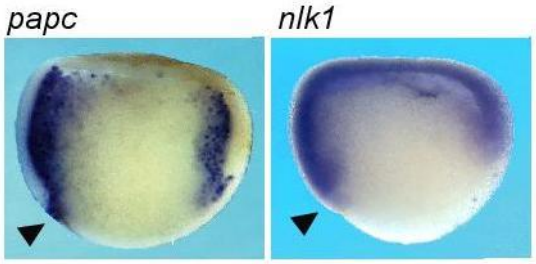

Figure 12. Expression overlap of *nlk1* and *papc* in the dorsal marginal zone (DMZ) and involuting mesoderm. Whole mount *in situ* hybridization using DIG-labelled anti-sense RNA probes (violet) for *nlk1* and *papc* on sagittally sectioned gastrula (stage10. 5) embryos. Both mRNAs are detectable in the DMZ and the involuting mesoderm. Arrowhead shows the position of blastopore.

4. 1. 3 Subcellular co-localization of PAPC and Nlk1

To further consolidate the physiological function of the PAPC-Nlk1 interaction during *Xenopus* embryogenesis, I investigated the subcellular localization of the two proteins. Since PAPC has a transmembrane domain, it is usually localized at the plasma membrane [20], while Nlk1 is known to be located in the nucleus and cytoplasm [79]. Therefore, it would be interesting to find if and where the two proteins co-localize in cells. As an antibody against endogenous *Xenopus* Nlk1 is not available, a tagged construct of Nlk1 was overexpressed in *Xenopus* animal caps (AC) along with membrane bound RFP (membraneRFP) to visualize plasma membrane. As expected, Nlk1 was found in the nucleus and in a dotted manner in the cytoplasm (Fig. 13,Ist column).Some of the Nlk1 dots were also localized close to or at the plasma membrane.

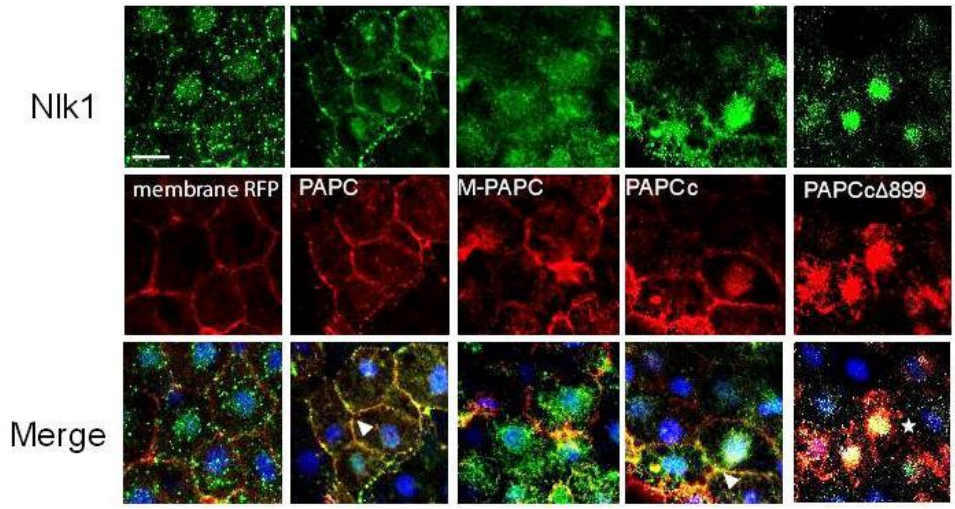

Figure 13. Co-localization of Nlk1 with PAPC depends on the cytoplasmic domain. Immunofluorescence staining of *Xenopus* animal cap (AC) explants injected animally at 4-cell stage with Nlk1-Myc (green) and membraneRFP or with the indicated PAPC-Flag constructs (red), DAPI (blue) was used for nuclear staining. *papc constructs, membranerfp: 300pg/embryo*. Scale bar=50 µm. White arrowhead indicates co-localization sites at the plasma membrane and asterix marks expression of both proteins inside the nucleus.

Interestingly, the subcellular localization of Nlk1, changes from the dotted pattern toward more continuous membrane localization in the presence of PAPC (Fig. 13 second column). This further supports that the two proteins interact in vivo. M-PAPC, which is lacking the cytoplasmic domain where Nlk1 should not bind is still localized at the membrane but not able to recruit Nlk1 to the membrane (Fig. 13 third column). The cytoplasmic domain of PAPC (PAPCc) is known to localize predominantly in the nucleus and partially at the plasma membrane to homophilic interactions (unpublished results Dr. C. Berger). When PAPCc was co-expressed with Nlk1, the two proteins co-localized in the nucleus and at the membrane, while there were less dots of Nlk1 found in the cytoplasm (Fig. 13, fourth column). Since PAPC and PAPCc but not M-PAPC co-localized with Nlk1, I also tested whether PAPCcΔ899 which is missing the putative interaction domain, lacked the ability to affect Nlk1 localization. However, since it too is localized in the nucleus, both Nlk1 and PAPCcΔ899 expression overlapped there.

Taken together, PAPC and PAPCc but not M-PAPC could recruit Nlk1 to the plasma membrane and co-localized with Nlk1, indicating that the cytoplasmic domain is involved in this process

4. 2 Nlk1 regulates gastrulation movements during *Xenopus* gastrulation

In the previous experiments I could show that Nlk1 and PAPC interact with each other via the C-terminal domain of PAPC. This suggests that Nlk1, like PAPC [47,80], might be involved in the regulation of gastrulation and Wnt/PCP signaling. However, the role of Nlk1 during *Xenopus* gastrulation has rarely been explored. Hence I investigated if Nlk1 has a role in regulating *Xenopus* gastrulation via Wnt/PCP signaling similar to PAPC.

4. 2. 1 Nlk1 is present as a maternal component during early development

There are two different isoform of Nlk that are present in *Xenopus*, Nlk1 and Nlk2 (Sec. 3.5). In most of the studies performed in *Xenopus,* a clear distinction between Nlk1 and Nlk2 is missing. In the present study I also primarily focused on Nlk1, since it was initially found to interact with PAPC and have a dominant expression during gastrulation (Fig. 14B).

I started analyzing the expression pattern of Nlk1 during early development. I performed *in situ hybridization* (ISH) on early developmental stages of *Xenopus* embryogenesis. ISH confirmed the presence of Nlk during early development (Fig. 14A). Followed by ISH, I performed qPCR with primer specific for Nlk1 and Nlk2. Both qPCR and ISH confirmed the presence of Nlk during *Xenopus* gastrulation. The experiment also shows that the expressions of Nlk1 far exceed Nlk2 expression in gastrulating embryo (Fig. 14 B). This is an important reason for the focus of current thesis on Nlk1.

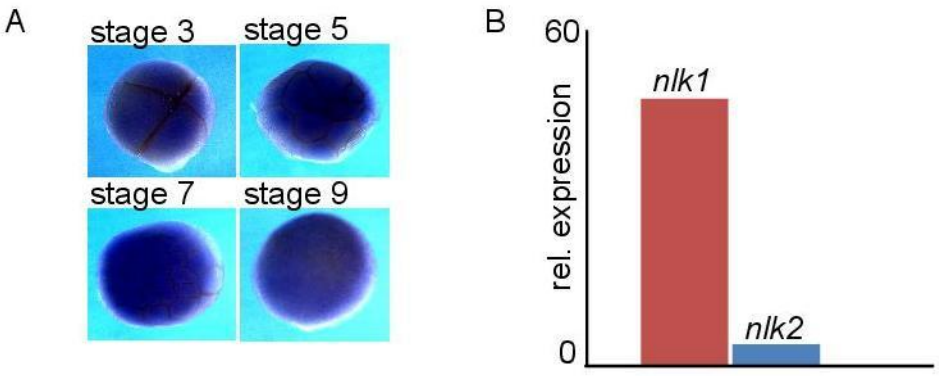

Figure 14. Maternal expression of Nlk1 in the animal hemisphere during early embryogenesis. A: WholemountISHfor*nlk1* on wild-type embryos of indicated stages. **B:** A representative qPCR analysis showing expression of *nlk1* and *nlk2* during gastrulation in dorsal marginal zone explants. The exp. shows a dominant expression of *nlk1* during gastrulation. Expression normalized to ODC as housekeeping gene).

4. 2. 2 Loss of Nlk1 causes gastrulation defects

To investigate the role of Nlk1during early development, I performed loss of function experiments using morpholino oligonucleotides (MOs) specifically targeting *nlk1* (Nlk1 MO) and first analyzed the phenotype of the morphants. Previous studies have shown that Nlk1 is required for anterior formation, however region of Nlk1 knockdown was not specified [66]. Nlk1 MO was injected dorsal equatorially at 4 cell stage, the embryos were cultured till stage 35 and fixed. The phenotypes of Nlk1 morphants were compared to PAPC MO injected embryos, to find potential similarities of loss of these two proteins. Loss of PAPC is known to induce gastrulation defect [19,46,47]. When compared to Control morpholino (Cont. MO) injected embryos, the majority of Nlk1 deficient embryos had gastrulation defects such as spina bifida (60%) or short body axis (30%)(Fig. 15). This roughly resembled the phenotype of PAPC MO injected embryos (spina bifida-45%, short body axis-40%) and for the first time indicates that Nlk1 might be involved in the regulation of gastrulation movements.

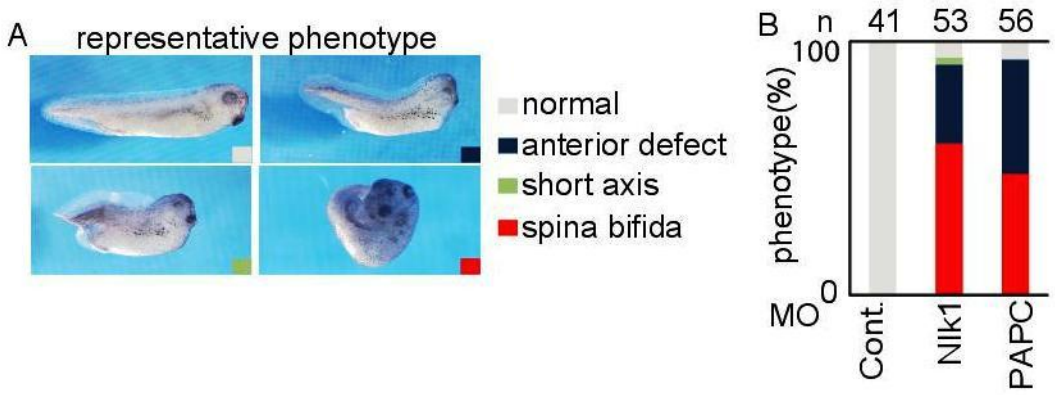

Figure 15. Loss of Nlk1 in the DMZ causes gastrulation defects. A: Representative phenotype of Stage 35 embryos injected dorsal equatorially with morpholino oligonucleotides (MOs) (15ng/embryo). **B:** Quantification of phenotypes of stage35 embryos shown in A.MO: injected morpholino, n: number of analyzed embryos.

4. 2. 3 Convergent extension (CE) movement are regulated by Nlk1

The occurrence of short axis and spina bifida in Nlk1 morphants indicated that Nlk1 might be involved in regulating gastrulation movements such as CE. To confirm the involvement of Nlk1 in regulating CE movement, I performed animal cap (AC) elongation assays. At blastula stage (stage 7-9) the animal hemisphere, which will later become the prospective ectoderm and neural tissue, is still a mass of pluripotent cells. These pluripotent cells can be induced to become mesoderm and undergo CE, leading to elongation of AC explants when they are treated with TGF-β factors. Due to this property and because they lack endogenous Wnt activity, AC explants have been widely used for analyzing the effect of different factor on CE movement [8].

Embryos were injected animally at 4 cell stage with Nlk1 morpholino alongside PAPC morpholino (positive control) and control morpholino. Bvg1 (a chimeric TGF-β receptor) is used for mesoderm induction in animal caps [11,81]. I injected *bvg1* along the samples to induce the TGF-β signaling in animal cap tissue. Animal caps were excised at stage 8. 5 and were grown for 14 hrs at room temp. Animal cap elongation was then quantified for every sample. As previously reported PAPC morpholino injected caps showed reduced elongation (35% compared to contr. MO) [11].Complementing our

hypothesis, Nlk1 morpholino injected samples also showed a reduced animal cap elongation (30% compared to cont. morpholino, Fig. 16A) and similarly Nlk1 morpholino injected samples showed reduced elongation (Fig. 16B).

Since the inhibition of mesoderm induction also results in non-elongation of animal caps, I analyzed the expression of the mesodermal marker gene *brachyury* by qPCR to confirm that at loss of Nlk1 does not impair mesoderm induction (Fig. 16C).

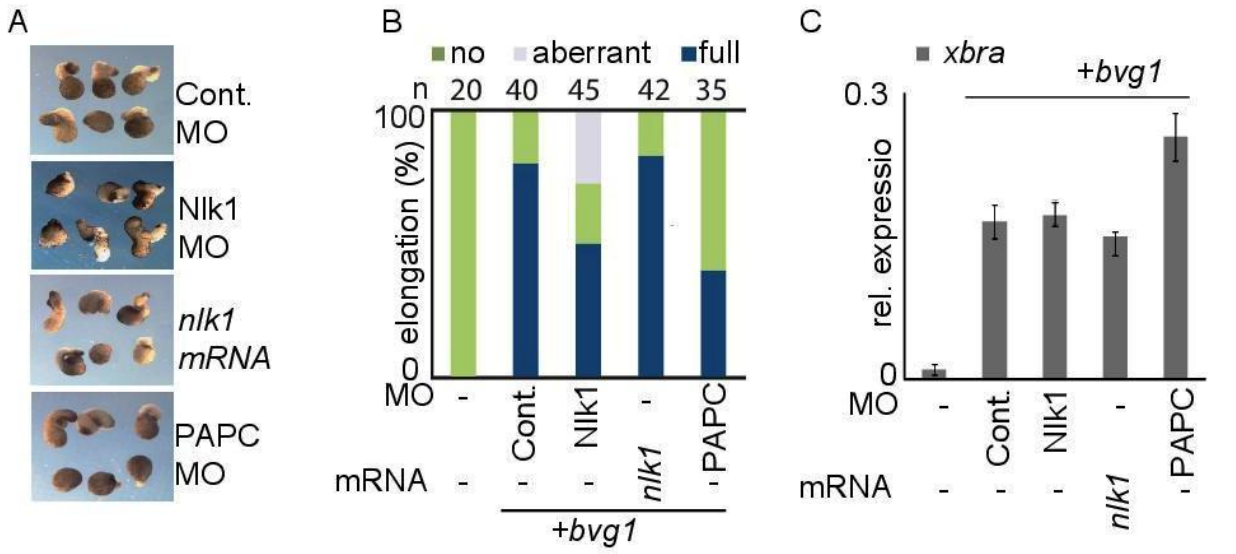

Figure 16. Nlk1 morphant show reduced animal cap elongation. **A**: Representative result of AC elongation assay of embryos injected animally at 4-cell stage with indicated MO (15ng/embryo) and synthetic mRNA (*bvg1*-100pg/embryo,*nlk1*-300 pg/embryo). **B:** quantification of the cap elongation for each sample shown in A.n= number of caps analyzed. **C:** Graph represents qPCR analysis *xbra (Xenopus brachyury)*expression normalized to ODC as housekeeping gene, confirming that mesoderm induction was not altered in MO injected ACs, Error bar represent standard deviation for technical triplicates of representative experiment.

