2019

Canonical Wnt Mechanisms in Neural Crest Induction

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Canonical Wnt Mechanisms in Neural Crest Induction

Mark Perfetto

Dissertation submitted
to the Eberly College of Arts and Sciences
at West Virginia University

in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy in
Biology

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Morgantown, West Virginia
December 2019

Keywords: Canonical Wnt Signaling, ADAMs, Akt signaling, neural crest

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ABSTRACT

Canonical Wnt Mechanisms in Neural Crest Induction

Mark Perfetto

Canonical Wnt signaling is a pathway that is critical for normal development and the progression of disease. The canonical Wnt signaling pathway was put together carefully by synthesizing decades of research. Over those decades, canonical Wnt signaling was found to be crucial for nearly all aspects of development, but of importance to this thesis, is a key regulatory factor for the development of the highly migratory multipotent stem cells, the neural crest. As our knowledge of the importance of canonical Wnt signaling grew, research is being conducted to further our understanding of how canonical Wnt signaling communicates with other signaling pathways. Over my Ph.D., we have provided new mechanisms for how canonical Wnt signaling cross-talks with Ephrin and Akt signaling. Here, I discuss how EphrinB signaling inhibits canonical Wnt signaling during neural crest induction and in human cell culture. Within that project, we found that the metalloproteinase ADAM19 protects ADAM13 during neural crest induction to inhibit EphrinB signaling, thus activating canonical Wnt signaling. I also discuss how Akt signaling is necessary for neural crest induction to activate canonical Wnt signaling. Within that project we found that the RNA-helicase DDX3 is required for Rac1 expression during neural crest induction to activate Akt signaling and thus activate canonical Wnt signaling. Finally, I discuss the impact of connecting canonical Wnt signaling with Ephrin and Akt signaling in neural crest, development, disease, and potential new discoveries and research.

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Acknowledgements

This was hard. This was really hard. I absolutely love science and writing this dissertation challenged me in ways I had not expected and I have grown as a scientist and person because of it.

To my advisor Shuo Wei. You have taught me so much about science, but more importantly to me, you taught me how to be a scientist and have a life. You have been my north star and shown me infinite patience over our seven years together. Your tutelage has been invaluable, I will be forever grateful, and I hope to continue to learn from you and your example in the future.

To Jen Gallagher. You told me once that you thought that you could have been a better mentor to me. I am not sure how that is possible. You have put so much time and effort into training and caring for me as a scientist and a person. I would not be the same person today without you.

To Andrew Dacks. Kristyn Lizbinski said it best, 'you are an amazing scientist and an even better person'. You and Jen cared for and protected me as though I were a member of your own labs. You are one of the most motivating figures in my life, and when I was in the lab at two in the morning, not wanting to continue an experiment, thinking of you would often push me to the finish line.

To Pete Mathers and Eric Tucker. Thank you for joining my committee, teaching me, and taking the time to help guide my experiments and writing throughout my career. To Mickey Howell and Betty Cowgill. None of this would be possible without you. So many times I would come to you both, stressed and confused, and you would calmly help me without judgement or pretense.

Chapter 1: Introduction

Chapter 1.1, Canonical Wnt signaling

Canonical Wnt is a signaling cascade that drives and contributes to many developmental and pathological processes, and most notably to this dissertation, is critical for neural crest development and the progression of colorectal cancer. Canonical Wnt signaling is a process that begins when the extracellular glyco-lipo proteins Wnts bind to their transmembrane co-receptors LRP5/6 and the Frizzled receptors. Upon binding, the Wnt receptors undergo a conformational change to inhibit the β -catenin destruction complex, allowing the transcriptional co-activator β -catenin to then accumulate in the nucleus to drive the transcription of canonical Wnt target genes.

The first experiment that unknowingly examined canonical Wnt signaling happened over eighty years ago. In 1935, Nobel prize winner Thomas Hunt Morgan at Caltech observed a mutation in *Drosophila* that had 'glazed' eyes (Morgan et al. 1935). It was later found that the *Drosophila* Glazed (Gla) mutant had a loss of photoreceptor cells and overproduction of eye pigment caused by a retrotransposon insertion of a gain-of-function allele, the *wnt1* homologue wingless (*wg*) (Brunner et al. 1999). The wingless name would be given about forty years after the discovery by Morgan et al. 1935 when Sharma 1973 and Sharma and Chopra 1976 characterized wingless flies that gave that hypomorphic allele its name.

Then in 1980, a series of Nobel prize earning experiments was published in *Nature* by Nüsslein-Volhard and Wieschaus. In that paper, Nüsslein-Volhard and Wieschaus classified and characterized a group of segmental defects in *Drosophila* mutants

including the previously discovered fused, wg, and cubitus interruptus and the newly discovered genes gooseberry, hedgehog, and patch. In their results, they note that wingless and hedgehog had a similar phenotype where the larvae lacked all segment boundaries. This paper started a hunt for more genes that would fit into the different segmental defect categories to find related or interacting genes. Wieschaus et al. in 1984 would be the first to find another possible wg related gene armadillo (the β -catenin homologue). Then arrow (the LRP homologue), dishevelled, porcupine, and shaggy/zeste-white 3 (the Glycogen Synthase Kinase 3 homologue) would be discovered, but how and if these genes interacted with one another were still a mystery (Perrimon and Mahowald 1987, Wieschaus and Riggelman 1987).

The first study in vertebrates on canonical Wnt signaling was again unknowingly done over seventy years ago. Simultaneously with Thomas Morgan's work characterizing the glazed phenotype, staff at the Jackson Memorial Laboratory in 1936 noticed that pups nursing on mice with breast cancer had a higher incidence of later developing breast cancer and leukemia themselves. This observation was later confirmed in studies done by Korteweg 1934, Korteweg 1935, MacDowell 1935, MacDowell and Richter 1935, Murray and Little 1935, Bittner 1936, Cloudman and Little 1936, Korteweg 1936, and Murray and Little 1936. Later it was found that this transmission occurs by exposure to the mammary tumor virus (MMTV) that causes an insertional activation in the int-1 gene. When MMTV was found to insert and activate other genes, including int-2 and int-3 (Peters et al. 1983, Gallahan and Callahan 1987), which are all unrelated to one another, the Int family genes were renamed. The int-1 gene was found to be similar to the wg gene (van Ooyen and Nusse 1984, Rijsewijk et al. 1987) and so Nusse et al.

1991 renamed int-1 as WiNgless-Type MMTV integration site (wnt). At the time, the scientific community only knew of at least 10 different Wnt genes in mice, but we would later go on to find that there are 7 in *Drosophila*, 5 in *Caenorhabditis elegans*, 11 in chicks, 12 in Zebrafish, 20 in *Xenopus* (including wnt7c), and 19 in mammals (Nusse et al. 2005, Garriock et al. 2007, Zhang et al. 2011, Holstein 2012).

The first developmental study in vertebrates was done in *Xenopus* by McMahon and Moon in 1989. In that paper they found that overexpression of mouse wnt1 mRNA caused an axis duplication (creating two dorsal structures) and dorsalized and posteriorized the embryo (McMahon and Moon 1989). Once a phenotype for Wnt was established, the *Xenopus* community confirmed the results seen in *Drosophila* showing that wnt, β -catenin, and dishevelled were somehow related (McCrea et al. 1993, Sokol et al. 1995). These studies started to put together the growing number of canonical Wnt signaling puzzle pieces and opened the door to exploring novel components.

The earliest study connecting some of these genes with canonical Wnt-like phenotypes was done by Bejsovec and Martinez-Arias in 1991. In that study they found that wg gene was required for the expression of engrailed (en) in *Drosophila*. Then in 1992, Siegfried et al. began to cross mutants categorized by Nüsslein-Volhard and Wieschaus 1980. Using en expression as a readout of wg activity, they found that zesty-white 3 inhibitory mutants increased En expression and that flies with both inhibitory mutations of wg and zesty-white 3 had increased expression of En. This indicates that zesty-white 3 is a negative regulator of wg signaling and that wg is upstream of zesty-white 3 (Siegfried et al. 1992). In similar experiments, it was also found that dishevelled,

frizzled, and armadillo were positive regulators of wg signaling and were downstream of wg (Noordermeer et al. 1994, Peifer et al. 1994, Krasnow and Adler 1995, Krasnow et al. 1995, Wang et al. 1996), mechanisms that are conserved in *Xenopus* (DeMarais and Moon 1992, Sokol et al. 1995, Pierce and Kimelman 1995, Dominguez et al. 1995, Funayama et al. 1995).

Then in 1996, a seminal paper by Snyder et al. came out in *Current Biology*. In that study they confirmed that frizzled is a positive regulator and downstream of Wnt in *Xenopus*. Snyder et al. continued to show that transmembrane protein Frizzled-1 could sequester Xwnt-8 and Dishevelled but not Xwnt-5a to the plasma membrane. These data indicated for the first time that Frizzled could act as a receptor for Wnt, that Frizzleds bind to specific Wnts, and that Dishevelled is sequestered to the membrane upon Wnt activation. Snyder et al. 1996 went on to show that Glycogen Synthase Kinase 3 (more specifically GSK3 β) is downstream of Frizzled in the canonical Wnt pathway.

One big missing piece of canonical Wnt signaling was how this pathway affects transcription. It was known that β -catenin enters the nucleus upon activation of Wnt, and that this nuclear accumulation is critical for Wnt signaling, but it structurally lacks the ability to act as a direct transcription factor (Moon et al. 1993, Lemaire et al. 1995, Peifer 1995, Guger and Gumbiner 1996, Munemitsu et al. 1996, Näthke et al. 1996, Korinek et al. 1997, Moon et al. 1997). Then in 1996, Molenaar et al. found that the high mobility group transcription factor T-Cell-Factor 3 (then called XTcf-3, but now Tcf711) could bind to β -catenin and together activate transcription in a reporter gene assay in

Xenopus. Originally found in mouse lymphoid cells, the T-Cell-Factor/Lymphoid Enhancing Factor (Tcf/Lef) were transcription factors that, when knocked out in mice, cause severe defects in lymphopoiesis, along with a lack of hair follicles, skin, appendages, teeth, trigeminal nuclei, as well as perinatal death (Verbeek et al. 1995, van Genderen et al. 1994). After the experiments done by Molenaar et al. in 1996, several studies over the next few years found that the other Tcf/Lef transcription factors also bound to β -catenin to activate canonical Wnt signaling and was conserved from Drosophila to humans (Nusse and Clevers 2017). In 1998, two seminal papers were published in Nature. Cavallo et al. 1998 and Roose et al. 1998 found that Tcf/Lef transcription factors interact with the co-repressor Groucho/Tle when not bound to β -catenin to repress transcription of canonical Wnt target genes in Drosophila and Xenopus. Later it was found that once in the nucleus, along with replacing Groucho/Tle from Tcf/Lef, β -catenin can also recruit chromatin modifiers including CBP, Brg-1, Legless, Pygopus, and Parafibromin/Hyrax to epigenetically open Wnt target genes for transcription (Logan and Nusse 2004, Städeli et al. 2006, Mosimann et al. 2006).

The idea that β -catenin modulates canonical Wnt signaling by entering the nucleus coincided with the idea that it might also modulate Wnt signaling by accumulation (Aberle et al. 1997, Behrens et al. 1997, Brunner et al. 1997). Previously Miller and Moon, Munemitsu et al., and Yost et al. in 1996 found that GSK3 β phosphorylated β -catenin leading to its degradation. Then Aberle et al. in 1997 showed that β -catenin was degraded by the proteasome in an ubiquitin-dependent manner, that Wnt expressing cells had greatly reduced ubiquitinated β -catenin, and that GSK3 β operated somewhere

upstream of ubiquitination. These observations led to the idea of a β -catenin destruction complex that becomes inhibited in the presence of canonical Wnt. In two innovative papers published in 1998, Behrens et al. and Nakamura et al. found that β -catenin, Adenomatous Polyposis Coli (APC), GSK3 β , Dishevelled, and novel in both papers canonical Wnt repressor Axin, all bind together in a multiprotein complex (later called the β -catenin destruction complex) to degrade β -catenin in the absence of Wnt. Also in 1998, Ikeda et al. found that Axin binding to GSK3 β facilitated phosphorylation and degradation of β -catenin. These new findings, together with the previous findings, gave the impression that extracellular canonical Wnt works by binding to its receptor Frizzled to sequester Dishevelled and, along with it, the rest of the β -catenin destruction complex, at the inner leaflet of the plasma membrane, preventing β -catenin from being phosphorylated, ubiquitinated, and then degraded by the proteasome. It wouldn't be until 2002 that we would find out that the membrane bound casein kinase 1 α (Ck1 α) interacts with the destruction complex when recruited to the membrane and primes β -catenin for phosphorylation by GSK3 β by phosphorylating β -catenin at S45 so that GSK3 β can phosphorylate β -catenin at T41, S37 and S33 (Liu et al. 2002).

Since the discovery that β -catenin was degraded through the ubiquitin-proteasome system, there was a fervent search to find its specific E3 ligase. Then in 1998, Marikawa et al. found that the E3 ligase β -TrCP was a negative regulator of canonical Wnt signaling and inhibited dorsal axis development in *Xenopus*. That following year four papers were published showing that β -catenin is ubiquitinated by β -TrCP after being phosphorylated by GSK3 β (Hart et al. 1999, Liu et al. 1999, Latres et al. 1999, Winston et al. 1999). To better complete the picture of the signaling pathway, two important

papers came out in the same article of Nature in 2000. Semenov et al. 2000 found that extracellular lipophilic transmembrane lipoprotein receptor (LDLR)-related protein 6 (LRP6) could induce dorsal axis formation in *Xenopus* and was necessary for neural crest development. They further found that LRP6 bound to Wnt1 directly and an inhibitory mutant of LRP6 could block Wnt or Wnt-Frizzled but not Dishevelled or β -catenin induced canonical Wnt signaling. This combined with the mouse work done by Pinson et al. 2000 showed that LRP6 acted as a co-receptor, heterodimerizes with Frizzleds upon Wnt binding, and shed light on why the lipid modification of extracellular Wnt is necessary for activation (Nusse and Clevers 2017).

Of importance to this dissertation, the small G protein Rac1 can modulate canonical Wnt signaling. This connection was hinted at in 1998, when Kuroda et al. found that IQGAP1, a target of Cdc42 and Rac1, increases nuclear pools of β -catenin by destabilizing β -catenin/E-cadherin interactions. Then, a breakthrough was made by Esufali and Bapat 2004 in *Oncogene*. Using the TOPFlash reporter construct with Tcf/Lef binding sites to drive transcription of luciferase as a canonical Wnt signaling readout, they found that Rac1 is sufficient to enhance and in some instances is necessary to activate Wnt signaling. In two colorectal cell lines with dysregulated and active canonical Wnt signaling, HCT116 (that has a stabilizing β -catenin mutation) and SW480 (that has an APC mutation), Esufali and Bapat 2004 found that overexpressing a constitutively active mutant of Rac1 (V12Rac1) increased, and dominant-negative Rac1 (N17Rac1) decreased, TOPFlash activity, the expression of canonical Wnt target genes cyclin1 and c-myc, and total and nuclear β -catenin. Interestingly, they also made another dominant-negative mutant of Rac1, which had the activating V12

mutation but could not enter the nucleus (V12Rac1PBRQ) and found that it had no effect on canonical Wnt signaling. These experiments for the first time made a strong connection between Rac1 and canonical Wnt signaling. This phenotype is also remarkably similar to the one we observed in our experiments with the ATP dependent RNA-helicase DDX3.