4. 2. 4 Nlk1 is involved in Wnt/PCP regulation

As mentioned before, gastrulation in *Xenopus* to a large extent is controlled by the Wnt/PCP and its component PAPC [18,47]. To investigate if Nlk1 affects gastrulation movements by interfering with Wnt/PCP signaling, I performed an ATF2 luciferase reporter assay [82] in Nlk1 or PAPC morphants. *Wnt11* was used to boost the Wnt/PCP pathway. Embryos were injected at 4-cell stage in the dorsal blastomeres and were processed for reporter quantification at late gastrula (stage 12). ATF2 reporter assay supported the hypothesis that Nlk1 is involved in regulation of Wnt/PCP pathway regulation similar to PAPC. A *wnt11* induced reporter activity is reduced to basal levels if Nlk1 or PAPC is knocked down in the embryos (Fig. 17). The same experiment when replicated without wnt11 induction (data not shown), again showed a reduced reporter activity for Nlk1 morphants.

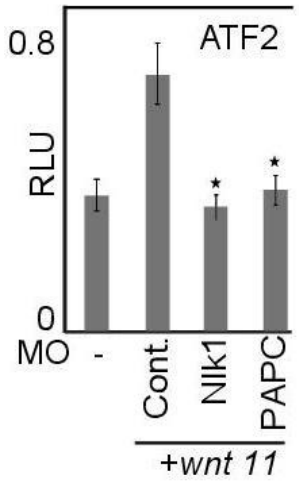

Figure 17. Nlk1 and PAPC knockdown inhibit wnt11 mediated Wnt/PCP signaling. ATF2 luciferase reporter assay of stage 12 embryos injected at 8-cell stage dorsally with indicated morpholinos (12 ng/embryo) and *wnt11* synthetic mRNA (250pg/embryo). The graph shows the mean ± SD of biological triplicates of a pool of 5 embryos (*p > 0. 95 to controls).

4. 2. 5 Nlk1 is required for tissue separation during gastrulation

Another important process regulated by PAPC during gastrulation is tissue separation. Knock down of PAPC is known to lead to improper Brachet's cleft formation [11]. Therefore, I analyzed if loss of Nlk1 also affects tissue separation and Brachet's cleft formation during *Xenopus* gastrulation. Nlk1MO and PAPC MO (as positive control), were injected dorsal equatorially in 4 cell stage. Embryos were fixed at early gastrula (stage 10. 5) and sagittally sectioned through dorsal midline. Brachet`s cleft formation was then examined and quantified. While in wild-type and control MO injected embryos the Brachet's cleft is clearly visible from anterior to posterior mesoderm (close to blastopore), in Nlk1 and PAPC morphants only the anterior Brachet's cleft is visible, while formation of the posterior cleft is disturbed (Fig. 18 A). Once again, a loss of Nlk1 mimicked loss of PAPC and confirmed that Nlk1is required for tissue separation (Fig. 18B).

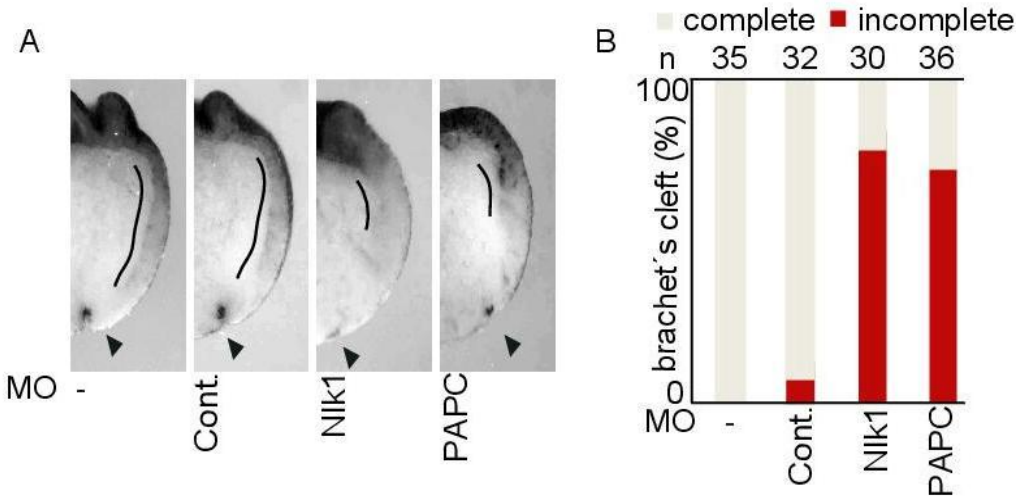

Figure 18. Brachet's cleft formation is impaired in Nlk1 morphants. A: Representative image for Brachet´s cleft of uninjected or embryos injected with either Cont.MO, Nlk1MO or PAPC MO (10ng/embryo). Dorsal is right, blastopore (posterior) is marked by arrowhead, dark line visualizes the length of Brachet's cleft for each sample. Nlk1 and PAPC morphants show incomplete Brachet's cleft formation. **B:** Quantification of the Brachet´s cleft formation of MO injected or wild-type embryos shown in A.

4. 3 Nlk1 and PAPC have additive effects during *Xenopus* gastrulation

As explained in the introduction (Sec. 3.4), PAPC is a known regulator of *Xenopus* gastrulation and Wnt/PCP pathway. Once the involvement of Nlk1 in gastrulation regulation was confirmed, I asked if Nlk1 and PAPC show any cooperative effect during *Xenopus* development. To address this question, a set of morpholino mediated loss-of-function experiments with combined knock-down of the two proteins were performed. In these experiments, a minimum amount of morpholino was used so that the individual knock-down should have a mild effect, and a synergistic effect will be easier to interpret.

4. 3. 1 Combined loss of Nlk1 and PAPC increases gastrulation defects

To investigate the effect of a double knockdown on development, I started to analyze the phenotype of double morphant compared to Nlk1 or PAPC, single knockdown. Morpholinos were injected equatorially in the dorsal blastomeres of 4 cell stage embryos. Total amount of morpholino injected in each embryo was adjusted by adding the respective amounts of Cont. MO. The phenotypes of the single and double morphants were analyzed at stage 35.As intended, using the lower doses of morpholino yielded only few (25 %) of embryos with gastrulation defects when either Nlk1 or PAPC was targeted. However, when both proteins where reduced simultaneously the amount of the embryos with gastrulation defects almost doubled (approx 52%). Hence, Nlk1 and PAPC double morphants displayed no synergistic, but an additive effect (Fig. 19A-B).

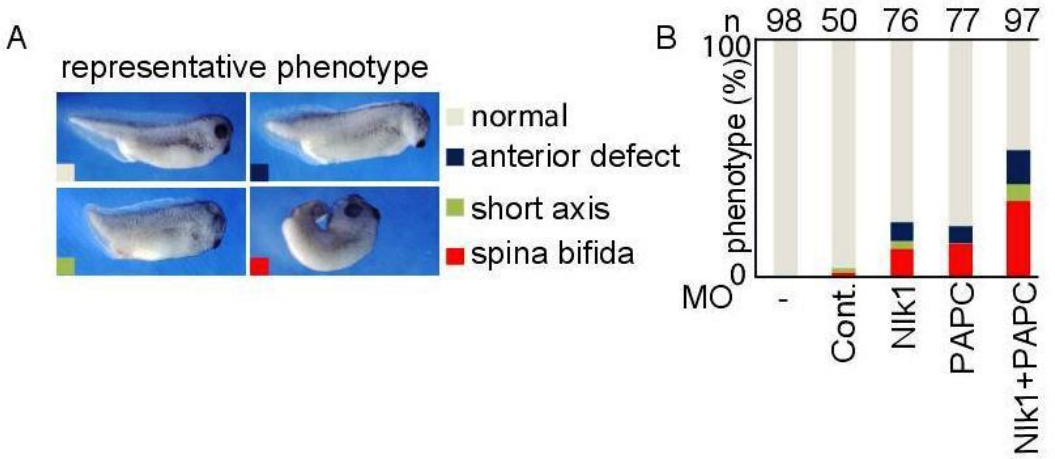

Figure 19. Double knockdown of Nlk1 and PAPC affects the phenotype in an additive manner. A: Representative phenotype of embryos (stage 35) injected dorsal equatorially with indicated morpholino (Cont.MO, Nlk1 MO, PAPC MO = 6ng/embryo), total amount of morpholino injected in each sample was adjusted to12ng/embryo with Cont.MO. **B:** Quantification of phenotypes of stage35 embryos shown in A.MO: injected morpholino, n: number of analyzed embryos.

4. 3. 2 Nlk1 and PAPC have an additive effect on CE movement

The previous experiment revealed an additive effect of PAPC and Nlk1 double knockdown on gastrulation phenotype. To confirm that this is due to disturbed CE movement an AC elongation assay was performed. As in the previous experiment, both Nlk1 and PAPC were knocked down individually and simultaneously. In this case, however, the MOs were injected animally in the ventral blastomeres at 4 cell stage. Animal caps were excised at stage 8. 5 and were grown in presence of Activin (TGF-β factor used instead of bvg1 in this case to induce AC elongation) and the elongation of the ACs was scored when sibling embryos reached stage 25. Once again an additive effect was observed in combinatorial loss of two proteins. In this experiment, the low doses of either Nlk1 or PAPC MO reduced the AC elongation in explants only by 15-20% compared to the control treated ACs. However, a double knockdown of both the proteins caused a 50% reduction in AC elongation (Fig. 20A-B).

Once again *brachyury* expression was quantified in ACs to reassure that inhibition of AC elongation was not caused by inhibited mesoderm induction, but by disrupted PCP signaling (Fig. 20C). The assay affirmed the additive effect of these two proteins on (CE) movements.

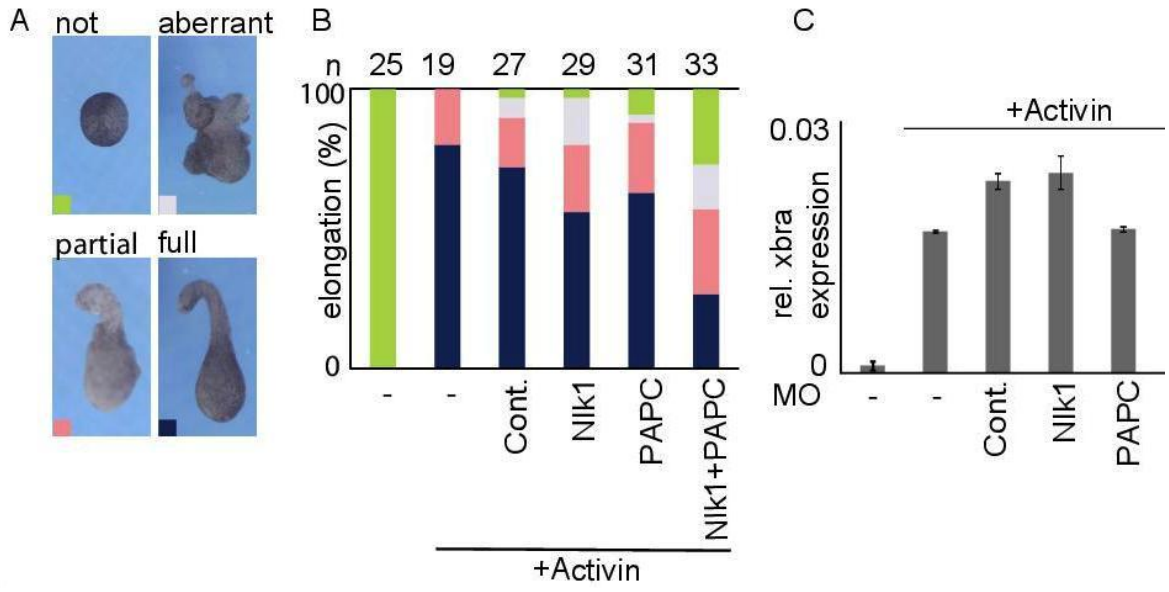

Figure 20. Additive effect of Nlk1 and PAPC double knockdown on AC elongation. A: Representative images of elongated or non-elongated ACs, **B:** Quantification of the elongation (as shown in A) of the ACs from embryos injected with corresponding MO (5 ng/embryo). Total amount of MO injected in each sample was adjusted by respective amount of Cont.MO.50ng/ml of Activin was used for AC elongation. **C:** Graph represents qPCR analysis *xbra (Xenopus brachyury)* expression normalized to ODC as housekeeping gene, confirming that mesoderm induction was not altered in MO injected ACs, Error bar represent standard deviation for technical triplicates of representative experiment.

4. 3. 3 Additive regulation of Wnt/PCP signaling by Nlk1 and PAPC

To confirm that the additive function of Nlk1 and PAPC observed in phenotype and CE movement is caused by disrupted Wnt/PCP pathway regulation, ATF2 reporter assays were performed in the double morphants. Morpholinos against Nlk1, PAPC, were injected alone or combined in the dorsal blastomere at 4 cell stage, along with *wnt11* mRNA to induce Wnt/PCP signaling. Injected embryos were collected at stage12 and processed to measure the ATF2 reporter activity. As reported before (Fig. 17) a loss of Nlk1 or PAPC reduced the reporter activity, albeit in this case less, since lower MO doses were used. However, a double knock down of both proteins further reduced the induction close to basal reporter levels (Fig. 21).

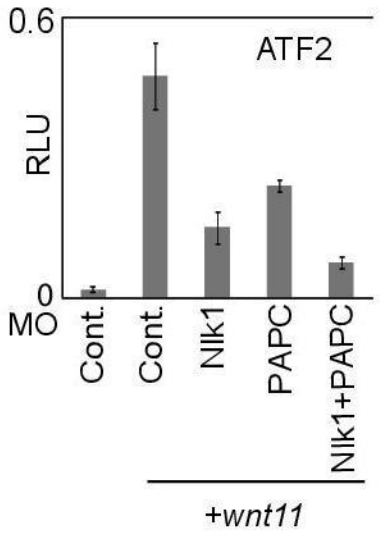

Figure 21. Nlk1 and PAPC double knock down repress Wnt/PCP signaling in an additive manner. Representative graph of ATF2 luciferase reporter assay of stage 12 embryos injected with indicated MOs (5 ng/embryo: Cont. MO, Nlk1 MO, PAPC MO).*wnt11*synthetic mRNA (250pg/embryo) was used to induce Wnt/PCP activation. Total amount of morpholino injected in each sample is adjusted by cont.MO. The graph shows the mean ± SD of biological triplicates of a pool of 5 embryos.

4. 4 Nlk1 regulates PAPC protein stability

4. 4. 1 Specificity of PAPC antibody

To better understand the molecular function of the interaction of Nlk1 with PAPC, detection of the endogenous proteins is essential. Since our group is focusing on elucidating the role of PAPC during gastrulation, we ordered a customized polyclonal antibody from Biogenes. The antibody was raised in rabbit against a peptide (amino acid 820-833 of PAPC) in the C-terminal region of the protein, which is highly conserved in protocadherin 8 family (PAPC homologs in higher vertebrates). To confirm the specificity of the custom antibody for endogenous PAPC in *Xenopus* embryos, I performed immunoblot and immunofluorescence experiments. Protein lysates from gastrula embryos injected with PAPC MO, Nlk1 MO and different *papc* mRNAs were analyzed on Immunoblot using the custom PAPC antibody to address if it can specifically detect endogenous PAPC protein. Since it is known that *papc* is not maternally expressed [16], but induced after MBT (mid blastula transition - stage 8), I assumed that the PAPC protein is only detectable at gastrula stages and used an embryo lysate from stage 5 as negative control. The custom PAPC antibody could indeed detect a band at the expected size of PAPC (approx-109 kDa) in early gastrula stage 10 embryos (Fig. 22A). Since this band was as expected absent in cleavage stage embryos (stage 5), and stronger in mid-gastrula stage 11 embryos, this is most likely the specific endogenous PAPC protein. Specificity of this antibody is further supported by the reduction of its intensity in lysates from PAPC MO injected embryos, when compared to Cont. MO. Notably, Nlk1 MO lead to a similar decrease in PAPC protein expression. A Myc-tagged overexpressed construct showed a slightly higher migration of an additional band (Fig. 22 A). Analyses of *papc* injected *Xenopus* AC explants revealed that also in Immunofluorescence stainings the custom antibody efficiently detects the overexpressed PAPC protein (Fig. 22B). M-PAPC, which lacks the C-terminal domain targeted by the custom antibody was not detected (Fig. 22B).Taken together, these results indicate, that the used antibody specifically detects endogenous PAPC by binding to the C-terminal epitope.

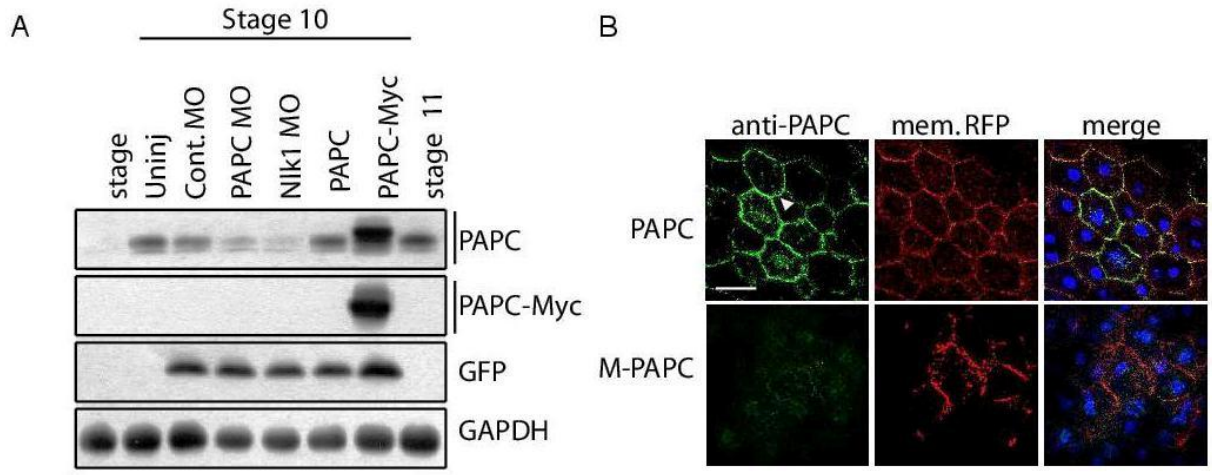

Figure 22. Custom designed antibody specifically detects endogenous PAPC protein. A: Immunoblot analysis of embryo lysate from different stages of *Xenopus* development and stage 10 gastrula embryo lysates injected with the indicated morpholinos and mRNAs (Cont. MO, Nlk1 MO and PAPC MO: 12 ng/embryo; *papc* and *papc-Myc*: 300 pg/embryo) were used to confirm the specificity of the band detected in the wild-type stage 10 and stage 11 gastrulae by the custom anti-PAPC antibody. Overexpressed PAPC-Myc was additionally detected using an antibody against Myc.GFP (300 pg/embryo) was used as injection control and visualized with the respective antibody. The housekeeping protein GAPDH showed equal loading of the samples. **B:** Immunofluorescence staining of overexpressed PAPC and M-PAPC protein in *Xenopus* AC explants using the custom PAPC antibody. Arrowhead shows PAPC protein (green) detected by the custom antibody targeting the C-terminus. MembraneRFP(red) and DAPI (blue) were used for membrane and nuclear staining, respectively. Scale bar = 50 µm.

4. 4. 2 PAPC is stabilized in the presence of Nlk1

In the previous section, I presented that a loss of Nlk1 reduced PAPC protein level (Fig. 22). To confirm this finding I repeated the immunoblot for endogenous PAPC in gastrula stage embryos, including both Nlk1 overexpression and knockdown. Since Nlk1 is a kinase, I also addressed in this experiment, whether Nlk1 gain or loss would cause a shift of the PAPC band due to a changed phosphorylation state. However, no change in migration of protein was observed in either a loss or gain of Nlk1, indicating that PAPC phosphorylation might not be affected by Nlk1. On the other hand, again a morpholino mediated loss of Nlk1 resulted in reduced amount of PAPC protein and accordingly a gain of Flag-Nlk1 increased the amount of endogenous PAPC (Fig. 23).

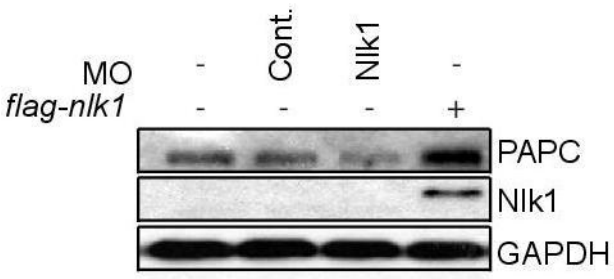

Figure 23. Endogenous PAPC protein is stabilized by Nlk1. Immunoblot analysis of lysates from stages 10 gastrula embryos injected with the indicated morpholinos (12 ng/embryo) or mRNA (300 pg/embryo).PAPC was detected using the custom antibody for the PAPC C-terminus and overexpressed Nlk1 was detected using an antibody against Flag. The housekeeping protein GAPDH showed equal loading of the samples.

4. 4. 3 PAPCc protein is destabilized by GSK-3β and proteasomal degradation

A recent publication has identified a GSK-3β dependent ubiquitination of PAPC [20]. GSK-3β dependent ubiquitination can induce proteasomal degradation of proteins [83]. Hence I investigated if PAPC stability is GSK-3β dependent and if proteasome mediated degradation is involved. In again of function experiment, HEK293 cells overexpressing PAPCc, were treated either with the GSK-3β inhibitor LiCl or with the proteasomal inhibitor MG132. Immunoblot for PAPCc-Flag of cell lysates reveal that MG132 increased the amount of PAPCc protein detected (Fig. 24A). There was a very slight stabilization in presence of LiCl. For a clear visualization of stability, an intensity graph of PAPCc normalized to co-injected GFP has been plotted (Fig. 24A, bottom).

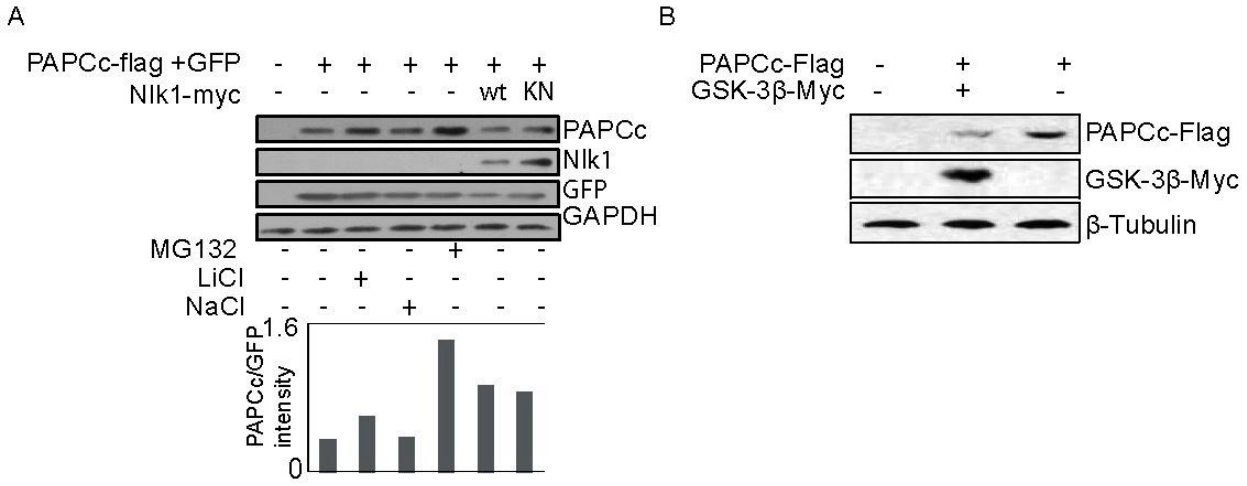

Figure 24. PAPCc is stabilized by GSK-3β activity and inhibition of proteasomal degradation. **A:** Immunoblot analysis showing that PAPCc is stabilized in presence of GSK-3β and proteasome inhibitors. Lysate from HEK293 cells transfected with indicated plasmid and treated with the indicated inhibitors (10 µM MG132, 10mMLiCl and NaCl). PAPCc and Nlk1 proteins were detected using antibody against Flag and Myc tag respectively. GFP was used as a transfection control. The housekeeping protein GAPDH showed equal loading of the samples. **B:** Immunoblot analysis showing that GSK-3β reduces PAPCc protein amount. Loaded were lysates from HEK293 cells transfected with indicated plasmid. PAPCc and GSK-3β proteins were detected using antibody against Flag and Myc tag, respectively. The housekeeping protein β-tubulin showed equal loading of the samples. In both the experiments transfection was normalized using an empty vector control.

As, LiCl is not a specific inhibitor for GSK-3β, I also overexpressed PAPCc-Flag in presence or absence of GSK-3β-Myc. Immunoblot analysis revealed that the PAPCc protein level is reduced in presence of GSK-3β-Myc (Fig. 24B). These experiments are in line with the previously described involvement of GSK-3β with PAPC ubiquitination and also indicate a probable proteasomal dependent degradation of PAPC [20].

4. 4. 4 Overexpressed Nlk1 is sensitive to PAPC

In my immunoblot analysis for overexpressed PAPCc protein in HEK293 cells, I came across another interesting finding which suggested a reciprocal stabilization of Nlk1 in presence of PAPCc (data not shown). To confirm this observation, I overexpressed Nlk1-Myc in *Xenopus* embryos co-injected with PAPC MO for knockdown or overexpressing PAPC-deletion constructs for gain of PAPC.

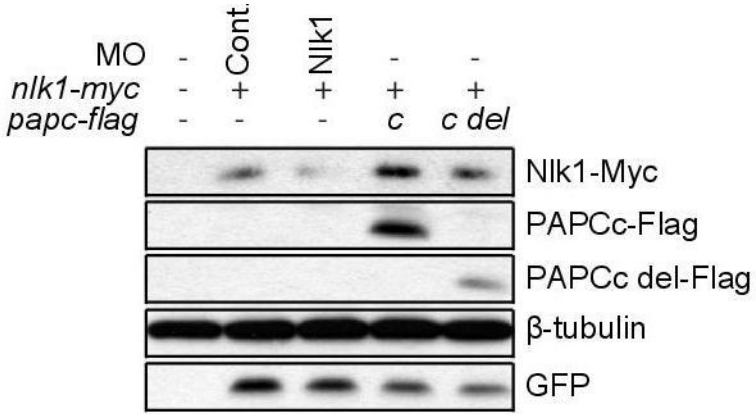

Figure 25. Nlk1 protein is stabilized by PAPCc. Immunoblot analysis of lysates from stages 10 gastrula embryos injected with the indicated MOs (12 ng/embryo) or mRNA (300 ng/embryo). Nlk1 and PAPC/PAPCc∆899 were detected by using antibody against Myc and Flag tag respectively. GFP was used as injection control. The housekeeping protein GAPDH showed equal loading of the samples.

Analysis of the Nlk1-Myc protein expression in lysates of stage 10.5 embryos confirmed that Nlk1 is sensitive to PAPC levels: PAPC MO injected sample showed reduced Nlk1-Myc, while Nlk1-Myc was increased in presence of PAPCc (Fig. 25). The negative control, PAPCcΔ899 which does not bind Nlk1 (Sec. 4.1.1) was consequently not able to stabilize Nlk1 expression, confirming that physical interaction of Nlk1 with PAPC is required for stabilization.