Canonical Wnt signaling plays critical roles in development, tissue homeostasis, stem cell maintenance, and cancer. In development, canonical Wnt signaling is necessary from sperm maturation, to dorsal-ventral and anterior-posterior axis formation, to even aging. Nearly every developing tissue from worms to mammals have been found to be affected by Wnt signaling. Human mutations in the canonical Wnt signaling pathway have been implicated in these developmental defects but not limited to bone density defects from LRP5, LGR4, SOST, Wnt16, Wnt1, and WTX (Gong et al. 2001, Brunkow et al. 2001, Boyden et al. 2002, Little et al. 2002, Jenkins et al. 2009, Zheng et al. 2012, Pyott et al. 2013, Styrkarsdottir et al. 2013), Familial exudative vitreoretinopathy from LRP5, FZD4, Norrin and TSPAN12 (Robitaille et al. 2002, Toomes et al. 2004, Xu et al. 2004, Poulter et al. 2010), Robinow syndrome from Wnt5a, Dvl1, and ROR2 (van Bokhoven et al. 2000, Person et al. 2010, White et al. 2015) and developmental tooth defects from LRP6, Wnt10a, Wnt10b, and Axin2 (Lammi et al. 2004, Adaimy et al. 2007, Massink et al. 2015, Yu et al. 2016). Of importance to this thesis, mutations in canonical Wnt signaling are associated with disorders caused by abnormal neural crest development known as neurocristopathies. Mutations in canonical Wnt target genes Snai2, Pax3, Sox10, Ret can lead to neurocristopathies such as piebaldism, Waardenburg syndrome, and Hirschsprung's disease. Knockout of *wnt1* and/or *wnt3a* in

mice leads to a reduction in neural crest derivatives (Ikeya et al. 1997), and indeed, in situ hybridization for Wnt1 and Wnt1-gfp transgenic mice label the neural crest and its derivatives (Danielian et al. 1998, Yu et al. 2005). The specific role of canonical Wnt signaling in neural crest development will be discussed in Chapter 1.3.

In colorectal cancer, aberrant canonical Wnt signaling is associated with 80% of pre-cancerous polyps. Although other mutations are required for malignancy, it is thought that disrupted canonical Wnt signaling is the first step toward most colorectal cancer. Along with controlling cell growth and stemness, canonical Wnt signaling is implicated in transitioning the intestinal cells from an Epithelial to a Mesenchymal (EMT) phenotype. As with neural crest, the specific role of canonical Wnt signaling in colorectal cancer will be discussed in Chapter 1.4.

Chapter 1.2, Akt signaling and Canonical Wnt Crosstalk

Over forty years ago, viral Akt was isolated from the Akt8 retrovirus found in the spontaneous thymomas of the high-leukemia AKR mouse strain (Staal et al. 1977). Then Staal in 1987 and Staal and Hartly in 1988 were able to clone the gene, map it to chromosome 14q32, and discovered a 20-fold amplification in five human gastric adenocarcinomas tested, hinting at a role for Akt in malignancies other than lymphoma, and also found that the virus could induce thymic lymphomas in nude mice. Over the next four years, through degenerate PCR and hybridization, Akt was identified to be a serine/threonine kinase (Bellacosa et al. 1991, Jones et al. 1991). It was recognized to be similar to Protein Kinase A and C and so Akt donned two new names; Related to A

and C kinase (RAC) and Protein Kinase B (PKB). It was also discovered that there were three isoforms of Akt from different genes, Akt1 (PKB α /RAC α), Akt2 (PKB β /RAC β), and Akt3 (PKB γ /RAC γ) (Staal 1987, Jones et al. 1991, Coffe and Woodgett 1992, Konishi et al. 1995). Akt was also discovered to have an SH2-like domain (Bellacosa et al. 1991). Bellacosa et al. surmised that Akt might likely interact with tyrosine kinases because of this, but later incorrectly found that Akt is only phosphorylated on Serine and Threonine residues in vivo and in vitro. While Serine and Threonine phosphorylation are of the utmost importance in the research found in this dissertation, Akt was later found to be phosphorylated by other kinases on Y176, Y315, and Y326 (Figure 1, Chen et al. 2001, Mahajan et al. 2010).

With about 40 percent of viral Akt being located to the membrane and viral Akt being found to be myristoylated, there was a hint that Akt may play a role on the cytoplasmic side of the plasma membrane (Ahmed et al. 1993). Then in 1995, 4 papers published in Nature, Cell, and EMBO Journal showed a connection between the much investigated Phosphoinositide 3-Kinase (PI3K) family of kinases and Akt (Burgering and Coffe 1995, Cross et al. 1995, Franke et al. 1995, Kohn et al. 1995). Class 1 PI3Ks were up to that point found to phosphorylate Phosphatidylinositol 4,5-bisphosphate (PIP₂) to Phosphatidylinositol 3,4,5-triphosphate (PIP₃) and class 2 PI3Ks convert Phosphoinositol-4-phosphate (PI(4)P) into Phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂). The SH2-Domain-Containing Inositol 5'-Phosphatase (SHIP) can also convert PIP₃ into PI(3,4)P₂. It was later found that PI3Ks activate Akt by making PIP₃ and PI(3,4)P₂. It was also found that PI(3,4)P₂ binds more readily binding to Akt over

PIP₃. These molecules bind directly to the Plakstrin-Homology (PH) domain of Akt and cause dimerization and auto-phosphorylation (James et al. 1996, Franke et al. 1997). PIP₃ and PI(3,4)P₂ are inserted on the cytoplasmic side of the plasma membrane and thus Akt binding to them also sequesters Akt to the membrane.

Exposure of cells to growth factors, insulin, or pervanadate activate Akt's kinase activity in a PI3K dependent manner (Burgering and Coffey 1995, Franke et al. 1995). However, the increase in PIP₃ and PI(3,4)P₂ is not enough to activate Akt and was speculated that PIP₃ and PI(3,4)P₂ activate Akt by bringing it to the plasma membrane (Franke et al. 1995, Andjelkovic et al. 1996, James et al. 1996, Frech et al. 1996). Treatment with growth factors, insulin, or pervanadate also hyperphosphorylate Akt1 and it was found that phosphorylation of serines and threonines are largely responsible for Akt1's activity (Figure 1, Andjelkovic et al. 1996). Then, a seminal paper by Alessi et al. 1996 showed that phosphorylation of T308 and S473 is critical for the activity of Akt. They further showed that this phosphorylation is done in a PI3K dependent manner (Alessi et al. 1996). It was later found that phosphorylation of T309 and S474 on Akt2 and T305 and S472 on Akt3 were necessary for their activation (Hodgkinson et al. 2002, Kumar et al. 2005). Future studies would use phosphorylation of Akt at these sites to measure Akt activity.

But what phosphorylates Akt at these residues? The fact that phosphorylation of Akt1 at T308 and S473 was PI3K dependent gave a clue that phosphorylation may be dependent on the presence of PIP₃ and PI(3,4)P₂ (Alessi et al. 1996). Then Alessi et al. 1997 and Stokoe et al. 1997 found a kinase that was only active in the presence of PIP₃

and PI(3,4)P₂ that they named phosphoinositide-dependent protein kinase 1 (PDK1). They also found that PDK1 phosphorylates Akt1 at T308. The discovery that PDK1 directly phosphorylates Akt1 at T308 also helped to solve the mystery of why Akt goes to the membrane to get activated. First, PDK1 is only active in the presence of PIP₃ and PI(3,4)P₂. Second, PIP₃ and PI(3,4)P₂ binding to the PH domain of Akt causes a conformational change in Akt, distancing the PH domain from the kinase domain and allowing PDK1 to phosphorylate it (Calleja et al. 2007, Calleja et al. 2009). But, phosphorylation of T308 on Akt1 is not enough to fully activate it (Alessi et al. 1996).

There was a lot of speculation as to what phosphorylated Akt1 at S473. In 2002 there were hints that it might be mTOR with the finding that mTOR can phosphorylate hydrophobic pockets like the one found around S473 (Hara et al. 2002, Kim et al. 2002). There was strong discouragement of this idea however when it was found that rapamycin had no effect on Akt phosphorylation at S473. However, in a seminal paper by Sarbassov et al. 2005, they found a new form of an mTOR complex that was insensitive to rapamycin. In looking at a large screen of different kinases, it was found that mTORC2 (formerly the mTOR, GβL, and rictor complex) phosphorylates Akt1 at S473. Sarbassov et al. 2005 also found that, similar to Akt, mTORC2 becomes active when stimulated by growth factors in a PI3K dependent manner. The full role and the order by which S473 phosphorylated is still debated today, but what is agreed upon is that phosphorylation of this site stabilizes phosphorylation of T308 (Manning and Toker 2017). Later it was found that under conditions of DNA damage, the DNA-dependent protein kinase (DNA-PK) can also phosphorylate S473 on Akt1 (Bozulic et al. 2008).

Interestingly, in a series of rictor knockout studies that inhibit mTORC2 activity, it was found that the noncanonical I ϵ B kinase ϵ (IKK ϵ) and the TANK-binding kinase 1 (TBK1) can phosphorylate both S473 and perhaps T308 in a PI3K dependent manner (Xie et al. 2011).

Of interest to this thesis, the small G-protein Rac1 seems to be a critical part of growth factor mediated Akt1 phosphorylation. This was first hinted at by Yamaguchi et al. 2001 and Shin et al. 2004 when they found that basic Fibroblast Growth Factor (bFGF), and Nerve Growth Factor (NGF) increase both active Rac1-GTP and phosphorylated Akt. Then in 2006, Gonzalez et al. found that Rac1 was required Sphingosine-1-Phosphate (S1P) mediated activation of Akt. They also found that this activation of Akt by Rac1 is done in a PI3K dependent manner. In that study, overexpression of Rac1 caused an increase in phosphorylated Akt that was greatly reduced by the PI3K inhibitor, wortmannin. They also found that knockdown of Rac1 reduced Akt phosphorylation. It was later found that other growth factors including Epidermal Growth Factor (EGF) and Insulin-like Growth Factor 1 (IGF-1) increase total Rac1 concentrations or active Rac1 and active Akt (Yamaguchi et al. 2001, Shin et al. 2004, Wu et al. 2007, Dise et al. 2008, Meng et al. 2008). The precise order of how PI3K, Rac1, and Akt interact with one another is still being studied. There is conflicting evidence of whether PI3K can activate Rac1 (Qian et al. 2005) or not (Gonzalez et al. 2006), but none-the-less, there are several studies giving evidence that Rac1 activates Akt in a PI3K dependent manner.

The half-life of T308 and S473 phosphorylated Akt is fairly brief. Upon stimulation with

50ng/mL EGF, phosphorylation of Akt1 peaks at 5 minutes and is half of that peak at 15 minutes and upon stimulation with 200nM insulin peaks at around 15 minutes and is half of that peak in 30 minutes (Xie et al. 2011). This leads to the question of how Akt is dephosphorylated and inactivated so quickly.

After finding that Akt is phosphorylated in 1995, there was a search for what dephosphorylates Akt. Then in 1996, it was found that Akt1 (called RAC-PK in the paper) phosphorylation was greatly increased by the sponge and shellfish poison okadaic acid (Andjelkovic et al. 1996). Okadaic acid is a specific inhibitor of the phosphatases Protein Phosphatase 1 (PP1) and Protein Phosphatase 2A (PP2A) (MacKintosh and MacKintosh 1994). Andjelkovic et al. 1996 through a series of overexpression studies, went on to show that it was specifically PP2A that affected dephosphorylation of Akt. Fascinatingly, it was found that HSP90 binds to Akt over amino acids number 229-309, and that this binding is necessary to prevent PP2A mediated dephosphorylation (Sato et al. 2000). Further experiments showed that PP2A operates preferentially on T308 of Akt1 instead of S473 (Gao et al. 2005). In that paper, Gao et al. 2005 then found that the phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP) specifically dephosphorylated Akt1 on S473. Surprisingly, PHLPP is the only phosphatase in the human genome that contains a PH domain leading to the idea that PHLPP might help dephosphorylate Akt at the plasma membrane (Bayascas and Alessi 2005). Along with PP2A and PHLPP that operate directly on Akt, another phosphatase was found to inactivate Akt indirectly. The Phosphatase and Tensin homolog (PTEN) was a deleted gene on chromosome ten that

was found to be mutated or lost in a multitude of cancers (Li and Sun 1997, Li et al. 1997, Steck et al. 1997). The following year it was discovered that PTEN could operate as a phosphatase of phosphoinositides at the 3' position (Maehama and Dixon 1998, Stambolic et al. 1998). This acted as a way of turning off PI3K signaling and reducing the amount of PIP₃ and PI(3,4)P₂. PTEN thus also acts to inhibit Akt by reducing PI3K signaling. Recently, there was some evidence given that PIP₃ and PI(3,4)P₂ release from Akt is the rate limiting step to deactivation and dephosphorylation, opening up the idea that dephosphorylation may start with the release of PIP₃ or PI(3,4)P₂ (Ebner et al. 2017).

Akt is now known to phosphorylate over 100 diverse substrates, affecting many different cellular pathways (Carracedo and Pantolfi 2008, Manning and Toker 2017). The first bona fide substrate of Akt was discovered in 1995. Previously, it was found that Glycogen-Synthase Kinase 3 β (GSK3 β) was inhibited by insulin (Hughes et al. 1992, Welsh and Proud 1993, Cross et al. 1994, Saito et al. 1994). Knowing that both GSK3 β and Akt are strongly affected by insulin, Cross et al. 1995 set to exploring if there was a connection between the two. In that paper, they found that insulin inhibited GSK3 β in an Akt dependent manner by overexpressing Akt, and Akt kinase dead mutant, and using the best known Akt activator at the time, pervanadate. They also found by ³²P radiolabeling and tryptic peptide analysis that Akt specifically phosphorylates GSK3 β on residue S9 and S21 on GSK3 α . Structural studies would later find that phosphorylation of GSK3 β by Akt causes a competition for the priming phosphate that is required for substrate binding. This then turns the N-terminal region of GSK3 β into a pseudo-substrate (Frame

et al. 2001). This event prevents substrates from binding and also blocks substrates from getting near the catalytic center of Akt. Phosphorylation of GSK3 β , FOXO transcription factors, and mTORC1 now used as confirmatory readouts for Akt activation (van der Vos and Coffey 2011, Webb and Brunet 2014, Saxton and Sabatini 2017).