4. 4. 5 Nlk1 and PAPC mutually inhibit their poly-ubiquitination

The observed mutual stabilization and the stabilization of PAPC by GSK-3β or proteasomal inhibitor indicate a probable proteasome mediated degradation of Nlk1 and PAPC. To confirm that the mutual stabilization of Nlk1 and PAPC is due to polyubiquitin dependent proteasomal degradation, I performed ubiquitination assays in HEK293 cells overexpressing HA-tagged Ubiquitin (Ubi-HA) along with Nlk1-Myc and PAPCc-Flag. Cell lysates were immunoprecipitated for both PAPCc and Nlk1 and probed against HA (Ubi-HA). The result showed that both the proteins undergo polyubiquitination, however a clear reduction of polyubiquitination can be seen when both proteins are expressed together (Fig. 26A-B). These experiments confirmed that PAPC and Nlk1 mutually stabilize each other by inhibiting each other's polyubiquitination and hence degradation.

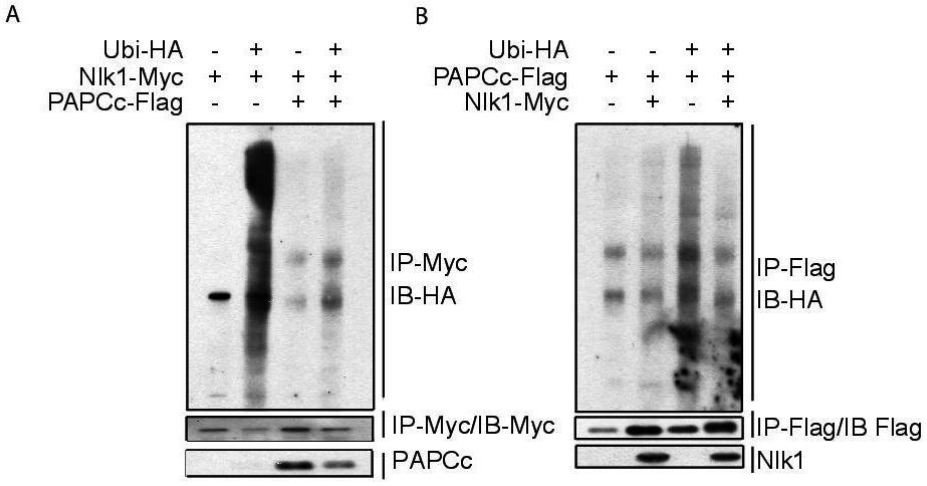

Figure 26. Nlk1 and PAPCc mutually reduce polyubiquitination of each other. A: Cell lysate from cells overexpressing Nlk1-Myc were immunoprecipitated with Myc antibody and immunoblotted for Ubi-

HA (ubiquitin-HA).Bottom panels show immunoblot for immunoprecipitated Nlk1-Myc and co-expressed PAPCc-Flag using the respective antibodies. **B:** Cell lysate from cells overexpressing PAPCc-Flag were immunoprecipitated with Flag antibody and immunoblotted for Ubi-HA (ubiquitin-HA).Bottom panels show immunoblot for immunoprecipitated PAPCc and co-expressed Nlk1-Myc using the respective antibodies. IP:immunoprecipitation, IB:immunoblotting.

4. 5 Characterization of putative phosphorylation sites of PAPC

I have so far presented data supporting that the protein stability of PAPC is regulated by the two proline directed Ser/Thr kinases Nlk1 and GSK-3β. Notably, the two kinases seem to have opposing effects on PAPC stability: Nlk1 enhancing (Fig. 23), and GSK-3β reducing PAPC protein levels (Fig. 24). Since GSK-3β dependent ubiquitination succeeds phosphorylation of the target protein I wondered if the phosphorylation status of PAPC has an impact on Nlk1/GSK-3β regulated stability of the protein. To address this question I planned to generate PAPC phosphorylation mutants and characterized their effects in different assays.