Given that Akt acts as a central kinase hub with the ability to be modulated by many cellular pathways and its own ability to modulate so many diverse substrates, and that at least one isoform of Akt seems to be expressed in every tissue, it is not surprising that Akt has many different functions in the cell. As stated above, the earliest known function of Akt was an association that was found between Akt and lymphoma (Staal 1977) and later gastric adenocarcinoma (Staal 1987). Staal and Hartley in 1988 then went on to show that the Akt8 virus could induce thymic lymphoma in nude mice. After these discoveries in the late 1980's, more studies were conducted exploring the function of Akt. Akt2 was found to be amplified and was a major contributor to forming several ovarian carcinomas (Cheng et al. 1992). Then in 1995, several breakthroughs were made elucidating the many functions of Akt. A *Drosophila melanogaster* homologue of Akt named DRAC-PK was found (Andjelkovic et al. 1995). In that paper they found that the DRAC-PK gene gave rise to two different transcripts and that each transcript encoded two different polypeptides due to an ACG codon upstream of the AUG (4 polypeptides total). They also found that DRAC-PK was expressed maternally and throughout the entire lifetime of the larva and fly. Then after the discovery that PI3K regulates Akt, within the remainder of that year Akt was associated or was found to be a major contributing factor in Platelet-Derived Growth Factor (PDGF), EGF, and bFGF in a

PI3K dependent manner. Akt was found to affect mTORC1 and insulin signaling (Franke et al. 1995, Burgering and Coffey 1995, Cross et al. 1995). With these diverse signaling pathways affected, Akt was implicated in development, glucose uptake, metabolism, diabetes, and cancer progression and migration (Franke et al. 1997, Brazil and Hemmings 2001). Within the next few years Akt was implicated in hypoxic/ischemic-induced injury and neurodegenerative disorders with the discovery that Akt enhanced neuronal survival in the absence of growth factors and a critical role in IGF-1 mediated neuronal survival (Dudek et al. 1997). This finding initiated a series of studies investigating Akt in cell survival and led the way to discovering that Akt was found to be crucial for the anti-apoptosis by inhibiting the pro-apoptotic factor Bcl-2 Associated Death Promoter (BAD) in Interleukin-3 induced hematopoietic cells (Del Peso et al. 1997), later an entire class of anti-apoptotic forkhead transcription factors, the FOX family (Brunet et al. 1999), and by inactivating Caspase-9 (Cardone et al. 1999). Akt is also highly conserved from Dictyostelium to humans (Suess et al. 2017) and Akt isolated from cows can rescue Drosophila Akt inhibitory mutants, indicating that many of these upstream and downstream events of Akt are conserved.

Along with discovering the cellular roles of Akt, developmental biologists wanted to continue the work done by Andjelkovic et al. on DRAC-PK. In 1998, Staveley et al. renamed DRAC-PK to Dakt1 and made an inhibitory Dakt1 D. Melanogaster mutant that had ectopic apoptosis during embryogenesis and a complete loss of head and dorsal structures (Staveley et al 1998). In 1999, Verdu et al. found that Dakt1 controls the size of cells in the eye imaginal disc but not cell cycle or proliferation by overexpressing or

knocking it out specifically in that tissue. Then in 2000, Scanga et al. confirmed the work found by Verdu et al. 1999 and also found that there is a PI3K, PTEN, Akt signaling cassette that controls cell size and is utilized multiple times throughout the development of *Drosophila*. In that investigation, they found that Dakt1 mutant and dominant-negative Dp110 (PI3K) have similar phenotypes found from Stavely et al. 1998 and Verdu et al. 1999 but also found a reduction in wing size. They also found that there is a high level of DNA fragmentation in the Akt mutant in dominant-negative Dp110 embryos (Scanga et al. 2000). This makes sense as there was a high level of apoptosis. These initial studies led to a series of papers over the next 5 years in *Drosophila* that showed a conservation in the molecular pathway that was being pioneered in cancer cells using cell size, loss of head and dorsal structures, apoptosis and DNA fragmentation as readouts (Gao et al. 2000, Rintelen et al. 2001, Cho et al. 2001, Miron et al. 2001, Brogiolo et al. 2001, Miron and Sonenberg 2001, Stocker et al. 2002).

The first *Caenorhabditis elegans* study on Akt came out in 1998. Paradis and Ruvkun found that there were two homologues of Akt in *C. elegans*, Akt-1 and Akt-2. They also found that Akt was indispensable for insulin signaling and that overexpression of Akt could ameliorate loss of PI3K (called AGE-1 in *C. elegans*) (Paradis and Ruvkun 1998). Paradis and Ruvkun further found that akt-1 and akt-2 have overlapping expression in *C. elegans* during the dauer stage (an alternative *C. elegans* larval state caused by overcrowding) and speculated that their main function in *C. elegans* was to repress metabolic shift and growth arrest associated with the dauer stage. A big breakthrough was found in *C. elegans* in 1999, when Brunet et al. found that Akt phosphorylates and

inhibits the forkhead family transcription factor member (FKHRL1). They found that Akt inhibits FKHRL1 by causing it to be sequestered in the cytoplasm. This inhibition prevented the expression of genes critical for apoptosis and cell death (Brunet et al. 1999). This seminal paper led to a series of discoveries showing that Akt regulates most forkhead family transcription factors by direct phosphorylation (Manning and Toker 2017). Beyond dauer stage regulation, Akt was shown to be critical for longevity, metabolism and stress response in *C. elegans* (Padmanabhan et al. 2009).

Like *C. elegans*, the *Xenopus* community did not start investigating Akt until 1998. Andersen et al. 1998 found that when a constitutively active Akt is overexpressed in *Xenopus* oocytes meiosis is continued. In 1999, Lopez-Hernandez and Santos found that Akt may be involved meiotic maturation in oocytes in an PI3K independent manner. They gave evidence that progesterone and Ras could activate Akt when PI3K was inhibited. In 2003, *Xenopus* Akt was cloned for the first time by Anderson et al. and they named it xAkt. Fascinatingly, Finkielstein et al. 2001 found that xAkt was responsible for preventing irradiation induced apoptosis in embryos after the mid- blastula transition. Previously, it was found that overexpression of a dominant-negative PI3K caused a ventralizing phenotype with a small head and overexpressing a constitutively active PI3K (p110a*) had little effect, where only 8% of the embryos had two heads and a double axis (Didichenko et al. 1996). PI3K was also shown to have an effect on gastrulation in an Erk/MAPK mediated manner (Reif et al. 1996).

Then in 2004, Peng et al. began to investigate PI3K and Akt in *Xenopus*. They

confirmed what was found in Didichenko et al. 1996 with PI3K. They also found that similar to overexpression of dominant-negative PI3K (p85 Δ SH2), overexpression of a dominant-negative Akt (DN-Akt) resulted in a ventralized phenotype with a small head.

They continued to show that overexpression of a constitutively active Akt (myristoylated-Akt) resulted in dorsalized phenotype with double heads and double axes (Peng et al. 2004). They further found that p110a* could be rescued with co-expression of DN-Akt or GSK3 β and overexpression of constitutively active Akt could be rescued with co-expression of GSK3 β (Peng et al. 2004). In their discussion, they surmised that PI3K and Akt worked through GSK3 β that modulated β -catenin to cause axis duplication, dorsalization, and neurogenesis. This was the first time that Akt was shown to affect Wnt signaling in *Xenopus*. In 2007, Wang et al. found that PI3K/Akt signaling may have a role in somitomere differentiation to somites but also found that inhibition of PI3K and Akt in *Xenopus* embryos results in inhibition of nuclear β -catenin and active β -catenin at neurula stage. These experiments were the first time that Akt was shown to affect Wnt signaling in *Xenopus*.

The earliest evidence that Akt signaling is required specifically for neural crest development, and not just mesoderm development, comes from studies done in *Xenopus*. In 2009, Neuner et al. found that A Disintegrin And Metalloproteinase 19 (ADAM19) was expressed in the dorsal mesoderm and neural ectoderm, including the neural crest, and was required for the expression of neural crest genes *snai2* and *sox8* that was confirmed by our own lab (Li et al. 2018). Neuner et al. 2009 also found that

knocking down ADAM19 by antisense morpholino inhibited the activation of Akt. We would also go on to show that ADAM19 is required for neural crest specification and was required for canonical Wnt signaling during neural crest induction (Li et al. 2018). This gives further evidence connecting Akt and canonical Wnt signaling in *Xenopus* embryos and for the first time gives evidence that this connection may be required for neural crest induction. Then in 2015, Pegoraro et al. explored the role of the bifunctional kinase/phosphatase family member PFKFB4 in dorsal ectoderm specification. Originally shown to affect glycolysis, Pegoraro et al. 2015 showed that *pfkfb4* is enriched on the dorsal side of frog and chick embryos and was required for the activity of Akt. They went on to show that PFKFB4 antisense morpholino caused a reduction in the expression of neural crest genes *snai2*, *pax3*, and *sox10* but an increase in *zic1* by qRTPCR and in situ hybridization in neurula stage embryos. In exploring PI3K/Akt signaling in more detail, Pegoraro et al. 2015 incubated *Xenopus* embryos in the PI3K inhibitor LY294002 and found that there was a reduction in active Akt and also a reduction in *snai2* and *pax3* but no effect on *zic1* by qRTPCR and in situ hybridization in neural stage embryos. Pegoraro further found that the phenotype seen in PFKFB4 morphants could be rescued with co-expression of CA-Akt.

Interestingly, recent evidence by Geary and LaBonne 2018 have shown that high Mitogen-Activated Protein Kinases (MAPK) and low Akt signaling are required for developing neural crest stem cells. In a series of animal cap studies in *Xenopus*, Geary and LaBonne 2018 gave evidence that Akt signaling but not MAPK is required for pluripotent blastula stage cells to adopt a neural fate. They further showed that

expressing Pax3 and Zic1 in animal caps induce them toward a neural crest fate.

Interestingly, there was reduced Akt activity in the neural crest fated animal caps. Also interestingly, co-expressing p110a* with Pax3 and Zic1 inhibited other neural crest genes (Geary and LaBonne 2018). This indicates that Akt signaling may be necessary to induce neural stem cells and perhaps neural border formation but only low amounts of Akt activity are necessary for specification of neural crest. This is consistent with canonical Wnt signaling where Wnt is required to dorsalize and posteriorize the embryo and, later on, only a moderate amount of Wnt signaling is required to specify the neural crest.

Seminal experiments in chick embryos have mostly recapitulated the results seen in other developmental models. Early zebrafish experiments on Akt were also replicating those found in other developmental models. One of the first seminal and novel experiments on Akt in zebrafish, not previously discovered in other models, came in 2008 when Liu et al. found that Akt was essential for hematopoiesis and angiogenesis in a k-ras/PI3K dependent manner. Zebrafish then became the preeminent model for exploring how Akt is necessary for hematopoiesis and angiogenesis (Zhao et al. 2010, Deng et al. 2011, Jing and Zon, 2011, Zhang et al. 2012, Jeffery et al. 2015).

Then recently in zebrafish, Ciarlo et al. 2017 gave more evidence that Akt is necessary for neural crest development. Using Caffeic Acid Phenethyl Ester (CAPE), which they showed inhibits FGF or insulin stimulated Akt activation, they inhibited neural crest genes sox10, pax7a, crestin, and dlx2a. They further showed that expressing myristoylated-Akt could rescue the neural crest phenotype observed by embryos cultured with CAPE. They continued to show that overexpressing PTEN also reduced

Akt activity and crestin (Ciarlo et al. 2017). This data combined with those done in *Xenopus* gave building evidence that Akt is necessary for proper neural crest development.

The first developmental studies of Akt in mice occurred in 2000 using adenoviruses to transfect constitutively active or dominant-negative Akt. It was found that Akt mediated nitric-oxide dependent vasomotor activity (Luo et al. 2000) and promotes angiogenesis in normocholesterolemic mice (Kureishi et al. 2000). Then in 2001 the first knockouts of Akt was done in mice (Chen et al. 2001, Cho et al. 2001a, Cho et al. 2001b). Chen et al. 2001 found that knockout of Akt1 in mice produce viable offspring but they are smaller, have more apoptotic cells, and are more sensitive to γ -radiation, UV-radiation, TNF, anti-Fas, serum withdrawal, or dexamethasone induced apoptosis. Cho et al. 2001a confirmed a similar phenotype, but also showed that Akt1 was not necessary for glucose homeostasis and mentioned that most of the Akt null embryos and pups died, somewhere between midembryonic development and when the pups were weaning, particularly 3 days post-birth. Cho et al. also made Akt2 knockout mice (Cho et al. 2001a, Cho et al. 2001b). In those two studies they found that while Akt null mice survived, Akt2 was critical for glucose homeostasis and loss of Akt2 resulted in insulin resistance and a diabetes-like syndrome. In 2005, Easton et al. and Tschopp et al. made an Akt3 knockout mouse line and found Akt3 null mice are also viable but have a reduced brain size. These Akt knockout phenotypes make sense as Akt1 is the most ubiquitously expressed, Akt2 being most highly expressed in insulin-related metabolic organs, and Akt3 found almost exclusively in the brain.

In 2016, Wilson et al. gave the first evidence that Akt modulates the neural crest in mice. It was previously found that human mutations in the cytoskeletal crosslinking protein SPECC1L caused facial clefting in human patients, zebrafish, and mice with several defects in *Drosophila* as well (Saadi et al. 2011). Saadi et al. 2011 went on to show that knockdown of SPECC1L in zebrafish by antisense morpholino resulted in the loss of neural crest observed by in situ hybridization of the neural crest gene *crestin*. Wilson et al. 2016 then continued the research done by Saadi et al. 2011 by knocking out SPECC1L in mice. They found that SPECC1L null mice have reduced neural crest delamination, migration, and apoptosis. Wilson et al. went on to show that SPECC1L knockout mice also have a reduction in the whole embryo levels of total and active Akt and a reduction in β -catenin levels at the neural fold (where the neural crest delaminates). These experiments gave the first evidence in mice that Akt may be necessary for neural crest development and the first hint that there may be a connection between Akt and canonical Wnt signaling in the neural crest.

Several human mutations in Akt have been found in patients. An E17K mutation that occurs in Akt1 and Akt3 causes them to preferentially bind to the inner leaflet and get activated (Carpten et al. 2007, Davies et al. 2008). This activation leads to breast, colorectal, endometrial, ovarian, and melanoma cancers (Carpten et al. 2007, Davies et al. 2008, Shoji et al. 2009). The Akt1 E17 mutation also leads to proteus syndrome (Lindhurst et al. 2011). Proteus syndrome is marked by overgrowth and hyperplasia of many tissues and organs, increased incidence of deep venous thrombosis and as noted above, increased risk of developing cancer. Patients with proteus syndrome also exhibit

craniofacial abnormalities, intellectual disability and vision loss that are also linked with abnormal neural crest development (Biesecker et al. 1999, Cohen et al. 2003, Turner et al. 2004, Cohen et al. 2005, Biesecker et al. 2006, Lindhurst et al. 2011). Similar to Akt1, there is also an activating mutation in the kinase domain of Akt2 that is associated with colorectal cancer (Parsons et al. 2005).