Therefore I first searched for putative Nlk1/GSK-3β phosphorylation sites within the last 80 amino acid of PAPC, which had been identified as the physical interaction domain for Nlk1. Using bioinformatic programs (phosphosite, netPhosK), I was able to identify two overlapping putative phosphorylation site (Ser residue) of proline directed MAPK in the last 80 amino acid of PAPC, Ser-949 and Ser-963.Sequence alignment (ClustalW) showed that these two sites are conserved in Pcdh8 (human homolog of PAPC) too (Fig. 27). However in *Xenopus* these sites constitute a stronger GSK-3β binding motif (SP/SXXXS) [84].

```
Xenopus   DPYYHINNPVANRMHAEYERDLVN---RSATLSPQRSSSRYQEFN------YSPQISRQL 970
Human     DNYYQAQLPKTVGLQSVYEKVLHRDYDRTVTLLSPPRPGRLPDLQEIGVPLYQSPPGRYL 1060
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Figure 27. Two putative Nlk1/GSK-3β phosphorylation sites in PAPC are conserved in Pcdh8. Two Serine residues marked in red at amino acid position 949 and 963 of PAPC represents putative Nlk1/GSK-3β phosphorylaiton site.

Once these putative sites were identified, site directed mutagenesis was performed to mutate these amino acid residues to either alanine (Ser-949/963A: PAPC-PN phospho-null), or to aspartic acid (Ser-949/963D: PAPC-PM phospho-mimic).Both the mutants were created for PAPC as well as PAPCc.

4. 5. 1 PAPC phosphorylation mutants are differentially stabilized by Nlk1

PAPC as well as PAPCc are sensitive to the presence or absence of Nlk1. To characterize the two PAPC mutants for their Nlk1 sensitivity (Sec. 4.4), I performed immunoblotting experiment in Hek293 cells overexpressing the phospho-mimic PAPCc-PM and the phospho-null PAPCc-PN mutants in presence or absence of Nlk1. PAPCc which binds to Nlk1 was used as a positive control for Nlk1 mediated stabilization, while PAPCcΔ899 which does not interact with Nlk1 served as a negative control. Immunoblot analysis for the PAPCc constructs showed that PAPCc was stabilized by Nlk1, while PAPCcΔ899 was not (Fig. 28). Of the two mutants PAPCc-PN was unaffected by co-expression of Nlk1, while PAPCc-PM mutant was considerably stabilized in presence of Nlk1 (Fig. 28). Analysis of the stability of these phospho-mutants in *Xenopus* Nlk1 morphants confirmed, that Nlk1 stabilizes PAPCc-PM, but not PAPCc-PN (data not shown). These findings suggest that PAPCc phosphorylation at these motifs is required for Nlk1 mediated stabilization.

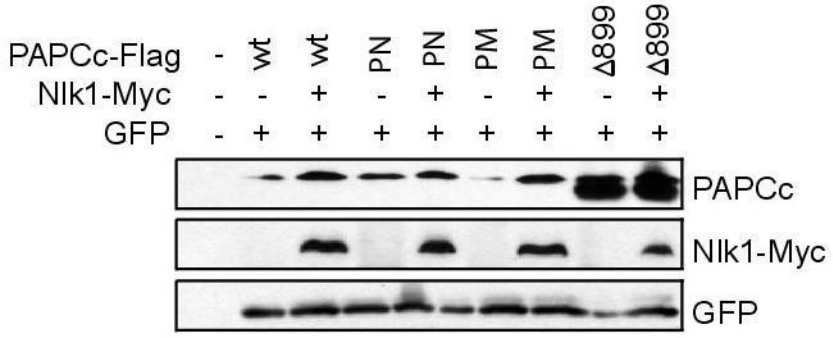

Figure 28. Phosphorylation at putative Nlk1Nlk1/GSK-3β phosphorylation site is required for PAPCc stabilization. Immunoblot analysis from HEK293 cell lysate transfected with indicated plasmid. All PAPC proteins were detected using Flag antibody while Nlk1 was detected using Myc antibody.GFP was used as a transfection control. An increase in PAPCc (wt) and PAPCc-PM protein stability is visible

4. 5. 2 PAPC phosphorylation mutants show normal membrane localization

Kai.et. al reported that phosphorylation of PAPC at GSK-3β sites is required for proper PAPC membrane localization [20]. Therefore, I investigated whether the membrane localization of the PAPC phosphorylation mutant used in this thesis is also impaired. Furthermore, I was also curious to see if there is a change in membrane localization of wild-type PAPC and mutants, if Nlk1 is knocked down. IF staining of the overexpressed mutants in *Xenopus* ACs revealed, that membrane localization of either PAPC or PAPC phospho-mutants used was not impaired (Fig. 29).Furthermore, there was no change in membrane localization of either PAPC or PAPC phospho-mutants observed when Nlk1 was depleted. However, these IF assays complemented my Western blot experiments (Sec. 4.5.1), since there was a decrease in the amount of PAPC and PAPC-PM in samples where Nlk1 was knocked down (Fig. 29A+C). No such decrease was observed for PAPC-PN (Fig. 29B). This experiment consolidates the fact that phosphorylation at these residues is needed for Nlk1 mediated PAPC stabilization. PAPC localization however seems unaffected by Nlk1.

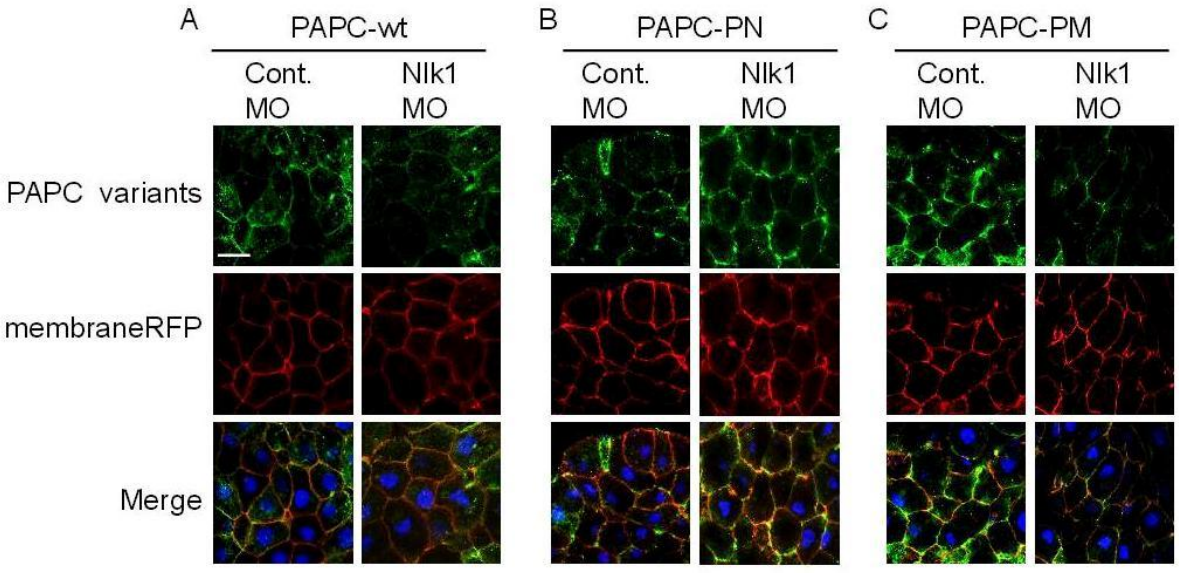

Figure 29. PAPC phosphorylation mutants show proper membrane localization. IF analysis of *Xenopus* AC explants injected animally at 4-cell stage with corresponding synthetic mRNA for *papc*(**A**), *papc-pn* (**B**) and *papc-pm* (**C**) *and membranerfp* (300 pg/embryo) and MOs (cont. ,Nlk1:12 ng/µl). Proper

membrane localization of PAPC constructs (green) is seen, membraneRFP (red) was used to visualize the plasma membrane (red). DAPI (blue) was used for nuclear staining. Scale bar = 50 µm.

4. 5. 3 PAPC phospho-mutants regulate Wnt/PCP signaling differently

Since the phosphorylation state of proteins is known to affect their functioning, the ability of the two phospho-mutants of PAPC to regulate Wnt/PCP signaling was analyzed. During my studies I observed, that gain, as well as loss of PAPC inhibits Wnt11 mediated Wnt/PCP signaling in ATF2 reporter assays (data not shown).Therefore, comparing the effect of PAPC wild-type and mutant overexpression on ATF2 reporter assay could lead to inconclusive results. Instead, a rescue experiment was used to explore the signaling ability of the mutants, where loss of PAPC, leading to pathway inhibition was rescued by PAPC or its phosphorylation mutant. The inhibition of Wnt11 induced ATF2 reporter activation by PAPC knock down was indeed restored partially by PAPC and by the phospho-mimic mutant of PAPC (Fig. 30). The PAPC-PN was not able to rescue the PAPC MO, although it was indeed expressed in the experiments (Fig 30, inset). This assay supports the previous findings, that phosphorylation at these sites is needed for proper PAPC function

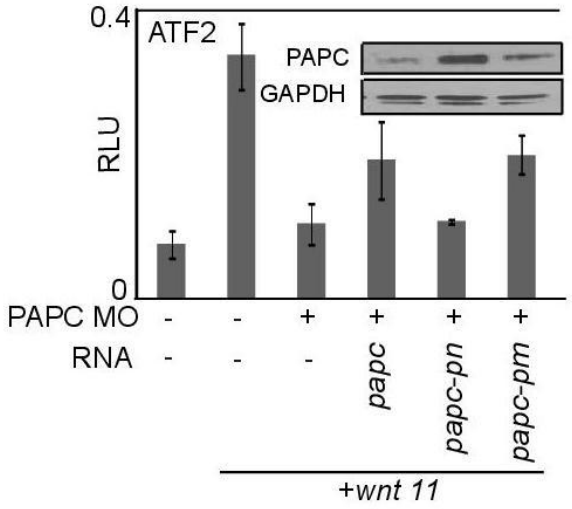

Figure 30. Loss of PAPC can be rescued by either PAPC or phospho-mimic PAPC. Representative graph for ATF2 luciferase reporter assay of stage 12 embryos injected with indicated morpholino (12 ng/embryo) or synthetic mRNA (300pg/embryo). *Wnt11*synthetic mRNA (250pg/embryo) was used to

stimulate reporter activation. The graph shows the mean ± SD of biological triplicates of a pool of 5 embryos. The assay was performed three times with same trend. Inset: Expression of PAPC and PAPC phosphorylation mutant was confirmed by immunoblot using an anti-Flag antibody and GAPDH for loading control.

4. 6 Nlk1 Kinase activity is dispensable for gastrulation regulation and PAPC interaction

I have shown that Nlk1 can regulate gastrulation movement and the Wnt/PCP pathway similar to PAPC (Sec. 4.2) and that Nlk1 and PAPC mutually stabilize each other (Sec. 4.4). Furthermore, mutation of the putative Nlk1 phosphorylation sites, differentially changed the behavior of PAPC in respect to stability, Nlk1 sensitivity and signaling ability (Sec. 4. 5). However, I could not observe any Nlk1 dependent shift of the PAPC band which would be expected if it is phosphorylated (Sec. 4.4.2). To study whether phosphorylation of PAPC by Nlk1 is required for the process, a kinase inactive (KN, kinase negative) mutant of Nlk1 should not be able to stabilize PAPC and regulate Wnt/PCP signaling and gastrulation movement. Therefore, I compared the effect of Nlk1 and its kinase negative mutant Nlk1 KN in overexpression experiments.

4. 6. 1 The kinase activity of Nlk1 is dispensable for PAPCc stabilization

To investigate, if the kinase activity of Nlk1 is required for PAPC stabilization, despite the lack of an observable phospho-shift on the endogenous protein, I analyzed the effect of the kinase negative mutant of Nlk1 (Nlk1 KN) on PAPC stabilization. In these experiments I used PAPCc for overexpression. This has the advantage that a potentially overlooked phospho-shift might be seen. It is possible that a potential shift of the endogenous PAPC band could not be detected due to its relatively large size and low expression. Since PAPCc is smaller and overexpressed, these shortcomings could be circumvented by using this deletion construct. Analyzing the protein expression and size of PAPCc in embryos depleted of Nlk1 or overexpressing Nlk1-Myc confirmed that the cytoplasmic domain of PAPC is stabilized by Nlk1 (Fig. 31). This experiment had two

interesting outcomes, firstly Nlk1 mediated PAPC stabilization is a post-translational event as an overexpressed construct behaved identical to the endogenous protein. Secondly, the kinase activity of Nlk1 is dispensable for PAPC stabilization.

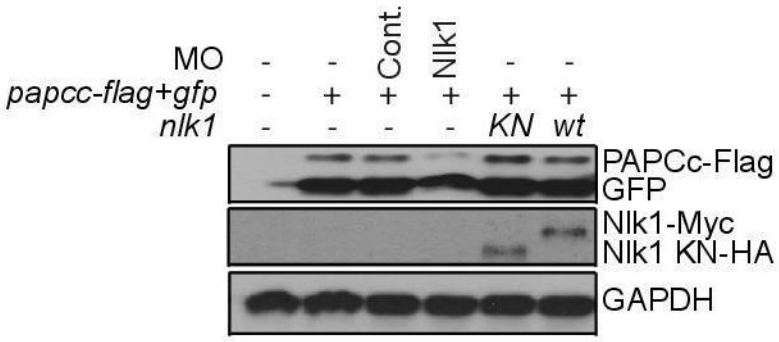

Figure 31. Overexpressed PAPCc is also sensitive to Nlk1 or Nlk1 KN. Immunoblot analysis of lysates from stages 10 gastrula embryos injected with the indicated MOs (12 ng/embryo) or mRNA (300 pg/embryo). PAPC was detected using an antibody against Flag. Lane 2 shows overexpressed Nlk1 and Nlk1 KN, detected using antibody against the indicated tag.GFP was used as injection control. The housekeeping protein GAPDH showed equal loading of the samples.

4. 6. 2 Nlk1 KN mutant is membrane recruited via the C-terminus of PAPC

In section 4.1.3, I have showed that Nlk1 co-localizes with PAPC. More precisely, overexpressed PAPC has changed the localization of Nlk1 from nucleus to membrane. To confirm that the same holds true for Nlk1 KN, an immunofluorescence experiment was performed in *Xenopus* ACs. Tagged constructs of Nlk1 KN alone or with wild type PAPC or its deletion constructs were overexpressed in the animal hemisphere. Nlk1 KN, like Nlk1 had a predominantly nuclear localization when expressed alone (Fig. 32, Ist column). However, when expressed along with PAPC or PAPCc, Nlk1 KN was recruited to the membrane, where it co-localized with both the proteins, respectively (Fig. 32, second and fourth column). Still, some Nlk1 KN protein remained in the nucleus, similar to wild-type Nlk1 (Sec. 4.1.3) when expressed with PAPC and PAPCc. Like observed for Nlk1, also Nlk1 KN does not change its localization when the PAPC mutants lacking the binding domain (M-PAPC and PAPCc∆899) where co-expressed

(Fig. 32, third and fifth column). In summary, these results indicate that the kinase activity of Nlk1 is not required for its interaction with PAPC.

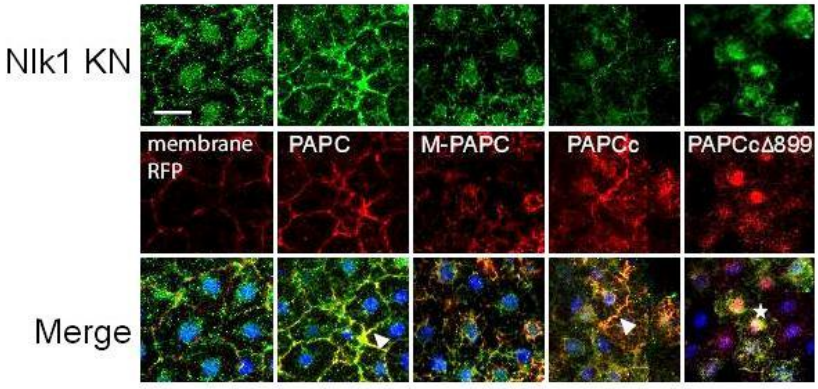

Figure 32. Nlk1 KN is recruited to the membrane in presence of PAPC. Immunofluorescence staining of *Xenopus* AC explants injected at 4-cell stage with Nlk1KN-HA (green) and the indicated PAPC-Myc constructs or membraneRFP as control (red). DAPI (blue) was used for nuclear staining. Scale bar = 50µm. White arrowhead indicates co-localization sites at the plasma membrane and asterix marks expression of both proteins inside the nucleus.

4. 6. 3 Overexpression of Nlk1 or Nlk1 KN cause gastrulation defects in *Xenopus* embryos

As mentioned in the introduction, a tight balance of Wnt/PCP pathway is required during gastrulation and if this balance is altered in either direction, gastrulation defect are observed in phenotypes (Sec. 3.3.2.1). As the requirement of Nlk1 during gastrulation has already been shown (4.2.1), a gain of Nlk1 was expected to cause gastrulation defects too. If phosphorylation of PAPC is required for the function of Nlk1 during embryonic development, overexpression of Nlk1 KN should have a different effect on the phenotype of *Xenopus* embryos than the wildtype Nlk1.

Equal amounts of synthetic mRNA for Nlk1 orNlk1 KN were injected dorsal equatorially at 4 cell stage. Overexpression of both Nlk1 and Nlk1 KN produced gastrulation defective phenotype (spina bifida and short axis), while preprolactin (ppl) mRNA

injected control embryos did not (Fig. 32). Notably, gain of Nlk1 and gain of Nlk1 KN yielded roughly equal values of gastrulation defects (65%). Surprisingly, this suggests that Nlk1 kinase activity is not required for gastrulation regulation.

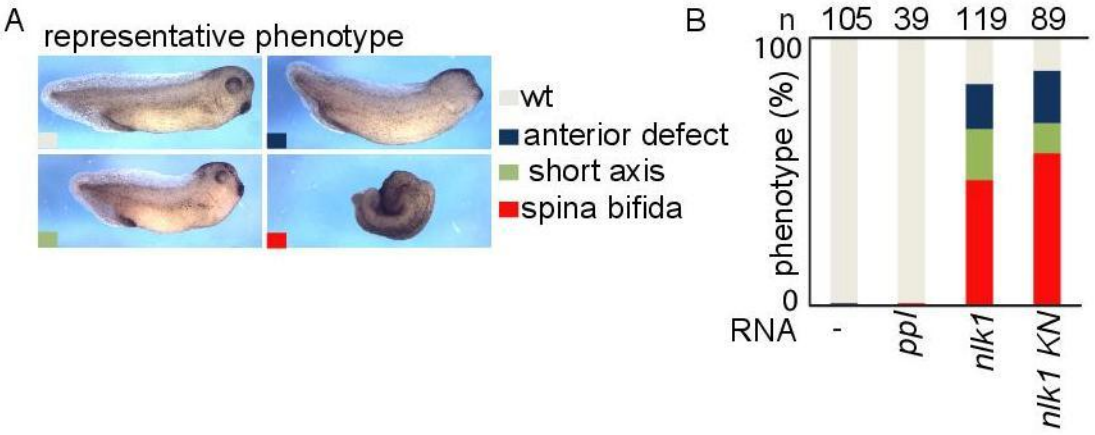

Figure 33. Overexpression of Nlk1 or Nlk KN cause similar gastrulation defects. A: Representative phenotype of embryos (stage 35) injected dorsal equatorially with synthetic mRNA for *ppl, nlk1, nlkKN* (250 pg/embryo). **B:** Quantification of phenotypes of stage 35 embryos shown in A. ppl: preprolactin, n: number of embryos analyzed.

4. 6. 4 Nlk1 KN interferes with Wnt/PCP pathway similar to Nlk1

I have demonstrated earlier, that Nlk1 affects gastrulation movements by interfering with Wnt/PCP signaling using the ATF2 luciferase reporter assay (Sec. 4.2.4). Therefore, I repeated the assay with Nlk1 KN, to explore in more detail if the kinase activity is necessary for Wnt/PCP and gastrulation regulation. ATF2 reporter assay of stage 12 embryos, injected dorsal equatorially with two different concentrations of Nlk1 or Nlk1 KN respectively, reveals a concentration dependent repression of Wnt11 induced ATF2 reporter activity (Fig. 34). This finding complements the previous finding that Nlk1 kinase activity is dispensable for Nlk1 mediated regulation of gastrulation and Wnt/PCP signaling.

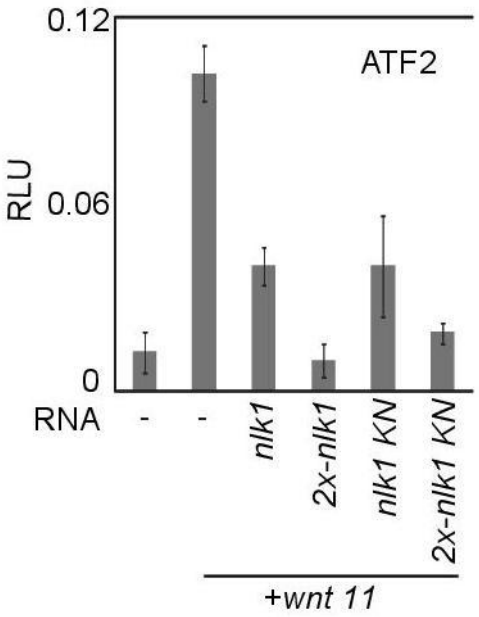

Figure 34. Equivalent Wnt/PCP pathway regulation by Nlk1 and Nlk1 KN. Representative ATF2 luciferase reporter assay of stage 12 embryos injected with indicated synthetic mRNA for *nlk1* and *nlk1kn* (1x-250pg/embryo, 2x-500 pg/embryo). *Wnt11* synthetic mRNA (250pg/embryo) was used to activate the reporter. Total amount of synthetic mRNA in every sample was adjusted with *gfp.* The graph shows the mean ± SD of biological triplicates of a pool of 5 embryos. The assay was performed three times with same trend.

Surprisingly, the analyses of the Nlk1 KN mutant indicate, that although the phosphorylation status of PAPC changes its stability (Sec. 4.5), the kinase activity of Nlk1 is dispensable for its interaction with and stabilization of PAPC, and hence its function during gastrulation and Wnt/PCP regulation.

5 Discussion

Paraxial protocadherin (PAPC) is a single pass transmembrane protein that plays a central role during vertebrate gastrulation [11,47,56,58]. Despite extensive studies, the mechanism of PAPC function and more importantly its regulation still remain elusive. In particular, PAPC binding partners that might regulate its function during early development are largely unknown. In this thesis, I confirm and explored previous observation made in our group, namely that PAPC interacts with the atypical MAP kinase Nlk1.