Chapter 1.3: Neural Crest

Over 150 years ago in 1868, Wilhelm His, Sr described a new strand of cells dorsal and running parallel to the spinal cord that he called the 'Zwischenstrang' (the between strand) (His 1868). Using the newly invented fixed microtome along with developing a method of hardening embryonic tissues with acids and salts, His was able to dissect chick embryos in very thin slices, where the tissue reliably maintained their structure. This mesenchymal tissue was then renamed as the neural crest by the cranial nerve zoologist Arthur Marshall in 1879 to more accurately describe that this tissue 'crested' the border of neural tissue (Hall 1999). The scientific community were immediately curious and baffled by the neural crest. Unlike the three germ layers that had been described for many years, this is a population of progenitor cells that seems to move and disperse and contribute to nearly every part of the embryo. This meant that the neural crest also disputed germ layer theory that stated ectoderm, mesoderm, and endoderm make unique and autonomous tissues in the body.

Determining the precise derivatives of the neural crest has always been a challenge, and indeed, is still hotly debated today. Originally, the neural crest was identified as

contributing to cranial nerves. Then in 1891 and 1898, Julia Platt, using *Acanthias* sharks and the *Necturus* mud puppy, meticulously documented the movements of the neural crest. She found that from the neural fold, neural crest cells migrate to make the pharyngeal arches along the lateral sides of the embryo. From the arches she then tracked the neural crest to the jaw and documented how they differentiated into cartilage and odontoblasts (Platt 1891, Platt 1898). This finding was confirmed in 1911 by Hill and Watson using marsupials (Hill and Watson 1958). These findings challenged the ideas that only mesoderm derived tissues could make bone or cartilage, of germ layer theory, and how vertebrate predators evolved. This caused a great amount of disbelief and scrutiny in the scientific community, and as you can see from the citation above, Hill and Watson did not get published for more than forty years because of it.

To determine the other derivatives of the neural crest, revolutionary experiments were performed before and after world war 2 by Stone 1932, Raven 1937, Triplett 1958, and Milaire 1959 to track neural crest cells in *Xenopus*, *Amblystoma*, and mice using cytoplasmic inclusions of yolk granules and pigments, Nile blue sulfate staining, high RNA content, nuclear staining, and differences in cell size. From these experiments, it was proposed that the neural crest could contribute to the peripheral nervous system, smooth muscle, cartilage, bone, pigment cells, and the adrenal medulla, to generally categorize.

Work by Weston in 1963 propelled the neural crest field into the modern era, and allowed the identification of individual cells that could be traced. Using Tritiated

thymidine as a marker, marked neural tubes and neural crest were grafted onto donor embryos and the cells were tracked with never before seen precision. This technique confirmed how the neural crest migrate and that they differentiate into spinal ganglia, sympathetic neuroblasts, and melanoblasts. This work was continued by LeDouarin and Teillet in 1973 and 1974 when they grafted quail neural ectoderm onto chick hosts. The quail cells can be distinguished from their hosts due to quail nuclei being morphologically distinct from chick nuclei. Using this technique, LeDouarin and Teillet showed that the neural crest gives rise to the parasympathetic enteric ganglia, with again, incredible precision (LeDouarin and Teillet 1973, LeDouarin and Teillet 1974).

With the identification of neural crest specific markers such as Hnk1, p75, snai2, and crestin, to name a few, later experiments using in situ hybridization, immunofluorescence, and transgenic models (to be discussed in Chapter 1.8) would go on to find many other tissues that neural crest cells contribute to, including the cardiac septa of the heart (Crane and Trainor 2006, Saint-Jeannet 2006).

The first experimental studies that demonstrated the ability of neural crest cells to differentiate in many tissues came from Siber-Blum and Cohen 1980. In that paper they were able to isolate trunk neural crest cells and found that the cells could be cultured to form melanocytes or adrenergic neurons. Transplanting those cultured quail neural crest cells into chicks then resulted in different populations of neurons, the sympathetic ganglia, adrenal gland, and the aortic plexus (Bronner-Fraser *et al.* 1980).

Up to this point in the early 1980's, the molecular mechanisms of how the neural crest

migrates and what the neural crest differentiates into were extensively studied (Ledouarin and Kalcheim 1999), but very little was known about the embryonic origins of the neural crest (Basch and Bronner Fraser 2006, Saint-Jeannet 2006). These cell culture and transplant experiments done by Siber-Blum and Cohen and Bronner-Fraser et al. started a fervor in the neural crest field wanting to know how neural crest cells get induced.

The induction of the neural crest is thought to be a classic example of secondary induction, whereby a competent tissue responds to the surrounding tissues to elicit differentiation. This thought comes from in vitro and in vivo transplantation experiments where non-neural ectoderm were placed on the neural fold, or neural plate cells on the paraxial mesoderm, and those cells took on a neural crest-like fate (Moury and Jacobson 1990, Liem et al. 1995, Selleck and Bronner-Fraser 1995, Mancilla and Mayor 1996, Woo and Fraser 1998). Interestingly, transplanted neural plate cells preferentially differentiate into melanocyte-like cells and non-neural ectoderm cells preferentially differentiate into spinal and cranial ganglia. This indicates signals from the surrounding tissues of the ectoderm and mesoderm are a strong driving force to turn competent tissue into the neural crest (Selleck and Bronner-Fraser 1995, Mancilla and Mayor 1996, LaBonne and Bronner-Fraser 1998, Monsoro-Burq et al. 2003). In 2004, Ragland and Raible were able to show in zebrafish that the signals from the ectoderm and mesoderm were able to compensate for one another to induce the neural crest, showing redundancy of the signals.

But what are these signals coming from the surrounding tissue that drive neural crest

formation? One of the most critical signaling pathways in developmental biology for setting up the dorsal/ventral axis is the Transforming Growth Factor β (TGF β) family subgroup, Bone Morphogenic Protein (BMP) signaling. BMPs were first found in the 'dead matrix' of developing bone and were found to induce cell growth (Urist 1965).

BMPs are extracellular dimers that bind to their transmembrane receptors to initiate a signaling cascade (Oelgeschläger et al. 2000). BMP signaling is highly controlled by the localized secretion of BMP antagonists by embryonic organizers like the Spemann organizer in *Xenopus* or the node in mice or chicks. These organizers set up a gradient of BMP signaling in the embryo, where there is high BMP signaling on the ventral side and low BMP signaling on the dorsal side to set up a dorsal/ventral axis that has been shown in *Xenopus*, zebrafish, and mice (De Robertis and Sasai 1996, Harland and Gerhart 1997, Schulte-Merker et al. 1997, Kisimoto et al. 1997, Connors et al. 1999, Hild et al. 1999, Dick et al. 2000, Schmid et al. 2000, Arias and Steventon 2018).

In 1995, Liem et al. found that BMP4 and BMP7 were secreted by the non-neural ectoderm, and that they were enough to induce neural plate cells toward a neural crest fate. LaBonne and Bronner-Fraser in 1998 found that inhibition of BMP is necessary for inducing a neural (dorsal) fate in *Xenopus* stem cells. These two experiments gave the impression that a moderate amount of BMP signaling is necessary to induce the neural crest. In support of this, while the non-neural ectoderm secretes BMP4 and BMP7, the neural plate secretes BMP antagonists Cerberus, Noggin, Chordin, and Follistatins, and loss of these inhibitors results in the loss of neural crest (Wilson et al. 1997, Tribulo et

al. 2003, Sauka-Spengler and Bronner-Fraser 2008). However, moderate levels of BMP signaling are not enough to induce neural crest cells from competent cells, indicating that other signals are required (Wilson et al. 1997, LaBonne and Bronner-Fraser 1998, Garcia-Castro et al. 2002).

Another signaling cascade associated with neural crest induction is Fibroblast Growth Factor (FGF) signaling. FGFs are a family of extracellular proteins that bind to their transmembrane receptor tyrosine kinase receptors (FGFRs). Activated FGFRs then modulate the PI3K-Akt, Ras-MAPK, PLC γ , and STAT pathways by phosphorylation (Kouhara et al. 1997, Schoorlemmer et al. 2002, Gotch 2008, Yu et al. 2011, Yang et al. 2013, Ornitz and Itoh 2015). FGFR signaling primarily comes from the underlying paraxial mesoderm, including FGF2 and FGF8, during neural crest induction (Monsoro-Burq et al. 2003). One of the first studies observing the role of FGFR signaling in the neural crest came from Mayor et al. in 1997. In that paper, they found that the neural crest specifier gene *snai2* was upregulated in *Xenopus* embryos that were overexpressing FGFR and downregulated in embryos that were expressing dominant-negative FGFR. They also found that FGFR signaling was not enough to induce competent cells toward a neural crest fate (Mayor et al. 1997). This work was continued by Villanueva et al. 2002 when they showed that basic FGF could transform anterior neural border cells into a neural crest fate and that dominant-negative FGFR could inhibit this process. Overexpression of FGF2 can also transiently induce the neural crest (Monsoro-Burq et al. 2003). Conflicting with this, mice overexpressing FGF8 or lacking FGFR have no defects in neural crest development (Noden and Trainor 2005, Jones

and Trainor 2005). Jones and Trainor in 2005 continued to show that zebrafish lacking mesoderm (and thus FGF signaling) or carrying inhibitory mutations in FGF have no effects on neural crest induction. The current thinking on FGF signaling in neural crest induction is that it is likely a redundant signaling pathway as there are many signaling pathways that converge on FGFR effectors, such as canonical Wnt on Akt signaling.

Canonical Wnt signaling is necessary at every point in neural crest induction including neural plate border specification, neural crest specification and, in our own experiments, neural crest differentiation (LaBonne and Bronner-Fraser 1998, Garcia-Castro et al. 2002, Lewis et al. 2004, Perfetto et al. unpublished). There are many canonical Wnt ligands that are expressed around or in the neural crest during induction, including Wnt1, Wnt3a, Wnt6, Wnt7b, and Wnt8 (Knecht and Bronner-Fraser 2002). Indeed, as stated in Chapter 1.1, Wnt1 is used to label the neural crest in mice. Wnts come from the paraxial mesoderm in *Xenopus* and the non-neural ectoderm in chicks (Chistian et al. 1991, Garcia-Castro et al. 2002). Inhibiting canonical Wnt signaling in *Xenopus*, zebrafish, and chicks results in a loss of neural crest and their derivatives in a multitude of experiments (LaBonne and Bronner-Fraser 1998, Schubert et al. 2002, Lewis et al. 2004, Betancur et al. 2010, Wei et al. 2010, Li et al. 2018). LaBonne and Bronner-Fraser in 1998 showed in *Xenopus* embryos that overexpression of Wnt8 caused anterior expansion of the neural crest and a more lateral expression of *snai2* and dominant-negative Wnt8 resulted in loss of *snai2*. This was recapitulated in chicks, where overexpressing Wnt6 expanded the neural crest (Garcia-Castro et al. 2002, Schubert et al. 2002). In mice, there have been very few studies examining neural crest

induction (Betancur et al. 2010). Double mutations in Wnt1 and Wnt3a results in a loss of many neural crest derivatives (Ikeya et al. 1997). However, many single Wnt knockouts in mice have no observable effect on neural crest induction. The current presiding theory is that Wnts act redundantly of one another in mice, and multiple knockouts will need to be examined (Jones and Trainor 2005). Together, BMPs, FGFs, and canonical Wnt signaling pathways interact to give the neural crest region the competency to transform into neural crest cells and respond to specifying signals.

Neural crest development occurs in several phases that are orchestrated by Wnt, BMP, and FGF signaling that conduct a tightly regulated and cross-talking gene network. First, at the end of gastrulation, the neural border is specified between the neural plate and non-neural ectoderm. Canonical Wnt signaling acts to turn on one of the earliest neural border specifiers *gbx2.2* (Li et al. 2009). Interestingly, *snail* is expressed at the neural border region at this time, and while its exact role in neural crest induction is unknown at this time, interesting work out of Carole LaBonne's lab hints at a role of maintaining stem cell potency (Buitrago-Delgado et al. 2015). Then *pax3*, *pax7*, and *msx1* get induced by BMP, FGF, Wnt, *gbx2.2*, *ap2 α* , and *dlx5* while concurrently *zic1* gets induced by BMP (Sato et al. 2005, Monsoro-Burq et al. 2005, Hong and Saint-Jeannet 2007, Nikitina et al. 2008). In *Xenopus*, overexpression of *pax3* and *zic1* are enough to reprogram non- neural ectoderm and animal caps into neural crest-like cells (Sato et al. 2005, Milet et al. 2013).

After establishment of the neural border, the neural crest is then specified. Some of the

genes that specify the neural crest persist through migration of the neural crest to help differentiate the cells, some will have biphasic expression where they are expressed during specification but not during migration and are then turned on again during differentiation, and still others are only present during the time of specification. There are many genes that are associated with neural crest specification, some with known roles in stem cell maintenance, anti-apoptosis, or Epithelial-to-Mesenchymal Transition (EMT) to name a few, but of importance to this dissertation, I will discuss *snai1*, *snai2*, *sox9*, *sox10*, *foxd3* and *twist*. BMP, Wnt, FGF, *zic1*, *msx1*, and *pax3/7* induce the transcription of *sox9*, *snai1*, and *snai2* (Mayor et al. 1997, Vallin et al. 2001, Spokony et al. 2002, Villanueva et al. 2002, Aoki et al. 2003, Honore et al. 2003, Luo et al. 2003, Tribulo et al. 2003, Glavie et al. 2004, Lee et al. 2004, Perez-Alcala et al. 2004, Sakai et al. 2005, Sato et al. 2005, Meulemans and Bronner-Fraser 2004, Bagheri-Fam et al. 2006, Li et al. 2009). Neural border specifying factors, *snai1*, and *snai2* then induce *foxd3* and neural border specifying factors, *snai1*, *snai2*, and *sox9* induce *sox10* (Wettstein et al. 1997, Aybar et al. 2003, Werner et al. 2007). Neural border specifying factors, *snai1*, *snai2*, and *foxd3* then induce *twist*. During neural crest specification, the neural crest starts undergoing EMT as it prepares to become migratory cells. Specifying genes like *snai1* and *snai2* act to repress e-cadherin, and *foxd3* acts to promote the more migratory cadherin7 to allow this to happen (Cano et al. 2000, Cheung et al 2005, Taneyhill et al. 2007). It is not currently known when neural crest cells become more differentiated, but after induction, 4 general species of neural crest exist, trunk neural crest, vagal/sacral neural crest, cardiac neural crest, and cranial neural crest. The cranial neural crest will then migrate as four separate groups to the pharyngeal arches

and eventually differentiate to make up the craniofacial cartilage and cranial nerves.

Chapter 1.4 ADAM metalloproteases:

A Disintegrin and Metalloproteases (ADAMs), ADAMTSs (ADAMs with a thrombospondin motifs), and Matrix Metalloproteases (MMPs) are, as their names denote, metal dependent proteases that cleave an incredibly diverse number of substrates that include ExtraCellular Matrix (ECM) molecules, cadherins, integrins, and signaling molecules (McCawley and Matrisian 2001, Porter et al. 2005, Edwards et al. 2008, Christian et al. 2013). ADAMs, ADAMTSs, and MMPs are the three central families of metzincins, so named because they are functionally dependent on zinc ions and a methionine-turn near the active site. The catalytic domain of metizincins are characterized as globular structures with the active site between two subdomains. The active site contains highly conserved histidines in the HEXGHXXXGXXHD motif that binds the zinc atom and, as noted above, a methionine residue C-terminal to the zinc-binding motif (Bode et al. 1993, Maskos et al. 1998, Gomis-Ruth 2003, Orth et al. 2004).

ADAMs are type 1 transmembrane proteins that have a well characterized modular structure that contains a cytoplasmic tail, a transmembrane domain, an epithelial growth factor-like (EGF-like) domain, a cysteine-rich domain, a disintegrin domain, and a protease domain with or without a catalytically active site, a prodomain, and an N-terminal signal sequence. To date, over 30 ADAM proteins have been identified in various species. ADAM proteins are subdivided into proteolytically inactive (due to an

evolutionary loss of the zinc binding motif) and proteolytically active members (Weber and Saftig 2012). Known proteolytically inactive ADAMs include ADAMs 2, 7, 11, 18, 22, 23, and 29, and known proteolytically active ADAMs include ADAMs 8, 9, 10, 12, 13, 15, 17, 19, 20, 21, 28, 30, and 33. From 1998 to 2002, critical discoveries were made about the pro- and cysteine-rich domains of ADAMs. It was found that the prodomain is essential for protein folding, maintains latency of the active site, and is then removed in the Golgi by proprotein convertase 7 or furin (Lum et al. 1998, Roghani et al. 1999, Howard et al. 2000, Anders et al. 2001, Schlomann et al. 2002). Indeed, isolated prodomains can act as selective inhibitors (Gonzales et al. 2004, Moss et al. 2007). Interestingly, proportionally only a small fraction of ADAM proteins makes it to the plasma membrane, whereas the majority is found in the Golgi (Lammich et al. 1999, Schlondorff et al. 2000, Gutwein et al. 2003). How ADAM proteins get to the cell surface is still a largely understudied part of ADAM biology. Along with the prodomain, the cysteine-rich domain also regulates ADAM catalytic activity, possibly by regulating the removal of the prodomain or by orienting potential substrates (Milla et al. 1999, Iba et al. 2000, Reddy et al. 2000, Smith et al. 2002, Janes et al. 2005). The cysteine-rich domain also works with the disintegrin domain to enact cell adhesion and substrate targeting (Zolkiewska 1999, Iba et al. 2000, White 2003).

Interestingly, ADAMs were first described for their adherence abilities. In 1992 and 1993, Blobel et al. and Wolfsberg et al. first described ADAM1 and ADAM2 (called sperm-egg fusion proteins in the paper). They found that ADAM1 and ADAM2 were very similar to viral fusion proteins and that ADAM1 and ADAM2 were critical for sperm-egg

fusion and fertilization by binding to integrins. It would later be found that ADAM1 and ADAM2 are proteolytically inactive and that they bind to integrins via their disintegrin domain (Cho et al. 1998, Weber and Saftig 2012).

In 1996 and 1997, Kuzbanian, the *Drosophila* ortholog of one of the most well studied ADAMs, ADAM10, was found to control differentiation and axon extension in neurogenesis (Fambrough et al. 1996, Rooke et al. 1996, Pan and Rubin 1997). The authors went on to find that Kuzbanian could cleave the Notch receptor. In 1999, Lammich et al. found that ADAM10 potentially had a significant role as an α -secretase for the Alzheimer's associated protein Amyloid Precursor Protein (APP) in a non-amyloidogenic way. Since then, extensive research has gone into studying ADAM10 where it has been found to cleave numerous substrates, tested for its therapeutic potential in Alzheimer's disease, and found a role for ADAM10 in neural crest development (Shoval et al. 2007, Edwards et al. 2008, Fahrenholz 2010, Saftig and Reiss 2011, Weber and Saftig 2012). In 2003, Hall and Erickson found in chickens that ADAM10 is expressed in many different developing tissues, including the neural crest cells. In 2007, Shoval et al. continued this work and demonstrated that ADAM10 is necessary for neural crest transformation from an epithelial to a migratory mesenchymal state (EMT) and delamination by mediating the cleavage of N-cadherin. Also in 2007, Huh et al. showed that CD44 cleavage by ADAM10 facilitates neural crest derived cell migration into the cornea of chicks.

In 1997, another well studied ADAM, ADAM17, was found to modulate infection and

immunity by causing the release of $\text{TNF}\alpha$ (Black et al. 1997, Moss et al. 1997). The next year, Peschon et al. 1998 studied ADAM17 in mouse development. In that paper Peschon et al. created mice that express dominant-negative ADAM17 where the zinc binding motif is removed. They found that most mice die at embryonic day 17.5 (e17.5).

The small percentage that survived to become newborns had open eyelids (normally closed at birth), curly and stunted whiskers, lacked a conjunctival sac, had attenuated corneas were up to 40% smaller than control by weight, and had dense irregular pigmentation in the skin and hairs, as well as disorganized hairs. Those mice that died at e17.5 had delayed maturation in nearly every tissue. Later it was found that ADAM17 knockout mice also have defects in the heart (Jackson et al. 2003, Shi et al. 2003).

ADAM17 has also been studied for shedding of epithelial growth factor receptor ligands such as epigen, heparin-bound epithelial growth factor, and transforming growth factor α , from the cell surface, to allow the ligands to bind to their receptors (Sahin and Blobel 2007, Weber and Saftig 2012). This may explain the reduced size and underdevelopment of tissues found by Peschon et al. 1998. In the intestines, enteric neural crest cells colonize the tissue by migrating as chains from the future duodenum to the rectum. In 2010, the neural adhesion protein and ADAM10 and ADAM17 substrate, L1, was shown to be critical for this process (Anderson 2010). This brings up the idea that ADAM10 and ADAM17 may also be necessary for neural crest migration in the intestines. While ADAM17 is expressed in the cranial neural crest cells of mice, its function in neural crest development is not well studied (Christian et al. 2013).

The first studied ADAM in neural crest development was ADAM13. Alfandari et al. in 1997 found that adam13 is expressed in migratory neural crest cells in *Xenopus*. It was later found in 2010 that adam13 is also expressed in pre-migratory neural crest cells as well (Wei et al. 2010b). In 2001, Alfandari et al. continued their previous work and overexpressed a proteolytically dead dominant-negative ADAM13 or a wild-type control in *Xenopus* embryos. They found that dominant-negative ADAM13 inhibited neural crest migration. They went on to show that ADAM13 could cleave Fibronectin in vitro and speculated that this cleavage allowed the neural crest to move through the surrounding mesenchyme (Alfandari et al. 2001). In 2009, McCusker et al. found that ADAM13 could cleave Cadherin 11, found in migrating neural crest, to facilitate neural crest migration. In 2010, Wei et al. found that ADAM13 was also necessary for neural crest specification during induction. They found that ADAM13 could cleave and inhibit class B Ephrin ligands to activate canonical Wnt signaling. Interestingly, knockdown of ADAM13 by antisense morpholino could be rescued by co-expressing canonical Wnt target gene *snai2*. In 2011, Cousin et al. found that ADAM13 could likely cleave itself to allow γ -secretase cleavage of ADAM13's cytoplasmic tail. They continued to find that the cytoplasmic tail was necessary for neural crest migration by possibly going into the nucleus to modulate the expression of an abundance of genes, many of which could affect migration (Cousin et al. 2011).

ADAM19 was first studied in neural crest development in 2007. Komatsu et al. 2007 found that adam19 is expressed in the cardiac neural crest cells of mice. They went on

to show that knockout of adam19 in mice had severe ventricular septal defects (VSD) and abnormal tricuspid valve development. However, they also found that there was no effect on cardiac neural crest induction, EMT, delamination, or migration. Komatsu et al. 2007 surmised that ADAM19 must then affect differentiation of cardiac neural crest cells in mice. In *Xenopus*, however, ADAM19 was shown to be critical for cranial neural crest induction and migration (Neuner et al. 2009). These potentially conflicting findings may be indicative of the functional redundancy of genes that are in mammals than lower vertebrates due to the smaller number of paralogs. In 2011, Cousin et al. gave the first hint that ADAM13 and ADAM19 may work in the same pathway to facilitate neural crest development. They showed that knockdown of both ADAM13 and ADAM19 in *Xenopus* resulted in a more severe neural crest defect and indeed, the migratory phenotype seen by knockdown of ADAM13 and ADAM19 could be rescued by either ADAM13's or ADAM19's cytoplasmic tail. This led to our lab's finding that ADAM19 protects ADAM13 from ubiquitination and degradation by the proteasome to induce neural crest specification (Li et al. 2018).

Chapter 1.5 DEAD-box RNA helicases in development, canonical Wnt, and Akt

DEAD-box RNA helicases are ATP dependent helicases that are multi-faceted in their functions and are critical for every aspect of RNA metabolism including transcription, storage, transport, annealing and secondary structure formation, translation, and degradation. They not only operate to unwind RNA, but can also allow protein scaffolding on RNA by serving as RNA clamps, can disassemble ribonucleoprotein (RNP) complexes, and even directly catalyze RNA annealing. As such, DEAD-box RNA

helicases are highly conserved and are found in all eukaryotes, most bacteria and Archaea, and even some viruses (Gorbalenya and Koonin 1993, Fairman-Williams et al. 2010, Jankowsky 2011, Linder and Jankowsky 2011). Recently, there has also been fascinating research geared toward understanding the RNA independent roles that DEAD-box RNA helicases have in cells.

There are 37 human DEAD-box RNA helicases that are so named for their conserved Asparagine-Glutamic Acid-Alanine-Asparagine (DEAD) sequence. DEAD-box RNA helicases are a part of the second superfamily of helicases (SF2) that are defined by having two α - β RecA-like domains, each containing a Walker A and a Walker B motif (Gorbalenya and Koonin 1993, Cordin et al. 2006). The DEAD sequence is a part of the Walker B motif, named after Walker et al. 1982, and is responsible for ATP hydrolysis by coordinating a magnesium ion that activates an attacking water molecule (Koonin 2003). Upstream of the Walker B motif is a Walker A motif that is responsible for coordinating the γ -phosphate of ATP during hydrolysis. The Walker A and B motifs are critical components of the two α - β RecA-like nucleic acid binding domains in DEAD-box RNA helicases (Caruthers and McKay 2002). The two α - β RecA-like domains operate by forming a cleft to hold ATP. This cleft must be in a very tight conformation in order to bind and hydrolyze ATP (Hilber et al. 2006). Generating this tight cleft conformation requires a very strict amino acid sequence, providing an explanation for why the helicase core, which includes the two α - β RecA-like domains, in DEAD-box RNA helicases are so highly conserved (Linder and Jankowsky 2011).

DEAD-box RNA helicases are unique members in SF2 in how they interact with RNA. DEAD-box RNA helicases can bind either strand of double stranded RNA and can bind internally or at either end of the double strand (Yang et al. 2007). And while the RNA associates with both α - β RecA-like domains similarly to other members of SF2, research into the Drosophila DEAD-box RNA helicase Vasa (homologue to DDX4 in humans) has shown that DEAD-box RNA helicases sharply bend the RNA to avoid a clash with the conserved N-terminal alpha helix motif (Sengoku et al. 2006). Sengoku et al. went on to define this interaction as a 'wedge helix' that interestingly disrupts the base pairs when double-stranded RNA is bound. The RNA binds to the helicase core exclusively by the sugar backbone over a 5 nucleotide span (Linder and Jankowsky 2011). While the core sequence and the interaction of RNA with the core is highly conserved, variability of RNA sequence recognition by DEAD-box helicases comes from other parts of the protein, including auxiliary domains, making contact with the RNA.

During the hydrolysis cycle of ATP, DEAD-box helicases have characteristic conformational changes. Like other SF2 members, the two α - β RecA-like domains move relatively freely when not bound to RNA or ATP (Jankowsky and Fairman 2007). When RNA or ATP is bound, the two α - β RecA-like domains of DEAD-box helicases become very ridged to form the tight cleft as noted above. This process occurs in two steps (Henn et al. 2008) and has been confirmed for most DEAD-box helicases (Andersen et al. 2006, Bono et al. 2006, Sengoku et al. 2006, Nielsen et al. 2008, Del Campo and Lambowitz 2009, Fan et al. 2009).

Due to the wedge helix, double stranded RNA will naturally separate when bound to

DEAD- box helicases. As such, hydrolysis of ATP is not necessary for strand separation and building evidence is showing that only binding of ATP is required to make the tight cleft that allows RNA binding, separation, and unwinding (Chen et al. 2008, Liu et al. 2008, Aregger and Klostermeier 2009). ATP hydrolysis however is critical for the release of ADP and subsequently RNA from the helicase (Henn et al. 2008, Henn et al. 2010). And indeed, ADP release is the rate limiting step for substrate turnover. Interestingly, only one ATP binding event is necessary for separation of double stranded RNA, regardless of RNA length, but not every ATP binding event will lead to complete unwinding (Chen et al. 2008, Henn et al. 2010). This leads to the idea that DEAD-box helicases are very good at conserving energy but not very efficient at unwinding double stranded RNA.

Outside of their helicase activities, DEAD-box proteins can also operate as RNA clamps and organizers of proteins on RNA. When hydrolysis is arrested, DEAD-box helicases can serve as immobile RNA clamps that allow for the formation of protein complexes on RNA (Nielsen et al. 2008). DEAD-box proteins can also remove proteins from RNA. A seminal paper by Fairman et al. 2004 in Science shows that several RNA binding proteins can be removed from RNA by DEAD-box proteins, but fascinatingly it seems like only those that bind less than eight nucleotides. Removal of these proteins is not dependent on the structure of RNA, indicating that helicase activity is not required. This was confirmed later with other DEAD-box helicases (Bowers et al. 2006, Tran et al. 2007).