Nlk is known to be involved in regulation of different signaling pathways [85–88]. Among these, Nlk mediated Wnt/β-catenin signaling is most extensively studied. Nlk mediated phosphorylation of TCF/LEF transcription factors leads to a modulation of Wnt/β-catenin signaling [74,89]. During *Xenopus* development Nlk is required for anterior structure formation by interaction with other MAPK proteins like p38, TAB2,TAK, etc., and by inhibiting the Wnt/β-catenin signaling pathway [67,73,85].

5. 1 Nlk1 is a novel interaction partner of PAPC

In this thesis I present evidence of physical interaction of Nlk1 with PAPC. Co-immunoprecipitation data confirmed earlier yeast two hybrid screen results, showing that Nlk1 interacts with PAPC via its intracellular domain (PAPCc; Fig. 11). I also performed Immunofluorescence (IF) experiments in *Xenopus* animal cap explants to confirm co-localization of two proteins. IF experiments supported the previous findings, as Nlk1 co-localized with PAPC and PAPCc but not with M-PAPC, a membrane-tethered PAPC construct lacking the intracellular domain. Intriguingly, PAPCcΔ899 (PAPCc mutant lacking binding site for Nlk1) also showed co-localization with Nlk1 inside the nucleus, possibly due to mis-localization of the truncated PAPCcΔ899construct (Fig. 13). Apparently the last 80 amino acids of PAPC are also required for homo-dimerization, since PAPCc, but not PAPCcΔ899, is partially localized at the plasma membrane, where it then can also recruit Nlk1.

PAPC-mediated gastrulation regulation is primarily attributed to the extracellular domain of PAPC and its interaction with Frizzled (Fz) and cadherin proteins [45–47]; however, there is mounting evidence of an alternative signaling mechanism of PAPCc, especially in context of Wnt/PCP pathway and gastrulation regulation. For example, PAPCc interaction with ANR5 regulates *Xenopus* gastrulation via Rho activation [57]. Similarly, PAPCc interaction with Sprouty2 modulates Wnt/PCP and gastrulation movements [56]. A recent publication from our lab has shown that not only Wnt/PCP but also Wnt/β-catenin can be regulated by PAPC. Kietzmann et al. showed that PAPCc interaction with CK2β negatively regulates Wnt/β-catenin pathway, presumably by inhibition of CK2 holoenzyme formation [58].

Hence the interaction of Nlk1 with PAPCc is highly interesting because of two reasons; firstly, PAPCc is a well-established regulator of gastrulation and Wnt/PCP signaling, while the role of Nlk1 in both contexts has never been examined. Secondly, Nlk1 exerts its Wnt/β-catenin regulation by phosphorylation of transcription factors (TCF/LEF) inside the nucleus. An interaction with PAPC might thus hinder Nlk1 nuclear localization, and hence Nlk-mediated functions in the nucleus will be reduced. The data I present here support the second hypothesis: In the absence of PAPC, Nlk1 strongly localizes to the nucleus (Fig. 13). However, in the presence of PAPC or PAPCc the nuclear localization of Nlk1 is highly reduced. This suggests that interaction of Nlk1 with PAPC can affect its function by changing its intracellular localization. I also have preliminary data showing Nlk1 mediated Wnt/β-catenin regulation is inhibited by PAPC in animal cap explants. Interestingly, a lack of nuclear localization of Nlk is required to induce apoptosis in breast cancer cells [90], and another reports supports a tumor suppressor role of Pcdh8 (PAPC homolog in human) in breast cancer patients [91]. These findings support the idea there might be a clinical relevance of PAPC and Nlk interaction and Nlk´s localization.

To assess the interaction of PAPC with Nlk1 *in vivo*, I performed ISH stainings in sagittal sections of *Xenopus* gastrula embryos, and a clear expression overlap between Nlk1 and PAPC was evident in dorsal marginal zone (DMZ) (Fig. 12). DMZ is also the site for expression and activity of multiple Wnt regulators [16,45,80,92], and expression of Nlk and PAPC in the same region suggests a possible role of this interaction in the

regulation of multiple Wnt pathways. This is particularly intriguing because the role of Nlk1 in early *Xenopus* development and especially Wnt/PCP has not been established.

5. 2 Nlk1 stabilizes PAPC independently of its kinase domain

Nlk is known to regulate the activity of different proteins in a phosphorylation-dependent manner. For example, Nlk1 regulates β-catenin dependent Wnt signaling by phosphorylating β-catenin, and Nlk can phosphorylate NotchICD1 to regulate neurogenesis during *Zebrafish* development [71,86]. Nlk1 can also regulate the proteasomal degradation of some proteins. For example, Nlk can induce NARF-dependent TCF/LEF degradation, thereby inhibiting Wnt signaling [72].

Because Nlk1 was found to interact with PAPC, it may thus similarly regulate PAPC stability by protein modification. However, I did not observe Nlk1-dependent PAPC phosphorylation (Fig. 23 + 24), suggesting that Nlk1 is not a PAPC kinase. Indeed, my data show that Nlk1 and PAPC mutually stabilize each other, even when Nlk1 is replaced with a kinase-negative variant (Fig. 25 + 31). A kinase-independent role of Nlk in protein stabilization has also been previously reported [93]. Zhang et al. showed that Nlk inhibits MDM2-dependent ubiquitination and degradation of p53, presumably by blocking the MDM2 docking site in p53. It is thus feasible that Nlk1 may similarly protect PAPC from proteasomal degradation.

A recent publication has shown that PAPC undergoes GSK-3β dependent polyubiquitination [20]. Polyubiquitination of a protein can induce its proteasomal degradation. In agreement with this study, PAPCc was indeed stabilized when GSK-3β or proteasome were inhibited (Fig. 26), which suggests that Nlk1 can block PAPC/GSK-3β interaction and thereby prevent its degradation. A previous publication also showed stabilization of Nlk in PC12 cell lines following GSK-3β inhibition with LiCl [94]. Consistently, a clear reduction in polyubiquitination of PAPC and Nlk1 was observed when both the proteins were expressed together (Fig. 26).

Apart from sending proteins to degradation, ubiquitination can also change a protein's localization and function. Indeed, Kai et. al. further showed that polyubiquitination is

needed for PAPC membrane localization[20]. As my data show a reduced ubiquitination of PAPC in presence of Nlk1, Nlk1 gain-of-function should therefore lead to a loss of PAPC membrane localization, which was not observed here. As a possible explanation, I suggest that Nlk1 not only inhibits PAPC ubiquitination, but also physically interacts with polyubiquitinated PAPC at the membrane to prevent its proteasomal degradation. Stabilized PAPC protein can then interact with multiple additional interaction partners and regulate gastrulation.

One aspect that was not investigated in this study is the known function of Nlk as a transcriptional co-regulator [65,89]. It may be possible that Nlk1 controls PAPC levels additionally by transcriptional regulation. However, the observations that Nlk1 and PAPC physically interact, and that this interaction reduces the ubiquitination of both proteins, makes this possibility unlikely.

5. 3 Nlk1 regulates *Xenopus* gastrulation via Wnt/PCP signaling

As mentioned before, the role of Nlk1 during *Xenopus* gastrulation has not been explored. Wnt pathways are important regulators of vertebrate gastrulation [21,39,95], and the Wnt/Planar cell polarity (PCP) pathway in particular has emerged as a key regulator of *Xenopus* gastrulation [96,97]. During *Xenopus* development, Nlk1 is involved in negative regulation of Wnt/β-catenin signaling and a downstream component of Wnt/Ca^{2+} signaling [53,65], but whether Nlk1 is involved in Wnt/PCP signaling is not known. In this thesis I demonstrate that Nlk1 regulates Wnt/PCP during *Xenopus* gastrulation (Sec. 4.2).

PAPC expression during *Xenopus* development starts soon after mid-blastula transition (MBT). Consistent with PAPC protein stabilization by Nlk1, endogenous PAPC protein levels were also found to be sensitive to Nlk1 gain and loss-of-function (Fig. 23). An expression overlap of PAPC with Nlk is already seen at gastrula (Fig. 12), which suggests Nlk-dependent PAPC stabilization early in development. Considering the central role of PAPC protein during gastrulation regulation [18,45,47,61], a loss of Nlk1 should therefore resemble a loss of PAPC, i.e. it should induce gastrulation defects.

Indeed, loss of Nlk1 phenocopied loss of PAPC in *Xenopus* embryos (Fig. 15). Conversely, overexpression of wild-type or kinase-dead Nlk1 stabilized PAPCc in *Xenopus* embryos and promoted gastrulation, showing that Nlk1 kinase activity is indeed dispensable for its regulation of Wnt/PCP signaling and gastrulation movements (Fig. 31, 33 and 34).

The data suggest that Nlk1 promotes *Xenopus* development by maintaining proper gastrulation and this property in part is attributed to Nlk1 interaction and stabilization of PAPC. It should be noted, however, that the involvement of Nlk in other signaling pathways, such as TGF-β and Notch signaling, may also contribute to the observed gastrulation defects [85,86].

5. 4 Putative competition of Nlk1 and GSK-3βto regulate PAPC protein stability

According to the evidence provided in this study, Nlk1 does not appear to phosphorylate PAPC itself, since there was no phospho-shift of PAPC observed (Fig. 23 + 24) and the kinase activity of Nlk1 seemed dispensable for PAPC stabilization (Fig. 31). On the other hand, mutating the putative Nlk1 phosphorylation sites in the C-terminus of PAPC affected PAPCs protein stability and function (Sec. 4.5). Here, the data seem to be conflicting. However, the sites altered in the PAPC phospho-mutants are not merely Nlk1 sites, but could also be targeted by other MAP kinases, such as GSK-3β. Interestingly, GSK-3β showed an antagonistic effect on PAPC compared to Nlk1 (Fig. 24) which points towards a possible competition between Nlk1 and GSK-3β for PAPC binding. Combining the preliminary results obtained using the PAPC phospho-mutants and the kinase inactive Nlk1, one could speculate that Nlk1 binding protects PAPC from being phosphorylated and primed for polyubiquitination mediated proteasomal degradation by GSK-3β. This is supported by the observation that the phospho-null mutant of PAPC, which also cannot be phosphorylated by GSK-3β, appeared to be more stably expressed and was not sensitive to Nlk1. If the PAPC mutant can't be phosphorylated by GSK-3β, it should not be polyubiquitinated and hence this PAPC mutant should not need to be protected by Nlk1. However, the data provided need to be confirmed by additional experiment, as for example I did not address if these residues

are indeed phosphorylated by GSK-3β. It is furthermore possible that other proline-directed kinases with well-defined roles in development, such as JNK, CDKs, or Nlk2 [98–100]may phosphorylate PAPC in addition to or instead of GSK-3β. However, this hypothesis remains to be addressed in future studies.

Nevertheless, the interaction of PAPC with GSK-3β and Nlk1 raises some very challenging questions. Being a central part of β-catenin destruction complex, GSK-3β is a key regulator of canonical Wnt/β-catenin signaling. If interaction of GSK-3β with PAPC is independent of GSK-3β localization in the destruction complex, then one might expect no effect on Wnt/β-catenin signaling. However, if GSK-3β interaction with PAPC disrupts the destruction complex, then there will be a positive regulation of Wnt/β-catenin signaling, but previous studies have shown a negative role of PAPC on canonical Wnt pathway [58]. There is a possibility that the interaction of GSK-3β with PAPC is independent of destruction complex and the effect on Wnt/β-catenin signaling is indirectly mediated because of upregulation of non-canonical Wnt signaling. This is an exciting issue which should be addressed in later studies.

5. 5 The Nlk1 and PAPC interaction regulates different Wnt pathways through multiple mechanisms

The presence of PAPC protein during early gastrulation is important, as the interaction of PAPC with different proteins fine-tunes Wnt signaling and hence regulates gastrulation [45,47,57,58]. Later in development PAPC is required for somitogenesis, which also involves PCP signaling [101,102]. Similarly, Nlk appears to be required for Wnt/PCP regulation (this work) and for canonical Wnt inhibition and anterior formation [66,67,71]. Our data show an early expression overlap of Nlk and PAPC and also, the two proteins physically interact and stabilize each other.

During gastrulation, PAPC and Fz7 interaction leads to Wnt/PCP activation and cytoskeleton rearrangement [46]. Dynamic interaction of this Wnt11+Fz7 complex with PAPC was found to be responsible for the down-regulation of cadherin-mediated adhesion [45]. Functionally, this interaction activates the JNK pathway, Rho GTPase

signaling, and thus the Wnt/PCP pathway, which leads to cytoskeleton rearrangement and cell migration. Down regulating cadherin-mediated adhesion induces proper tissue separation [80]. Considering a positive role of PAPC in regulating the Wnt/PCP pathway, the interaction of Nlk1 and PAPC would therefore serve as a positive regulator of Wnt/PCP pathway and gastrulation.

PAPC intracellular domain, which interacts with Nlk1, also interacts with other proteins including ANR5, CK-2β, Spry2 to regulate gastrulation movements [56–58]. ANR5 is a FGF target gene and its interaction with PAPC leads to a proper Rho-JNK activation and hence cell movement [57]. Spry2 inhibits FGF and Wnt/PCP signaling [103,104]. PAPC interacts with Spry2 and this interaction inhibits Spry2-mediated Wnt/PCP inhibition [56], and may induce FGF signaling. It is thus possible that FGF pathway activation would then induce ANR5 expression and hence promote PAPC interaction with ANR5. All of these events may eventually help to maintain proper gastrulation and positive regulation of Wnt/PCP pathway.