DEAD-box RNA helicases are also unique members in SF2 in that they do not have

auxiliary domains inserted into their helicase core (with the exception of DDX1 and DDX24) (Fairman-Williams et al. 2010). Instead, all DEAD-box helicases have auxiliary domains that flank the core on the N- and C-terminal ends and are responsible for much of the variability and selective binding of the proteins (Fairman-Williams et al. 2010, Linder and Jankowsky 2011). The auxiliary domains can be anywhere between several or hundreds of amino acids in length and are largely understudied. Recent work on the auxiliary domains in yeast have shown that they not only affect what RNA or proteins bind by affecting the RNA or protein recognition motifs, but also affect how DEAD-box helicases interact with these molecules. Del Campo and Lambowitz in 2009 showed that the DEAD-box protein Mss116 actually causes a second bend in the RNA after the bend caused by the helicase core hinting at the idea of DEAD-box helicases can chaperone double stranded RNA into a secondary structure (Del Campo and Lambowitz 2009).

The functions of the helicase core and auxiliary domains of DEAD-box helicases are critical for RNA transcription, splicing, export, translation, and decay. Of importance to this dissertation, they also allow for selective RNA translation. In a paper by Chen et al. 2015, they showed that the DEAD-box helicase DDX3 is required for selective translation of the small G protein Rac1, likely by binding the 5'-UTR. Knockdown of DDX3 in colorectal cancer cells caused a reduction in Rac1 protein levels. Loss of Rac1 led to a subsequent decrease in β -catenin levels and canonical Wnt signaling as described in Chapters 1.1 and 1.2. This is the first mention of a DDX3-Rac1- β -catenin axis that is a critical idea for our DDX3-Rac1-Akt- β -catenin model, giving evidence for how DDX3 affects Rac1 in neural crest induction.

Only recently have DEAD-box helicases started to be studied in canonical Wnt and Akt signaling. The first study observing a role for DEAD-box proteins in Wnt signaling came from Yang et al. in 2006. In a study published in *Cell*, Yang et al. showed that DDX5 (called p68 in the paper) in HT29 colorectal cancer cells was necessary for nuclear import of β -catenin in a Wnt-independent manner. They went on to show that DDX5 binds directly to β -catenin to enhance nuclear import. They also demonstrated that knockdown of DDX5 leads to a reduction in phosphorylation of β -catenin on the GSK3 β and Ck1 α sites. While Yang et al. gave evidence that this increase in active β -catenin could be caused by DDX5 competing with GSK3 β to bind with both Axin and β -catenin, they did not investigate the in vivo phosphorylation of GSK3 β or Akt. Phosphorylation of DDX5 by the Platelet Derived Growth Factor (PDGF) signaling enhanced DDX5 mediated β -catenin nuclear import (Yang et al. 2006). The next year, Shin et al. 2007 showed that knockdown of both DDX5 and DDX17 led to a more severe canonical Wnt phenotype. This data combined with evidence that PDGF activates Akt signaling (Franke et al. 1995) and that DDX5 affects Akt signaling (Sarker et al. 2015), leads to the intriguing idea that DDX3, DDX5, and DDX17 may operate in a similar manner to activate canonical Wnt signaling. Interestingly, the only study of DEAD-box helicases in neural crest development came from Seufert et al. 2000 where they showed that there was high expression of DDX5 in migrating neural crest and in the neural ectoderm during neural crest induction in *Xenopus* and, to a lesser degree, in chicks.

In 2012, three articles in *Nature* were published studying mutations in subgroups of the

medullablastomas (Jones et al. 2012, Pugh et al. 2012, Robinson et al. 2012).

Interestingly, activating mutations in DDX3 were linked with Wnt type medullablastomas.

The next big breakthrough connecting DEAD-box helicases and canonical Wnt signaling came from Cruciat et al. 2013. In that paper, Cruciat et al. showed that DDX3 was critical for canonical Wnt signaling in *Xenopus* animal caps and Human Embryonic Kidney (HEK293T) cells. DDX3 modulates canonical Wnt signaling by binding to and activating Casein Kinase 1 ϵ , which in turn phosphorylates and inhibits Dishevelled to increase nuclear β -catenin and β -catenin-mediated transcription. They went on to show that DDX3 was necessary for neural plate development in a canonical Wnt mediated manner. They however did not investigate the neural crest and this study inspired our lab to investigate the possible role of DDX3 in neural crest induction and to take a closer look at how DDX3 may affect canonical Wnt signaling.

In 2015, Blok et al. studied human mutations in DDX3X that cause intellectual disabilities, skin abnormalities, hearing loss, cleft lip/palate and microcephaly, to name a few. Many of these disabilities are associated with abnormal neural crest development in zebrafish and all of these mutations fell within the helicase core. They went on to show that expression of Wnt3a (or Wnt8) caused a ventralizing phenotype in zebrafish and that co-expression with wild-type DDX3 enhanced ventralization. Blok et al. then co-expressed Wnt3a (or Wnt8) with the different mutants and found some that did not enhance ventralization, inferring that those mutations inhibit, or at least do not enhance, canonical Wnt signaling. However, this data could not be recapitulated in TOP-Flash assays in HEK293T cells and overexpression of individual mutants without Wnt3a (or

Wnt8) had no effect on ventralization in zebrafish nor TOPFLASH in HEK293T cells.

While these experiments are a great start in observing the potential role these mutants have on canonical Wnt signaling, more detailed knockdown or knockout and rescue experiments using these DDX3 mutants may be necessary.

In that same year, Heerma van Voss et al. 2015 explored the small molecular inhibitor of DDX3, RK33, in colorectal cancer. RK33 operates by binding to the ATP-binding domain in the helicase core of DDX3 to inhibit helicase activity. In their studies they found that RK33 inhibited canonical Wnt signaling in TOPFLASH experiments similar to knockdown of DDX3 in colorectal cancer cell lines (HCT116 and HT29). This data was confirmed in lung cancer by Bol et al. 2015. Interestingly, Voss et al. 2015 found that RK33 also reduced the expression of DDX5 but not DDX17 even though RK33 does not bind to DDX5 (Bol et al. 2015). As DDX3 and DDX5 can bind together (Choi and Lee 2012), it would be interesting to study DDX5 and DDX17 in *Xenopus* development.

One of the first studies on DEAD-box helicases and Akt came from Linley et al. 2012. In that paper they found that knockdown of DDX43 (called HAGE in the paper) in melanoma causes a decrease in the small G-protein RAS and subsequently a decrease in Akt activation through phosphorylation at S473. Previously it was known that RAS activates Akt by activating receptor tyrosine kinases and thus PI3Ks (Castellano and Downward 2011). This loss in Akt activity then led to a decrease in GSK3 β and FOCO phosphorylation. And in 2015, Sarkar et al. showed that DDX5 is necessary for the transcriptional activation of Akt in colorectal cancer. They went on to show that this loss in Akt caused decreased activity and protein levels of β -catenin and FOXO transcription

factors to induce oncogenesis. Interestingly, knockdown of DDX10 in ovarian cancer led to an increase in Akt activation through an unknown mechanism. In 2016, DDX3 was also shown to affect RAS in colorectal cancer cells (Wu et al. 2016). Wu et al. went on to show that knockdown of DDX3 causes a reduction in Akt activation and β -catenin levels. In high DDX3 expressing lung tumors, inhibition of Akt (perifosine) or β -catenin (XAV939) reduced tumor proliferation (Wu et al. 2016). This was the first paper showing that DDX3 affects Akt and led us to explore Akt in understanding how DDX3 activates canonical Wnt signaling in neural crest induction.

Chapter 1.6: Figures

Figure 1: Akt Signaling Overview taken with permission from Manning and Toker 2017, Elsevier Publishing, License #4722690229548. A. Canonical Signaling Cascade of Akt whereby activation of PI3K from Receptor Tyrosine Kinases (RTK) and G-Protein Coupled Receptors (GPCR) lead to increased amount of PIP₃ to then activate PDK1

and mTORC2 which then activate and phosphorylate Akt1. B. Known and predicted modification sites of Akt and the proteins that modulate them.

Chapter 2: Xenopus ADAM19 regulates Wnt signaling and neural crest specification by stabilizing ADAM13

Xenopus ADAM19 regulates Wnt signaling and neural crest specification by stabilizing
ADAM13

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Keywords: Neural crest (NC); neural plate border (NPB); disintegrin metalloproteinase (ADAM); Wnt signaling; Xenopus

Abstract

During vertebrate gastrulation, canonical Wnt signaling induces the formation of neural plate border (NPB). Wnt is also thought to be required for the subsequent specification of neural crest (NC) lineage at the NPB, but the direct evidence is lacking. We found previously that the disintegrin metalloproteinase ADAM13 is required for Wnt activation and NC induction in *Xenopus*. Here we report that knockdown of ADAM13 or its close paralog ADAM19 severely downregulates Wnt activity at the NPB, inhibiting NC specification without affecting earlier NPB formation. Surprisingly, ADAM19 functions nonproteolytically in NC specification by interacting with ADAM13 and inhibiting its proteasomal degradation. Ectopic expression of stabilized ADAM13 mutants that function independently of ADAM19 can induce the NC marker/specifier *snail2* in the future epidermis via Wnt signaling. These results unveil the essential roles of a novel protease-protease interaction in regulating a distinct Wnt pathway, which directly specifies the NC lineage.

Introduction

The vertebrate neural crest (NC) cells are multipotent embryonic cells that give rise to the craniofacial cartilages and bones, sensory neurons and glia, pigment cells, cardiac tissues, and many other cells and tissues. Deficiencies in NC development are a major cause of human birth defects, ranging from relatively subtle craniofacial abnormalities to more severe diseases such as CHARGE and DiGeorge syndromes (Bronner and Simões-Costa, 2016; Mayor and Theveneau, 2013). NC-based cell therapies are also being developed to repair tissues that are damaged by injuries or deteriorate with age (Kunisada et al., 2014; Zhu et al., 2016). Understanding the basic mechanisms that control the specification of NC lineage is crucial for developing new methods to prevent NC-related birth defects and to regenerate NC-derived tissues for clinical applications.

In vertebrate embryos, the NC cells are induced at the neural plate border (NPB) during gastrulation. NC induction requires coordinated actions of multiple signaling pathways, which activate the expression of transcription factors such as Pax3/7, Zic1 and Msx1 that induce NPB formation (i.e., the NPB specifiers). The NPB specifiers in turn activate the expression of another set of transcription factors (i.e., the NC specifiers), such as Snail2, FoxD3 and Sox9, which specify NC lineage in certain NPB cells. Once specified, the NC cells undergo proliferation while maintaining their multipotency. Around the time of neural tube closure, the NC cells delaminate and migrate to specific destinations, where they differentiate into various types of cells and tissues (Bronner and Simões-Costa, 2016; Simões-Costa and Bronner, 2015; Stuhlmiller and Garcia-Castro, 2012).

A major signaling pathway that is essential for NC induction is the canonical Wnt pathway. In *Xenopus* embryos, Wnt seems to be important for both the initial formation of the NPB and the subsequent specification of NC lineage. During NPB formation, Wnt is required for inducing the expression of Pax3 and Msx1, which cooperate with Zic1 to set up the NPB (Li et al., 2009; Monsoro-Burq et al., 2005). Gain-of-function studies suggest that the combination of Pax3 and Zic1 is sufficient to specify the NC lineage *ex vivo* in the animal cap or ectopically in the ventral ectoderm, and this effect is again Wnt-dependent (Milet et al., 2013; Monsoro-Burq et al., 2005; Sato et al., 2005). The direct Wnt target in NC specification is probably Snail2, as snail2 is the only NC specifier gene whose enhancer is known to contain a Wnt-responsive LEF/TCF binding site (Simões-Costa and Bronner, 2015; Vallin et al., 2001). However, it remains unclear whether the specification of endogenous NC lineage also requires Wnt activity.

The disintegrin metalloproteinases (ADAMs) are multi-domain transmembrane proteins that play important roles in development and disease. Most of the functional domains of ADAMs are extracellular, including a metalloproteinase domain that is catalytically active in some but not all ADAMs, as well as disintegrin and cysteine-rich domains that can mediate protein-protein interactions (Wei, 2013). About half of the ADAMs are expressed predominantly in the testis and are mainly involved in reproduction, whereas the others have significant presence in somatic tissues. Several somatic ADAMs are known to control cell signaling through their protease activities. For example, proteolytic processing of the Notch receptors by ADAM10 is necessary for Notch signal activation, and shedding of the ligands for epithelial growth factor receptors (EGFRs) by multiple

ADAMs is an essential regulatory event in EGFR signaling (Edwards et al., 2008; Reiss and Saftig, 2009). We have also shown that another ADAM, ADAM13, induces NC in *Xenopus* embryos by regulating canonical Wnt signaling. Knockdown (KD) of ADAM13 leads to reduced expression of markers for pre-migratory NC and inhibition of Wnt8-induced axis duplication. We further identified ephrin-B1 and –B2, two cell-surface ligands for EphB receptor tyrosine kinases, as the *in vitro* and *in vivo* substrates for ADAM13 and antagonists of Wnt signaling. By cleaving these ephrins, ADAM13 permits Wnt activation, which in turn induces the NC (Wei et al., 2010b). While these results clearly demonstrate the importance of ADAM13 in Wnt signaling and NC induction, direct evidence showing the regulation of endogenous Wnt signaling by ADAM13 in one or both stages of NC induction was still missing.

In the current study we investigated further the roles of ADAM13 and its close paralog ADAM19 in Wnt signaling and NC induction in *X. tropicalis* embryos. Using a transgenic Wnt reporter line, we detected high endogenous Wnt activity at the NPB at late gastrula stages. KD of either ADAM13 or 19 caused strong inhibition of Wnt activity at the NPB and reduced expression of NC markers; however, the expression of NPB markers remained unaffected. Thus ADAMs 13 and 19 are both required for a Wnt pathway that directly specifies the NC lineage, and this Wnt pathway appears to be different from the one that induces NPB formation. We further show that the function of ADAM19 in NC specification is independent of its protease activity but via protection of ADAM13. ADAMs 13 and 19 co-localized in the ER and physically interacted with each other, and KD of ADAM19 resulted in reduction of ADAM13 protein levels that was caused by

proteasomal degradation. Such proteasomal degradation was mediated by a conserved C-terminal lysine residue of ADAM13, as mutation of this lysine residue to arginine or deletion of the whole cytoplasmic tail generated stabilized ADAM13 mutants that were resistant to ADAM19 KD. Ectopic expression of these stabilized ADAM13 mutants could not only rescue the NC specification defects caused by ADAM19, but also induce robust expression of *snail2* in the future epidermis in a Wnt-dependent manner. Together our data suggest a novel non-proteolytic function of ADAM19 in regulating the turnover of ADAM13 and a distinct Wnt pathway that specifies the NC lineage.

Results

Knockdown of ADAM19 phenocopies ADAM13 loss of function in *X. tropicalis* embryos. Our phylogenetic and syntenic (gene linkage) analyses suggest that ADAMs 12, 13 and 19 form a subfamily of proteolytically active ADAMs that is conserved among vertebrates (note that the mammalian ortholog of *Xenopus* ADAM13 is ADAM33) (Bahudhanapati et al., 2015; Wei et al., 2010a). Similar to its *X. laevis* ortholog (Neuner et al., 2009), *X. tropicalis* *adam19* was found to be expressed in the dorsal mesoderm during mid-gastrulation, and broadly in the dorsal ectoderm at the end of gastrulation (Figs. S1A and S1B). At the same stages, transcripts of *adam13* were detected in the mesoderm and pre-migratory NC, respectively (Wei et al., 2010b). By contrast, *adam12* did not display any specific expression patterns (data not shown).