The PAPC interaction with CK2β is the first direct evidence of PAPC negatively regulating canonical β-catenin Wnt signaling [58]. CK2 is a positive regulator of canonical Wnt pathway, and it is composed of two catalytic active "α" or “α´” and two regulatory "β" subunits[105]. Positive regulation of canonical β-catenin Wnt signaling requires the holoenzyme[58,59]. An interaction of CK2β with PAPC inhibits holoenzyme formation and thus inhibits canonical Wnt pathway. There are multiple reports confirming an antagonism between canonical and non-canonical Wnt pathways [106–108]. Hence a negative regulation of CK2β might positively regulate Wnt/PCP pathway which is the key regulator of vertebrate gastrulation [19,96,97]. In summary, PAPC and Nlk1 interaction during gastrulation should have a positive impact on the Wnt/PCP pathway through CK2, although this hypothesis remains to be formally tested.

In a cell culture based model, Wnt5a has been implicated in induction of both Wnt5a/Ror as well as Wnt-Ca2+ signaling depending on the receptor context [109]. Wnt5a/Ror signaling induces *papc* expression [55]. Since Nlk1 increased the level of PAPC protein, it might thus enter into a negative feedback loop that negatively regulates Wnt5a/Ror signaling. However, this question has not been addressed in the present

thesis. Conversely, Nlk1 has been reported to be a downstream activator of Wnt-Ca^{2+} signaling and hence a negative regulator of canonical Wnt pathway[53].As PAPC stabilizes Nlk1 protein, it might thus indirectly act as a positive regulator of Wnt-Ca^{2+} signaling.

In summary, the data I present here suggest that PAPC and Nlk1 can be considered as regulators of gastrulation in *Xenopus*. The identification of Nlk1 as a PAPC stabilizing factor is therefore of considerable interest for developmental biology, but may also be of relevance for other biological questions as well. For example, human homologs of these proteins are implied in different pathologies. There is increasing evidence of Nlk involvement in disease [110], and particularly in cancer (Sec. 3.5.1). Similarly, there is growing data suggesting the involvement of protocadherins, specially pcdh8 (human PAPC homolog), in human pathologies like cancer [91,111–113]. Therefore, in depth study of PAPC/Nlk1interaction in humans might serve as crucial step in understanding the molecular mechanism of pathology and some probable therapeutic target.

6. Materials and Methods

6. 1 Materials-

6. 1. 1 Antibodies

Antibody	Source organism	Provider
α-Digoxigenin-AP	Sheep	Roche
α-Flag M2	Mouse	Sigma
α-Flag OctA probe	Rabbit	Santa Cruz
α-Myc 9E10	Mouse	Calbiochem
α-Myc	Rabbit	Merck
α-HA	Mouse	Sigma
α-Rabbit alexa 488	Goat	Invitrogen
α-Mouse alexa-488	Goat	Molecular probes
α-Rabbit alexa-555	Goat	Invitrogen
α-Mouse alexa-555	Goat	Invitrogen
α-HRP-Rabbit	Goat	Bio Rad
α-HRP-Mouse	Goat	Bio Rad
α-GFP polyclonal	Rabbit	Abcam
α-GAPDH	Rabbit	Synaptic system
α-Tubulin	Rabbit	Cell Signaling

6. 1. 2 Buffers and Solution

Buffer	Composition
10% BBR	10 % Boehringer blocking reagent, 1x MAB
6X SDS loading buffer	40% glycerol, 0.25% Bromophenol Blue
Agarose gel- TBE (tris-borate EDTA) buffer (1x)	8.9mM Tris, 8.9mM Boric acid, 0.2mM EDTA
DMEM high medium	Sigma
Elution buffer-magnetic beads	0.2M glycine, pH 2.8
Embryo dejellying solution	2% L-cysteine, pH 7.8-8
Embryo lysis-NP 40 buffer	0.5% NP-40, 20% glycerol, 50 mM-Tris-cl, 150 mMNaCl
IF-Blocking solution	20% Normal goatserum in TBS
IP Cell lysis buffer	1% Triton X 100, 2 mM beta mercaptoethanol and 1mM $MgCl_2$
ISH-Blocking solution	2% BBR, 20% sheep serum in MABT
ISH-Hybridization Buffer	1% BBR, 1 mg yeast tRNA, 0.1 mg heparin, 5x SSC, 50% formamide, 0.1 % Tween-20, 0.1 % Chaps, 5 mM EDTA
ISH-MAB (1x)	0.1 M maleic acid, 0.15 M Nacl, pH 7.5
ISH-MABT	100 mM Maleic acid, 150 mM NaCl,0.1% Tween, pH 7.5
LB-media	1% NaCl, 1% Bactotrypon, 0.5% yeast extract
MBSH (1x)	88mM NaCl,1mM KCl,2.4mM $NaHCO_3$, 0.82mM $MgSO_4$, 0.41mM $CaCl_2$,0.33mM $Ca(NO_3)_2$, 10mM HEPES pH 7.4,10ug penicillin
MEM	2mM EGTA, 0.1M MOPS, 1mM MgSO4 pH 7.4
MEM (1x)	0.1 M MOPS, 2mM EGTA, 1mM MgSO4 pH 7.4
MEMFA	MEM (1x), 3.7% formaldehyde
Mowiol-IF	20 mg Mowiol, 50 ml Glycerol
PBS (1x)	2.7mM KCl, 2 mM KH2PO4, 13.7mM

	NaCl, 10mM Na2HPO4 x 2H2O
pH 9 buffer	50mM MgCl2, 0.1 M NaCl, 0.1 M Tris pH 9.5
TBS (1x)	5 mMTris-HCl, pH 7.4, 15mMNaCl
Ubiquitination Assay- cell lysis buffer	50 mMTrisCl pH 7.5, 0.8%NP 40, 1 mM beta mercaptoethanol, 10 mMNaf, 150 mM NaCl,10 mMN-Ethylmaleimide
Western blot- Blocking buffer	5% Skim milk in TBST (0.1%-tween)
Western blot- Running buffer	0.1% SDS, 24.8mMTris, 192mM glycine
Western blot- Transfer buffer	24.8mMTris, 192mM glycine, 15-20% methanol

6. 1. 3 Cells and Bacteria

1. Hek293 cells- protein overexpression experiments
2. E. coli XL1 (chemocompetent bacterial cell) - plasmid transformation
3. DH10B (electrocompetent bacterial cell) - plasmid transformation

6. 1. 4 Enzymes, inhibitors,Kits and markers

Enzymes, inhibitors, Kits and markers	Provider
10x protease inhibitor complete Mini	Roche
Absolute QPCR SYBR® Green	Thermo Scientific
BM Purple	Roche
DIG-RNA labelling mix	Roche
DNAse I	Fermentas
Dual luciferase reporter kit	Promega
Fast digest™ Restriction enzyme	Fermentas
GeneRuler™ 100bp and 1kb Ladder	Thermo Scientific

Human chorionic gonadotropin (HCG)	Sigma
Lumi-Light^{PLUS} Western Blotting Substrate®	Roche
MasterPureTM Complete DNA and RNA Purification	Epicentre Biotechnologies
Midori Green Advanced (DNA staining dye)	Nippon
mMessagemMachine Capped RNA transcription kit (Sp6,T7)	Ambion
N-Ethylmaleimide	Fermentas
Nucleotide removal kit	Qiagen
PageRulerPrestained protein ladder	Fermentas
PCR purification kit	Quigen
Pierce® BCA Protein Assay Kit	Thermo Scientific
Proteinase K	Sigma
RiboLockRNAse inhibitor	Fermentas
RT Maxima Reverse Transcriptase	Fermentas
T4 DNA Ligase	Fermentas
Trypsin	PAA
TurboFect™ Transfection reagent	Thermo Scientific
CHROMASOLV	Sigma

6. 1. 5 Oligonucleotide

Site directed mutagenesis primers

For PAPC Phospho-Mimic

Primer Name	Primer Sequence (5' to 3')
A2887G_G2888A_ANTISENSE	5'-TGTCTTGATATCTGCGGATCGTAATTGAATTCTTGGTATCTGCTAGACG-3'
T2845G_C2846A_ANTISENSE	5'-ACGATCTCTGCGGATCTAACGTTGCACTTCTGTTGACTAAATC-3'
T2845G_C2846A	5'-GATTTAGTCAACAGAAGTGCAACGTTAGATCCGCAGAGATCGT-3'
A2887G_G2888A	5'-CGTCTAGCAGATACCAAGAATTCAATTACGATCCGCAGATATCAAGACA-3'

For PAPC Phospho-null

PRIMER NAME	PRIMER SEQUENCE (5' TO 3')
A2887G_G2888C_ANTISENSE	5'-GTCTTGATATCTGCGGAGCGTAATTGAATTCTTGGTATCTGCTAGA-3'
T2845G_C2846C_ANTISENSE	5'-ATCTCTGCGGAGCTAACGTTGCACTTCTGTTGAC-3'
A2887G_G2888C	5'-TCTAGCAGATACCAAGAATTCAATTACGCTCCGCAGATATCAAGAC-3'
T2845G_C2846C	5'-GTCAACAGAAGTGCAACGTTAGCTCCGCAGAGAT-3'

Above, numerals represent the position of amino acid to be mutated. Alphabet on left of numeral shows the nucleotide to be modified into nucleotide on right.

qPCR primers

nr3 fwd: 5´CCAAAGCTTCATCGCTAA AAG3´
nr3 rev: 5´AAAAGAAGGGAGGCAAATACG3´

siamois fwd: 5´TCTGGTAGAACTTTACTCTGTTT3´
siamois rev: 5´AACTTCATGGTTTTGCTGACC3´

xbra fwd: 5´TTCAGCCTGTCTGTCAATGC3´
xbra rev: 5´TGAGACACTGGTGTGATGGC3´

nlk1 fwd: 5´TGCCCTGGCCCACCCCTATC3´
nlk1 rev: 5´GTGGCCGTGGGCTCGAAGTC3´

***odc* fwd:** 5'GTCAATGATGGAGTGTATGGATC3'
***odc* rev:** 5'TCCATTCCGCTCTCCTGAGCAC3'

6. 1. 6 Antisense Morpholino oligonucleotides

The translational blocking morpholino oligonucleotides used in the study were ordered from Gene Tool, LLC. Both the morpholino(PAPC MO and Nlk1 MO) used in the study were already reported in previous publications [47,66].

6. 1. 7 Plasmids

Plasmid	Source
ATF2 reporter	Ohkawara and Niehours, 2011
pCS2+ membrane-RFP	Iioka et al., 2004
pCS2+ Nlk1-Flag	Dr. Shibuya
pCS2+GFP	Prof.BarbaraHölscher
pCS2+M-PAPC	Kim et al., 1998
pCS2+M-PAPC-Myc	C.Berger (Steinbeisser)
pCS2+Nlk1	BeseiOkhawara
pCS2+Nlk1 KN	BeseiOkhawara
pCS2+Nlk1 KN-Myc	Ligated Nlk1 KN into pCS2-Myc vector
pCS2+PAPC	Medina et al., 2004
pCS2+PAPCc-Flag	Wang et al., 2008
pCS2+PAPCc-Myc	Dr. C.Berger (Steinbeisser)
pCS2+PAPCcΔ899	Wang et al., 2008
pCS2+Wnt11	Du et al., 1995
pSP64T-Bvg1	D. Melton

6. 1. 8 Equipment

Name	Supplier
5415 D tabletop centrifuge	Eppendorf
Agarose gel chambera	PeqLab
Axiophot stereomicroscope	Zeiss
Blotting apparatus	PeqLab
Cell culture dishes, flasks and plates	Greiner Bio-One
Cold plate	Julabo
Conical tubes	Falcon
DC350FX camera	Leica
HERAcell 150i CO_2-incubator	Thermo Scientific
IM300 Microinjector	Narishige
Lucy 2	AnthosMikrosystems GmbH
Micromanipulator	Micro Instruments
ND-1000 Spectrophotometer	NanoDrop
Pipettes	Gilson
PROTRAN Nitrocellulose Transfer Membrane	Whatman
Thermoshaker	Biometra
Ti inverted microscope	Nikon
Waterbath WB 10	MemMert

6. 1. 9 Software and programs

Microsoft Word	Microsoft
Image J	NIH
Adobe Illustrator	Adobe
Adobe Photoshop	Adobe
Microsoft Excel	Microsoft
MendeleyRef.Manager	Mendeley
Phosphosite	Prediction of phosphorylation modification
netPhosK2	Kinase site prediction
ClustalW	Protein sequence alignment

6. 2 Molecular biology methods

6. 2. 1 Transformation

Chemical transformation was preferentially done to amplify freshly ligated or mutated plasmid constructs. 50-100 µg of ligation reaction/plasmid was mixed with XL1 chemically competent bacterial cells and kept on ice for 40 minutes. Then, after heat shock at 42ºCfor 90 sec., they were cooled down on ice for 5 minutes. Bacterial cells were then mixed with 250µl of prewarmed SOC medium or LB medium for bacteria to start growing and. After growing for 45 minutes to 1 hour, cells were spreaded on LB ampicillin agar plate to start the amplification of the antibiotic resistance carrying vector and cultured for 12 to 4 hours at 37ºC.

6. 2. 2 Plasmid DNA isolation

Plasmids or ligation products were transformed in XL1 bacterial cells with amp^R gene. A mini culture (5ml LB media) is established by growing bacterial colony for 6 hours at 37ºC with 250 rpm shaking followed by overnight culture in 200 ml LB media at 37ºC with 250 rpm shaking. Plasmid extraction was done using Midi-prep kit (QiaQuick DNA extraction kit) by following instruction manual provided. The concentration of the DNA was measured using nanodrop.

6. 2. 3 Restriction digestion

Restriction enzymes are prokaryotic endonucleases used by bacteria for defense against bacteriophage. They are widely used in molecular biology for cleaving DNA in a sequence dependent manner. Plasmid digestions for the study were done using Fast Digest restriction enzyme and following product usage manual. Time and temperature for digestion and subsequent heat inactivation were dependent on the amount of plasmid and the enzyme used.

6. 2. 4 Phenol Chloroform extraction of nucleic acid

Phenol chloroform extraction is the preferred method to separate nucleic acid from proteins and lipids. To the aqueous solutioncontaining nucleic acid, protein, lipids, 1/10 volume sodium acetate (3M) was added and mixed. Equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to the tube and mixed. Samples were centrifuged at 13000 rpm for 10minat 4ºC. Supernatant was transferred to a new tube and equal amount of chloroform:isoamylalcohol (24:1) was added. Centrifugationwas performed again at 13000 rpm for 10 minutes. The upper phase was transferred to a new tube and equal amount of isopropanolwas added to the tube. Samples were kept at -20ºC for 2hours (at least). Afterward, centrifugation was done for 30 minutesat 4ºC, and the resultant pellet was mixed with 1ml 70% alcohol followed by centrifugation again at 4ºC for 20 minutes

Pellet was air dried and mixed with desired amount of water or TE buffer.

6. 2. 5 Synthetic mRNA (Cap RNA) synthesis

Synthetic mRNA was used for protein overexpression in *Xenopus* embryo. I usedmMessage®mMachine (Ambion) RNA transcription kit for cap RNA synthesis by following the user manual for 10µl reaction.

Linearized plasmid	1 µg
dNTP mix (2x)	5 µl
Reaction buffer (10x)	1 µl
Enzyme (SP6/T7)	1 µl
Water	x µl to total 10µl

The reaction was performed at 37ºC for 3hours. After 3 hours the DNA template was degraded using DNAse I. The reaction was then stopped using 1/10 volume ammonium acetate. Followed by stopping; precipitation of RNA was done using phenol chloroform extraction method.

6. 2. 6 Dig RNA synthesis

Dig RNA was used to detect the endogenous RNA during ISH. A complementary RNA against target RNA is made and probed with digoxigenin, the complementary RNA is called Dig RNA. The Dig labelling mix and RNA polymerases were purchased from Roche. Linearized DNA was used as template and following reaction was performed:

linearized plasmid (raise volume to 18µl)		1 µg
10x labeling mix		2.5 µl
10x polymerase buffer		2.5 µl
RNAse inhibitor		0.5 µl
RNA polymerase		1.5 µl
Total volume	=	25 µl

The reaction was carried out for 3hours at 37ºC. After the completion of reaction, sampleswere precipitated by adding 1.5 µl 7.5M LiCl and 70 µl ice cold 100% ethanol. The samples were incubated at -20ºC for 1hr followed by centrifugation at 13000 rpm. The Dig RNA pellet is dissolved in 20µl 2x SSC/10% formamide solution.

6. 2. 7 Agarose gel electrophoresis

0.8-2% of agarose gels were used in different experiment to separate and visualize different DNA or RNA fragments of samples. Gels were prepared using TBE buffer. Midori green direct (DNA staining dye) was added directly to the gel to visualize the samples using UV light exposure.

“100bp plus” or “1kbp”DNA ladder (Fermentas)were used as reference. Samples were diluted with 6x loading dye (Fermentas). The samples were run at 120V for 30 minutesfollowed by gel analysis under UV light.

6. 2. 8 Reverse transcription reaction

For obtaining cDNA to perform qPCR on the RNA sample, I performed reverse transcriptase reaction. The reaction was performed using a thermocycler (MJ-Research, DNA Engine). RNA was reverse transcribed using random hexamer and the reverse

transcriptase from RT-Maxima kit (Fermentas) was used. The components were mixed according to the user manual and the following program was used for the reaction-

Temp	Time
25 ºC	10 min
50 ºC	30 min
85 ºC	10 min
4 ºC	∞

cDNA samples were stored at -20ºC for future use.

6. 2. 9 Quantitative real time PCR

qPCR was performed to investigate the transcription expression of certain targets. SYBR green was used for detection of amplified protein. Real time PCR thermocycler from Analytic Jena was used to carry out the qPCR reaction. Componenets were mixed as per instinctual manual and following program was followed for the amplification of target gene.

Cycle	**Temp**	**Time**
	95ºC	2 minute
40x	95 ºC 60 ºC	5 second 15 second
	Melt	6 second

6. 2. 10 PCR - site directed mutagenesis

Site directed mutagenesis was performed for creating the phospho-null and phospho-mimic mutants of PAPC. The primers used are listed in Section 6.1.5. The Quikchange kit (Agilent technologies) was used to induce desired mutations at two different locations

according to the kit manual. Primer designing was also done using online program from Agilent technologies.

6. 2. 11 Cell culture and cell transfection

Hek293 cells were used in the present study for overexpression studies. DMEM high glucose media with antibiotics (1% PS-penicillin/streptoMycin) and fetal calf serum (10%) was used for culturing cells. Trypsin EDTA (1x) was used for reseeding cells after they attained 80-90 % confluency.

Cells were transfected using TurboFect Transfection reagent (Thermo Scientific) by following the provided protocol. Amount of plasmid transfected in each well in all experiments was normalized using empty vector or GFP carrying vector. Protein isolation was done 24 to 48 hours post transfection. For some experiments certain inhibitors were used, for example, MG132 (proteasome inhibitor) or LiCl (GSK-3β). For the amounts added see the respective figure legends. Inhibitors were added 4hours prior to protein isolation.

6. 2. 12 Protein isolation from Hek293 cell

Protein isolation was done 24-48 hours. post transfection. Cells were washed with PBS (2x-2minutes). Lysissolution with protease and phosphatase inhibitor (both 1x- diluted as manufacturers recommendation) was poured on to cells and incubated on shaker for 10 minutes at 4ºC. Cells were then scraped with lysis buffer in 1.5 ml tubes and centrifuged for 20 minutes at 13000 rpm at 4ºC. The supernatant containing protein was collected and placed at -80ºC for future use.

6. 2. 13 Protein isolation from embryos

7-10 *Xenopus laevis* embryos were selected for isolation of protein. NP-40 lysissolution was preferred for embryo lysis. Protease and phosphatase inhibitors were added to lysis solution. Embryo lysate was placed at -80ºC for at least 1hr for improved protein

extraction. Lysate from -80ºC were thawed and centrifuged at 13000 rpm for 10 minutes. Supernatant was collected and mixed with an equal amount of CHROMOASOLV (sigma). Centrifuged was done again and the clear supernatant with protein was collected and stored at -80ºC for future use.

6. 2. 14 Protein immunoprecipitation

Protein immunoprecipitation was done using either magnetic bead or with agarose beads with already coupled antibody. For magnetic beads, protein lysate was mixed with corresponding antibody (1µg) and incubated for 2-3hours at 4ºC. Magnetic beads were equilibrated with lysis buffer and mixed with protein lysate. The mixture was then incubated at 4ºC overnight. Agarose beads were equilibrated with protein lysate solution directly and then mixed and incubated overnight at 4ºC.

Next day, proteins with beads were washed 3x with lysissolution. Proteins were eluted with elution buffer (50µl), mixed with 6x SDS laemmli buffer and heated at 95ºC for 3 minutes. The immunoprecipitated proteins were then loaded on a SDS-PAGE gel.

6. 2. 15 SDS-PAGE and Immunoblotting

Proteins were separated using denaturing SDS polyacrylamide gel electrophoresis (PAGE). 4% stacking and 10% or 15% separating gels were used for protein separation. Samples were loaded along with pre-stained molecular weight standard (Fermentas). Proteins were separated in stacking gel using a constant voltage of 100V and with 180V in separation gel.

Once the proteins had sufficiently migrated, the electrophoresis was stopped. The western blotting was performed using wet blot transfer method. Proteins from the gel were transferred onto nitrocellulose membrane for 90 minutes at 120ºC. After the blotting was completed the membrane was blocked for 1hr using blocking solution (5% skim milk in TBST-0.1%tween). Membrane was then incubated overnight at 4ºC with primary antibody. Next day, the blot was washed 4x10 minutes with TBST, followed by

secondary antibody incubation for 1hr in blocking solution. Membranes were washed again for 4x10 minutes. The membrane with secondary antibody was then incubated with Lumi-Light^{PLUS} Western Blotting Substrate. The blots were developed using ECL for protein visualization.

6. 3 *Xenopus* embryological methods

6. 3. 1 *Xenopus* priming

Xenopus laevis were used as animal model in the present study. The frogs were obtained from nasco.12-15 hours before collecting eggs, *Xenopus* female frog was primed by injecting with 400-500 IU of human chorionic gonadotropin (Sigma). The female was then kept in dark for approximately next 12 hours.

6. 3. 2 Collecting eggs, *in vitro* fertilization (IVF) and culture

Xenopus eggs were obtained from primed female the day after priming by squeezing the female frog around belly before IVF in a petridish. The sperms were obtained by dissecting a small piece of testis (previously isolated and preserved) in 1xMBSH solution. The sperm solution was mixed thoroughly with the eggs and allowed to stand for a minute. Followed by this, water was filled in the petridish submerging the embryos below. Successfully fertilized embryos oriented themselves as vegetal (light side) down and dorsal (lighter side) up about 20-30 minutes later. 1hr after fertilization, embryos were washed with 2% cysteine solution (pH 7.8-8) for a few minutes to remove the jelly from the embryos.

Unjellyed embryos are then selected for microinjections.

After microinjection embryos were cultured till MBT (stage 8) in 1xMBSH and then transferred to 0.1xMBSH. The embryos were grown at 14ºC to 20ºC. Embryo for phenotypic analysis were collected at stage 35 (unless otherwise stated) and fixed in MEMFA.

6. 3. 3 Microinjections

In the present study embryos were injected with either morpholino oligonucleotide or synthetic mRNA or a combination of both, as indicated in the respective figure legends. Embryos were cultured till stage 4 and embryos with clear dorsal light and ventral dark zone were selected for injections. Injections were made using IM300 microinjector. For injections embryos were placed in petridish filled with 1% agarose (1x MBSH). The injections were made in 1xMBSH solution. The Injected embryos were cultured in 1xMBSH for atleast 1 hr or till MBT and then transferred to a low salt 0.1xMBSH agarose-dishes and buffer.

6. 3. 4 Animal cap explants

Animal cap explants were used for animal cap elongation assay or RNA extraction. Injections were made into two ventral blastomere at 4 cell stage and embryos were cultured till stage 9. Using fine forceps first the vitelline membrane was removed followed by animal caps excision, caps were growntill gastrula stage in a separate petridish. These caps are then either processed for immunofluorescence assay or animal cap elongation assay.

6. 3. 5 Immunofluorescence staining of *Xenopus* animal caps

1. Excised animal caps were fixed in 1% PFA (paraformaldehyde in PBS)

2. Caps were washed with TBST (0.1% tween) for 5 minutes, two times

3. After washing caps were blocked using 20% goat serum in TBST for 2hours.

4. Caps were incubated overnight with primary antibodies diluted in blocking solution (1:1000).

5. Next day, caps were washed with TBST (3x-10 Minutes), followed by 1x wash with 300 mM NaCl in TBST and again wash with TBST (3x-10 Minutes)

6. Washed caps are incubated overnight with secondary alexa antibodies.

7. Next day the secondary antibody is washed similar to point 5.

8. After washing, caps are incubated for 5 min in DAPI (2µg/ml).

9. Caps are washed with 2xTBS and fixed in mowiol on coverslip for analysis.

6. 3. 6 Animal cap elongation assay

For animal cap elongation assay, excised caps were placed in 1xMBSH (10ng/µl gentaMycin) and cultured overnight at 18ºC. For caps that werenot injected with bvg1 to induce mesoderm formation, Activin (50ng/µl) was added to 1xMBSH solution. Cap elongation was analyzed after 14hours after excision and quantified.

6. 3. 7 Whole mount in situ hybridization

Whole mount in situ hybridization is a well established method to identify the spatial expression of particular gene in embryo. In this method, a synthetic RNA which was probed with DIG/Fluorescein label is hybridized to target RNA. The probe is then identified using an antibody, and substrates like BM purple is used to localize the hybridization site. In the present study we have used ISH to identify the spatial location of PAPC and Nlk1 in sagittally sectioned gastrula embryo. After the collection of gastrula stage embryo, following protocol was followed:

DAY1-

1. Embryos were fixed in MEMFA (1xMEM+Formaldehyde) for 2 hrs, followed by overnight incubation at -20ºC in 100% methanol. Embryos can be left here until the start of protocol.

2. Embryos were rehydrated by sequential washing in 75%,50%,25% methanol.

3. Embryos were washed in PBST (2x).

4. Followed by washing embryos were treated with Proteinase K (10µg/µl in PBST) for 10 minutes

5. Proteinase K treated embryos were fixed again in 4% PBS/PFA.

6. Fixed embryos were washed again-, 4x PBST for 10 minutes , 1x PBST+hybridization buffer (1:1) for 10minutes , 1x hybridization buffer at 65ºC for 3 hr.

7. The hybridization buffer was mixed with DIG-probe (1µl/1ml) and added on respective samples for overnight at 65ºC.

DAY2-

Wash time	**Wash buffer**
1x-5min	(preheated-65ºC)50% formamid/0. 1% Chaps/5x SSC
1x-5min	25% formamid/0. 1% Chaps/3,5x SSC
1x-5min	(preheated to 37°C)2x SSC/0. 1% Chaps
2x 25min	2x SSC/0. 1% Chaps at 37°C
1x 5min	0. 2x SSC/0. 1% Chaps at 60°C
2x 30min	0. 2x SSC/0. 1% Chaps at RT
2x 5min	0. 2x SSC/0. 1% Chaps at RT
2x 10	0. 2x SSC/0. 1% Chaps:MABT (1:1) at RT
1x 10	2x 10 min MABT at RT
1x 1h	1x MABT
1x 1h	1hr in blocking solution

Incubate overnight with α-Dig-AP (1:10000) antibody in blocking solution.

DAY3:

1. Embryos washed with 1xMABT at RT 2x 30 minutes

2. Embryos washed with 4x MABT for 1hr.

3. Embryos were stained using: pH 9-buffer/BMPurple (1:1) in dark.

4. Once the embryos were stained, the staining reactio was stopped with 2xPBS - 5 minutes

5. Stained embryos were fixed in 3.7 % formaldehyde/PBS at 4ºC.

To have a better and confined staining, the embryos werebleached using 1% H_2O_2/5% formamide in 0.5x SSC under intensive light.

6. 3. 8 ATF2 reporter assay

For ATF-2 Luciferase reporter assay, 4 cell stage embryos were injected dorsally with 100pg of ATF-2 luciferase reporter in combination with 20 pg of TK-Renilla luciferase reporter plasmid. The reporter plasmid were injected either alone or in combination with morpholino or synthetic RNA (as indicated in Fig. legends). Embryos were collected at stage 12 in triplicate of 5 embryo from each sample and lysed using 100 ul of Passive Lysis buffer (Promega). 20ul of the lysate was used to quantify the luciferase reporter activity using the Dual luciferase system (Promega).

6. 3. 9 Brachet's cleft analysis

For brachet's cleft analysis, injected embryos were cultured till stage 10.5 when blastopore lip clearly appears. Embryos were fixed in MEMFA (1xMEM+Formaldehyde) for 2 hours. Fixed embryos are equilibrated in PBS before cutting. A sagittal cut is made passing through the centre of blastopore. Any half of the embryo can be analyzed for brachet's cleft.

6. 3. 10 RNA extraction

RNA extraction was done from either the whole embryo or animal caps. RNA extraction was done using MasterPure™ RNA Purification Kit (Epicenter Biotechnologies) following the manual instructions. DNAse digestion was however prolonged to 1hr.The quality of extracted RNA was analyzed on agarose gel, and the quantification was done using nanodrop according to manufacturer's instructions. The RNA was stored at -80ºC for future use.

7 Appendix

7. 1 List of Abbreviations used:

Abbreviation	Expansion
Ab	Antibody
AC	Animal Cap
Amp	Ampicillin
APC	Adenomatous Polyposis coli
APS	Ammonium per sulphate
ATF2	Activating transcription factor 2
BMP	Bone morphogenetic protein
CaM Kinase	Ca2+ /calmodulin-dependent protein kinase
cDNA	complementary Deoxyribonucleic acid
CREB	*cAMP response element-binding* protein
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DMZ	Dorsal marginal zone
DNA	Deoxyribonucleic acid
Dvl	Dishevelled
E. coli	*Escherichia coli*
EC	Extracellular cadherin (domain)
FA	Formaldehyde
Fz	Frizzled
GFP	Green fluorescent protein
GSK	Glycogen synthase kinase

hCG	Human chorionic gonadotropin
HDAC	Histone Deacetylase
Hek	Human embryonic kidney
HRP	Horse radish peroxidase
IF	Immunofluorescence
IP	Immunoprecipitation
ISH	*in situ* hybridization
JNK	c-jun N-terminal kinase
LDLR	Low-density-lipoprotein receptor
LEF	Lymphocyte enhancer factor
LRP	Low-density-lipoprotein receptor related protein
MAPK	Mitogen activating proteinkinase
MBT	Mid-blastula transition
MEM	Modified Eagle´s Medium
Mo	Morpholino
mRNA	messenger Ribonucleic acid
NARF	Nlk associated ring finger protein
NEM	*N-Ethylmaleimide*
NFAT	Nuclear factor associated with T-cells
Nlk	Nemo like kinase
NPC	Neural progenitor cells
nr3	Nodal related 3
NSCLC	Non small cell lung cancer
ODC	Ornithine decarboxylase
PAGE	Polyacrylamide gel electrophoresis
PAPC	Paraxial protocadherin

PBS	Phosphate buffer saline
Pcdh8	Protocadherin8
PCP	Planar cell polarity
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcription-PCR
RFP	Red Fluorescent protein
RhoA	Ras homolog gene family member A
RNA	Ribonucleic acid
rpm	Rotation per minute
SDS	Sodium dodecyl sulfate
STAT	Signal transducer and activator of transcription
TAB	TGFβ-activated kinase 1 (TAK1)-binding proteins (TAB)
TAE	Tris/acetate/EDTA
TAK	TGFβ-activated kinase
TBE	Tris/borate/EDTA
TBS	Tris buffer saline
TCF	T-cell factor
TEMED	N,N,N',N'-Tetramethylethylendiamine
Ubi	Ubiquitin
VMZ	Ventral marginal zone
Wnt	*Wingless*; *Int*
wt	Wild type
Y2H	Yeast-two-Hybrid

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