We reported previously that KD of ADAM13 causes defects in craniofacial and eye development in *X. tropicalis* embryos (Wei et al., 2012; Wei et al., 2010b). To

understand the functions of ADAMs 12 and 19, antisense MOs were designed to block the translation of individual ADAMs (Fig. 2A)(Wei et al., 2010b). Morphology of the craniofacial structures was characterized using a transgenic *X. tropicalis* reporter line that expresses enhanced green fluorescent protein (eGFP) driven by a ~4 kb fragment of the *snail2* enhancer/promoter, which allows direct imaging of NC derivatives in live embryos (Li et al., manuscript in preparation). We injected individual MOs to target the anterodorsal region of the *snail2* reporter embryos, and raised these embryos (“morphants”) to tadpole stages. Although ADAM12 morphants displayed no apparent phenotype (data not shown), injection of either ADAM19 MO (MO 19-1 or 19-2) led to reduction in head cartilage structure on the injected side, a phenotype similar to that caused by ADAM13 MOs (MOs 13-3 and 13-1; Fig. 3B)(Wei et al., 2010b). Because the head cartilage structures are NC derivatives and ADAM13 is known to induce the NC, we tested if loss of ADAM19 also affects NC induction. Indeed, in situ hybridization results show that KD of either ADAM13 or 19 drastically reduced the NC marker/specifier *snail2* at stage ~12.5 (Fig. 2C), when NC specification just started, suggesting that both ADAMs are essential for NC induction.

Another phenotype caused by ADAM13 KD is the impaired eye structures (Wei et al., 2012), which was also manifested in ADAM19 morphants (Fig. 10A). Examination of the eye field markers *pax6* and *rx1* reveals that both markers were downregulated by stage ~12.5 (Figs. S2B and S2C)(Wei et al., 2012), indicating that depletion of either ADAM13 or 19 interferes with early eye field induction. Therefore, KD of ADAM19 phenocopies ADAM13 loss of function in interfering with the induction of both the NC and eye field.

Both ADAMs 13 and 19 are required for Wnt signaling and NC specification but not NPB formation

Because NC induction occurs in two steps, the initial formation of the NPB and the subsequent specification of NC lineage, we asked which step(s) are controlled by ADAMs 13 and 19. In addition to *snail2*, *sox9* and *foxd3*, two other NC markers/specifiers, were both downregulated at stage ~12.5 by KD of ADAM13 or 19; however, little or no effect was detected for NPB markers/specifiers *pax3*, *zic1* or *msx1* (Fig. 2D). We conclude from these results that both ADAMs 13 and 19 are required for NC specification but not earlier NPB formation.

The pivotal role of ADAM13 in NC specification has been attributed to its ability to regulate the activation of canonical Wnt signaling, as KD of ADAM13 inhibits Wnt8-induced axis duplication, and restoration of Wnt signaling rescues the NC defects caused by ADAM13 KD (Wei et al., 2010b). Wnt is thought to be essential for both the earlier NPB formation and the subsequent NC specification (Abu-Elmagd et al., 2006; Simões-Costa and Bronner, 2015; Stuhlmiller and Garcia-Castro, 2012). The inhibition of NC specification but not NPB formation by KD of ADAM13 or 19 suggests that these ADAMs may be required for Wnt activation at the established NPB to specify the NC lineage. To test this hypothesis, we took advantage of another transgenic *X. tropicalis* eGFP reporter line, which has been shown to faithfully report the endogenous Wnt activity (Tran et al., 2010). At stage 12.5, we detected strong eGFP signal at the NPB in the Wnt reporter embryos (Fig. 2E, white arrowheads), confirming that Wnt signaling is highly active at the NPB. Depletion of ADAM13 drastically downregulated eGFP

expression at the NPB in all injected embryos, and this effect was again mimicked by ADAM19 KD (Fig. 2E). Thus both ADAMs 13 and 19 are required in the right place and at the right time for Wnt activation and for the specification of NC lineage.

Function of ADAM19 in NC specification is independent of its protease activity

The striking similarity between the loss-of-function phenotypes of ADAMs 13 and 19 strongly implies a connection between the in vivo roles of these two metalloproteinases. Because ADAM19 is one of the closest paralogs of ADAM13 (Bahudhanapati et al., 2015; Wei et al., 2010a), the simplest explanation is that these two ADAMs have redundant functions. To examine if this is the case, we first compared the in vivo roles of the protease activities of ADAMs 13 and 19.

As shown in Fig. 3A, ectopic expression of wild-type ADAM13 caused anterior expansion of the expression domains of *snail2* and *sox9*, effects similar to those caused by ectopically expressed β -catenin or a dominant-negative EphB receptor (Hong et al., 2008; Li et al., 2009; Wei et al., 2010b). These results provide further support for our model that ADAM13 induces the NC by inhibiting forward ephrin-B signaling and promoting Wnt signaling (Wei et al., 2010b). Conversely, overexpression of the protease-dead E340A mutant of ADAM13 greatly reduced both *snail2* and *sox9* (Fig. 3A), suggesting that this mutant dominant-negatively inhibited NC specification. The dominant-negative effect of the E/A mutants has been reported for several ADAMs including ADAM13 (Alfandari et al., 2001), and is presumably caused by the mutants' abilities to bind and sequester substrates from the endogenous ADAM proteases. The

expansion and inhibition of the NC caused by wild-type ADAM13 and the E340A mutant, respectively, confirm the importance of ADAM13 protease activity in NC specification. Surprisingly, ectopic expression of wild-type ADAM19, as well as the protease-dead E345A mutant, slightly expanded the expression domains of *snail2* and *sox9* (Fig. 3A), indicating that ADAM19 may function independently of its protease activity. We further tested if the E345A mutant can rescue the NC specification defects caused by ADAM19 KD. Indeed, co-injection of the mRNA encoding either the wild-type ADAM19 or the E345 mutant with the MO 19-1 rescued the reduction in *snail2* and *sox9* expression (Fig. 3B). This is in stark contrast with our previous observations that wild-type ADAM13, but not the E340A mutant, rescued the NC specification defects caused by ADAM13 KD (Wei et al., 2010b). These results indicate a fundamental difference in the mechanism of action for the two ADAMs: unlike ADAM13, the protease activity of ADAM19 is dispensable for its function in NC specification.

ADAM19 non-proteolytically regulates ADAM13 protein levels in vivo

The ability of wild-type ADAM19 and the E345A mutant to rescue the NC specification phenotypes caused by MO 19-1 confirms that these phenotypes are specific for ADAM19 KD. To further examine the efficacy and specificity of ADAM13 and 19 MOs, we assessed the abilities of these MOs to inhibit the translation of corresponding mRNA targets as well as their closest paralogs. Western blot analyses show that each MO knocked down its target protein (Fig. 4A, Lanes 4, 11 and 12). However, both MOs 19-1 and 19-2, two ADAM19 MOs with no overlap in their sequence (Fig. 2A), drastically

downregulated the exogenously expressed ADAM13 (Fig. 4A, Lanes 5 and 6). By contrast, the ADAM12 MO (MO 12-1) did not have a significant effect on ADAM13 (Lane 3), and neither MO 12-1 nor 13-1 downregulated ADAM19 (Lanes 9 and 10). Moreover, ectopically expressed ADAMs 10 and 12 were also unaffected by MO 19-1 (data not shown), suggesting that the loss of ADAM13 caused by MO 19-1 or 19-2 was specific. Western blot analyses using an anti-ADAM13 antibody further show that whole-embryo injections of MO 19-1 or 19-2 resulted in over 60% reduction of endogenous ADAM13 in stage ~12.5 *X. tropicalis* embryos; a similar effect was observed in *X. laevis* embryos (Fig. 4B and data not shown). It is unlikely that the loss of endogenous and exogenous ADAM13 in embryos injected with MO 19-1 or 19-2 was caused by off-target effects, as the transcripts of *adams* 13 and 19 do not share any significant sequence similarity despite the moderate homology between the two proteins (~43% identities); we were also unable to detect any potential binding sites for either MO 19-1 or 19-2 on ADAM13 mRNA. Rather, these results imply that ADAM19 somehow post-transcriptionally regulates ADAM13 levels in *Xenopus* embryos. This is further supported by our observation that exogenous ADAM19 increased the levels of co-expressed ADAM13 in *X. tropicalis* embryos (Fig. 4C). Notably, a comparable increase in ADAM13 was observed with both wild-type ADAM19 and the protease-dead E345A mutant (Fig. 4C), suggesting that the regulation of ADAM13 is independent of ADAM19 protease activity.

ADAMs 13 and 19 co-localize in the ER and interact with each other

To further investigate the interaction between ADAMs 13 and 19, we determined the

sub-cellular localization of these proteins in *Xenopus* XTC cells. Due to the low expression levels of ADAM19 in XTC cells, we overexpressed this protein. Immunocytochemistry results reveal that the exogenous ADAM19 was predominantly localized to the ER, as shown by the overlapping patterns of ADAM19 and the ER marker Calnexin (Fig. 5A). Our bioinformatic analyses also suggest that there are multiple possible ER-localization signals in the cytoplasmic tail of ADAM19 (Neuner, 2011). The endogenous ADAM13 co-localized with the exogenous ADAM19, but there was also a significant portion of ADAM13 that was found in the lamellipodia (Fig. 5B). The localization of ADAM13 to the lamellipodia is consistent with the function of this protease in cleaving cell-surface substrates such as cadherin-11 to promote migration (McCusker et al., 2009). Similar co-localization of ADAMs 13 and 19 was also detected in 293T cells (data not shown).

We further examined if ADAMs 13 and 19 physically interact with each other by overexpressing both ADAMs in 293T cells. Immunoprecipitation (IP) of either ADAM13 or 19 pulled down the other ADAM that was co-expressed, indicating that these two ADAMs were associated with each other (Fig. 5C). To identify the domain(s) of ADAM13 that are responsible for interacting with ADAM19, we co-expressed ADAM19 with full-length ADAM13, or the disintegrin and cysteine-rich domains individually and combined, in 293T cells. Results of co-IP experiments show a strong association of ADAM19 with the cysteine-rich domain of ADAM13 (Fig. 5D), suggesting that this domain plays a major role in the interaction between ADAMs 13 and ADAM19.

ADAM19 protects ADAM13 from ubiquitin-proteasome-mediated degradation

We next asked how the interaction between ADAMs 13 and 19 results in the increase of ADAM13 levels, as seen in Fig. 4C. Because ADAM13 is known to cleave itself (Gaultier et al., 2002), it is possible that binding of ADAM19 to ADAM13 prevents the autocleavage of ADAM13. If this were the case, KD of ADAM19 should have no effect on the protease-dead E340A mutant of ADAM13. As shown in Fig. 6A, ectopic expression of wild-type ADAM13 with a C-terminal myc₆ tag gave rise to a smaller fragment (indicated by an asterisk) in addition to the pro- and mature forms of ADAM13. This fragment was not present in embryos expressing the E340A mutant, indicating that it was the C-terminal autocleavage product. Both the E340A mutant and the autocleavage product of wild-type ADAM13 were effectively downregulated by MO 19-1, arguing against the possibility that ADAM19 inhibits the autocleavage of ADAM13. By contrast, KD of ADAM13 resulted in an increase of the autocleavage product of ADAM19 (Fig. 10). Together these data suggest that the interaction between these two ADAMs inhibits the autocleavage of ADAM19 but not ADAM13. Interestingly, MO 19-1 had little effect on the levels of an ADAM13 mutant with the cytoplasmic tail deleted (ΔC ; Fig. 6A). Thus the cytoplasmic tail of ADAM13, but not the protease activity, is critical for the regulation by ADAM19.

We found previously that deletion of the cytoplasmic tail results in ~8-fold increase in cell surface levels of *X. laevis* ADAM13 (Alfandari et al., 2001). A similar effect was observed for *X. tropicalis* ADAM13 (Fig. 6F), raising the possibility that the cytoplasmic

tail may regulate the turnover of ADAM13, and that such turnover may be blocked by ADAM19. One of the most common mechanisms for membrane protein turnover is the ubiquitination of the cytoplasmic tail, which targets the proteins for proteasome-mediated degradation (MacGurn et al., 2012). Due to difficulties in detecting endogenously ubiquitinated ADAM13, we co-expressed HA- or myc₆-tagged ADAM13 with FLAG-tagged ubiquitin in 293T cells. Co-IP assays detected FLAG-tagged ubiquitin that was associated with ADAM13 (Figs. 5B and 5E), suggesting that ADAM13 can be ubiquitinated. To test if ADAM13 undergoes proteasome-mediated degradation in vivo, we dissociated *X. tropicalis* embryos expressing ectopic ADAM13, and cultured the cells with and without the proteasome inhibitor MG132. Western blot analyses show significantly higher levels of ADAM13 upon MG132 treatment (Fig. 6C). Notably, MG132 rescued the effects of ADAM19 KD on the levels of ADAM13 (Fig. 6C), indicating that ADAM19 regulates the proteasomal degradation of ADAM13.

The C-terminal K911 residue is essential for ADAM13 turnover and regulation by ADAM19

There are 20 lysine residues in the cytoplasmic tail of *X. tropicalis* ADAM13, and our prediction using the BDM-PUB algorithm suggests that the most C-terminal K911 residue has a very high probability of being ubiquitinated (Table I). This lysine residue is conserved in ADAM13 from fish to opossums, but not in the orthologs from higher mammals such as mice or humans. Certain other ADAMs, including the closely related ADAM12 from all known animal species, also contain a lysine residue at the C-terminus

that is likely ubiquitinated (Table I and data not shown), suggesting a possible common mechanism of regulation. To examine the role of the K911 residue of *X. tropicalis* ADAM13, we mutated this residue to arginine. When the same amount of expression plasmids was transfected into XTC or the mammalian 293T or 3T3 cells, the K911R mutant was expressed consistently at higher levels than wild-type ADAM13 (Fig. 6D and data not shown). Importantly, as compared with wild-type ADAM13, the K911R mutant had reduced ubiquitination when co-expressed with FLAG-tagged ubiquitin in 293T cells (Fig. 6E).

To assess the effects of ubiquitin on ADAM13 function in NC specification, we injected the same amount of transcripts encoding different variants of ADAM13, with and without the plasmid encoding ubiquitin, into *X. tropicalis* embryos. Similar to wild-type ADAM13, both the ectopically expressed K911R and ΔC mutants were able to induce an expansion of the *sox9* expression domain. Although co-expressed ubiquitin completely inhibited the expansion of *sox9* expression induced by wild-type ADAM13 and even caused a reduction of endogenous *sox9*, the effect was much weaker on the K911R mutant and nearly nonexistent on the ΔC mutant (Fig. 11). Finally, we determined the responses of different ADAM13 variants to ADAM19 MO in vivo. As shown in Fig. 6F, both the K911R and ΔC mutants were expressed at higher levels than wild-type ADAM13; unlike wild-type ADAM13, neither the K911R nor the ΔC mutant was reduced upon ADAM19 KD. Therefore, the K911 residue of ADAM13 is likely ubiquitinated and is essential for the regulation by ADAM19.

Stabilized ADAM13 mutants rescue the NC specification defects caused by ADAM19 KD

The requirement of ADAM19 for maintaining the protein levels of ADAM13, along with the similarity between the phenotypes of ADAM13 and 19 KDs, strongly implies that ADAM19 may function through regulation of ADAM13. To test this hypothesis, we evaluated the abilities of both ADAMs to rescue each other's KD phenotypes. The ΔC and K911R mutants, but not wild-type ADAM13 that was depleted upon ADAM19 KD, effectively rescued the NC specification phenotypes caused by ADAM19 KD, as shown by in situ hybridization for *snail2* and *sox9* (Figs. 6A and S5A). We also noticed that there was ectopic *snail2* expression in embryos injected with either the ΔC or the K911R mutant even in the presence of ADAM19 MO (Fig. 12A), suggesting that both mutants are sufficient to induce *snail2* expression outside of the NC territory independently of ADAM19 (to be further investigated below). To rule out the possibility that the myc₆ tag interferes with the biochemical properties of ADAM13, we generated constructs encoding untagged ADAM13 variants. The untagged ΔC and K911R mutants were indistinguishable from their myc₆-tagged counterparts in rescuing the NC specification defects caused by MO 19-1 and in inducing ectopic *snail2* (Fig. 12B), confirming that the myc₆ tag does not affect ADAM13 function. By contrast, neither wild-type ADAM19 nor the E/A mutant was able to rescue the reduced *sox9* expression caused by ADAM13 MO (Fig. 7B). Taken together, these data indicate that the in vivo function of ADAM19 in NC specification is dependent on ADAM13, but not vice versa. We further tested if the eye field induction defects caused by ADAM19 KD can also be

rescued by the ΔC and K911R mutants of ADAM13. Indeed, expression of the eye field markers *pax6* and *rx1* was restored when mRNA encoding either ADAM13 mutant was co-injected with MO 19-1 (Fig. 13), suggesting that ADAM19 also functions in eye field induction by regulating ADAM13.

Ectopic expression of stabilized ADAM13 mutants induces *snail2* in the future epidermis. We previously identified *Snail2* as the major downstream target that mediates ADAM13 function in NC specification, as exogenous *Snail2* rescued the NC defects caused by ADAM13 KD (Wei et al., 2010b). Our current finding that the ΔC and K911R mutants of ADAM13 can induce ectopic *snail2* in the dorsal ectoderm (Figs. S5A and S5B) not only validates these results, but also points to the possibility that the ADAM13-Wnt signaling axis may partially re-specify non-NC cells to adopt some NC-like properties. To test this possibility, we injected transcripts encoding different variants of ADAM13 into the posterior-ventral region of *X. tropicalis* embryos, where the cells are destined to become epidermis and do not express detectable levels of endogenous *snail2*. As shown in Fig. 7C, a low dosage of mRNA encoding wild-type ADAM13 induced weak ectopic expression of *snail2* in the ventral ectoderm, whereas the same amount of the ΔC and K911R mutant transcripts caused a robust induction. A similar induction of *snail2* expression by wild-type ADAM13 was obtained in animal cap assays using *X. laevis* embryos (Khedgikar et al., submitted). By contrast, the E/A mutant failed to induce ectopic *snail2*, and co-injecting a β -catenin MO completely abolished the *snail2*-inducing activity of the K911R mutant (Fig. 7C). These results are consistent with our previous observations that ADAM13 protease activity and downstream Wnt signaling

are required for NC specification (Wei et al., 2010b). However, we were unable to detect any ectopic expression of other NC or NPB specifiers that were tested, including *sox9*, *foxd3*, *pax3*, *zic1* and *msx1*, in any of these embryos (data not shown). Thus the ADAM13-Wnt axis is sufficient to induce *snail2* in the ventral ectoderm but not a complete conversion to the NC lineage.

Discussion

Many extracellular metalloproteinases, including several ADAMs, have been implicated in NC development (Christian et al., 2013). The ADAMs are key regulators of cell signaling, mainly due to their abilities to cleave signaling molecules such as ligands, receptors, agonists and antagonists. However, some ADAM proteases may function through their non-proteolytic activities in certain physiological contexts, and there are also non-proteolytic ADAMs that play important roles in development and disease (Edwards et al., 2008; Reiss and Saftig, 2009). As compared with the well-characterized functions of ADAM protease activities, little is known about the non-proteolytic roles of ADAMs in vivo. Here we report that *Xenopus* ADAM19 specifies the NC lineage through non-proteolytic protection of ADAM13. To our knowledge, there is no published example of one protease protecting another in vivo. A possible mechanism for this novel protease-protease interaction is discussed below.

The first two testicular ADAMs, ADAMs 1 and 2, were discovered as a heterodimer that is involved in sperm-egg fusion (Blobel et al., 1992). However, the physiological

consequence of this dimerization is unclear, and similar interaction has not been reported for somatic ADAMs. Instead, ADAMs 10 and 17, two somatic ADAMs, are known to interact with tetraspanins and non-proteolytic members of the Rhomboid family (iRhoms), respectively, which regulate the trafficking, stability and activity of these ADAMs (Adrain et al., 2012; Dornier et al., 2012; Haining et al., 2012; Matthews et al., 2017; McIlwain et al., 2012). The activity of *Xenopus* ADAM13 has also been shown to be regulated by other proteins such as PACSIN2 and XFz4 (Abbruzzese et al., 2015; Cousin et al., 2000). In the current study we observed a protective effect of ADAM19 towards ADAM13, which underlies the *in vivo* function of ADAM19 in NC specification. Our data suggest that in the absence of ADAM19, ADAM13 is ubiquitinated at the C-terminal K911 residue and degraded through the proteasome pathway. When ADAM19 is present, it binds to the cysteine-rich domain of ADAM13 and possibly masks the K911 residue in the cytoplasmic tail of ADAM13, resulting in the stabilization of ADAM13 that subsequently specifies the NC lineage (Fig. 7D).

Interestingly, although ADAM13 inhibits the autocleavage of ADAM19, ADAM19 does not seem to cleave ADAM13 or interfere with its protease activity (Figs. S3 and 5A). Therefore, the heterodimer is likely asymmetric: while the protease activity of ADAM19 may be blocked by ADAM13, ADAM13 remains free to cleave its substrates (Fig. 7D). This structural asymmetry is likely critical for the difference in the mechanism of action for the two ADAMs in NC specification.

Most of the studies on the functions of ADAMs in early NC development have been carried out in two *Xenopus* species, *X. laevis* and *X. tropicalis* (Christian et al., 2013).

Our previous work unveiled the essential roles of ADAM13 in NC induction in *X. tropicalis* embryos (Wei et al., 2010b), which is confirmed by the current study. However, KD of ADAM13 in *X. laevis* did not affect any NC markers, raising the question whether the function of ADAM13 in NC induction is conserved (Cousin et al., 2011). A major difference between the two frog species is that *X. tropicalis* is diploid, whereas *X. laevis* is allotetraploid (Session et al., 2016). The *X. laevis* genome contains a pseudoallele of *adam13* (a.k.a. *xmdc13*), which encodes a protein that is ~88% and 87% identical to *X. laevis* and *X. tropicalis* ADAM13, respectively (Cai et al., 1998). KD of both ADAM13 and xMDC13 did result in reduction of *snail2* expression prior to NC migration in *X. laevis* embryos (our unpublished data), indicating that ADAM13 function in NC specification is conserved between the two frog species. Unlike *adam13*, there is only one copy of *adam19* in *X. laevis*, and KD of this gene also causes reduction of pre-migratory NC markers at stage ~15 (Neuner et al., 2009). However, it was unclear if this effect was caused by inhibition of induction or post-induction maintenance of the NC. Combined with the current study, these results suggest that ADAM19 is required for NC specification in both *Xenopus* species. At later stages, ADAM13 is required for NC migration through its protease activity as well as the cytoplasmic tail, which is cleaved and translocalizes into the nucleus to regulate target gene transcription (Cousin et al., 2011). By contrast, we show here that the function of ADAM13 in NC specification is independent of its cytoplasmic tail, as the ΔC mutant can effectively rescue the early NC defects caused by ADAM19 KD and induce ectopic *snail2* (Figs. 6A, 6C, S5A and S5B). Thus the mechanisms of action for ADAM13 in NC specification and migration appear to be different. Previous research has implicated more than one canonical Wnt pathway in

NC induction. In *Xenopus* embryos, several Wnt ligands and receptors have been reported to have NC-inducing capacity, and loss-of-function studies have confirmed that at least two canonical Wnt ligands, Wnt3a and Wnt8, as well as two Wnt receptors, Xfz3 and Xfz7, are required for endogenous NC induction (Abu-Elmagd et al., 2006; Deardorff et al., 2001; Elkouby et al., 2010; Hong et al., 2008). These Wnt ligands and receptors seem to be expressed in different regions at the time of NC induction (Abu-Elmagd et al., 2006; Steventon et al., 2009), suggesting that instead of functioning redundantly, they may form separate Wnt pathways to regulate different aspects of NC induction.

Indeed, NC induction can be divided into two steps, the formation of NPB and the specification of NC lineage, both of which are thought to depend on Wnt signaling (Simões-Costa and Bronner, 2015). However, while the regulation of NPB markers/specifiers by Wnt signaling has been well studied, if and how Wnt controls endogenous NC specification remained elusive. This is probably because NC specification immediately follows, and is difficult to be separated from, NPB formation. In particular, it was unclear if the two steps of NC induction are controlled by the same Wnt pathway or separate ones. In this study we provide three important lines of evidence suggesting that a distinct Wnt pathway, which is controlled by ADAMs 13 and 19, is required for endogenous NC specification but not NPB formation in *X. tropicalis* embryos. First, by using a transgenic Wnt reporter line, we found that canonical Wnt signaling is highly active within the NPB at late gastrula stages (Fig. 2E). To our knowledge this is the first direct evidence indicating that there is strong Wnt signal at the NPB during NC

specification, although previous studies indicate that Wnt activity in NC explants increases between gastrula and neurula stages (Steventon et al., 2009). This spatiotemporal pattern of Wnt activity is consistent with its function in NC specification. Second, KD of either ADAM13 or 19 inhibits Wnt signaling and NC specification at the NPB, but the formation of NPB itself is unaffected (Figs. 1C-1E). These results differ from previous reports that Wnt inhibition causes loss of both NPB and NC markers (Li et al., 2009; Monsoro-Burq et al., 2005). Thus unlike the “core” Wnt components that are required ubiquitously for Wnt signal activation, ADAMs 13 and 19 are needed only in a subset of tissues, probably where the Wnt signal is cross-antagonized by ephrin-B signaling. Such differential requirement for these ADAMs allows us to separate the Wnt pathway that specifies NC lineage from the one that establishes the NPB. Last, we show that ectopic expression of ADAM13 induces the NC specifier *snail2* in the ventral ectoderm in a Wnt-dependent manner (Fig. 7C). This gain-of-function phenotype provides additional evidence for the specification of NC lineage by the ADAM13-Wnt signaling axis. Among the NC specifier genes, *snail2* is the only one that is known to contain a cis-regulatory element that binds LEF/TCF transcription factors, and has been speculated to be the direct Wnt target during NC specification (Simões-Costa and Bronner, 2015). Interestingly, ADAM13 cannot induce ectopic expression of any of the other NC or NPB markers that were examined, suggesting that *snail2* is indeed the direct target of the ADAM13/19-Wnt axis. This is further supported by our previous finding that exogenous *Snail2* can rescue the reduction of other NC markers, such as *sox9* and *twist*, caused by ADAM13 KD (Wei et al., 2010b).

The potential application of NC cells in tissue regeneration for the treatment of related birth defects or damages has drawn a great deal of research interest. Methods are being actively developed to differentiate pluripotent stem cells or reprogram non-NC lineages to generate NC cells (Kunisada et al., 2014; Zhu et al., 2016). In this study, we found that the stabilized ADAM13 mutants that can function independently of ADAM19 induce robust expression of the key NC specifier *snail2* in the ventral ectoderm; however, the lack of other NC or NPB markers/specifiers indicates an incomplete conversion to the NC lineage (Fig. 7C). By contrast, overexpression of ADAM13 on the dorsal ectoderm causes expansion of both *snail2* and other NC markers such as *sox9* along the NPB (Fig. 3A), suggesting that additional molecules, which are normally present at the NPB, are needed to cooperate with ADAM13 to induce a complete conversion of the ventral ectoderm cells to NC lineage. Potential candidates include inhibitors of bone morphogenetic protein (BMP) signaling, such as Chordin and Noggin, because high BMP signal on the ventral side of the embryos is known to inhibit NC induction (LaBonne and Bronner-Fraser, 1998; Steventon et al., 2009). Our results also call for caution in interpreting some of the published data on ectopic NC induction, where *snail2* is used as the only marker for the NC lineage. Finally, Wnt signaling and Snail2 are known to be key regulators of the epithelial-to-mesenchymal transition (EMT) in development, wound repair and cancer. In the traditional models of EMT, Wnt and Snail2 control the downstream expression of metalloproteinases, which in turn modify cell-cell and cell-matrix adhesion through their proteolytic activities to promote cell migration and invasion (Lim and Thiery, 2012; Powell et al., 2013). Here we show that both the proteolytic and nonproteolytic activities of ADAMs play important roles

upstream to regulate Wnt signaling and snail2 expression. It would be of tremendous interest to examine if similar mechanisms are also involved in developmental and/or pathological EMT.

Materials and methods

Plasmids and antibodies

X. tropicalis cDNA clones encoding adams 12, 13 and 19 were obtained previously (Wei et al., 2010b). Constructs encoding C-terminally myc₆- or HA-tagged wild-type ADAMs were generated by subcloning into pCS2+ vectors modified to append corresponding epitopes, and mutations were introduced using a QuickChange mutagenesis kit (Stratagene). Rescue constructs were made by introducing sense mutations downstream of the translational start codon in corresponding myc₆-tagged expression constructs. Constructs for preparing the in situ hybridization probes for snail2, sox9, foxd3, pax3, zic1, msx1, pax6 and rx1 were obtained previously (Li et al., 2011; Wei et al., 2012; Wei et al., 2010b). The FLAG-ubiquitin construct was a gift from Dr. Carole Labonne. In vitro transcription was carried out as described to generate mRNA transcripts and in situ hybridization probes (Sive et al., 2000). Commercial primary antibodies that were used in this study include: mouse anti-myc (DSHB 9E10), mouse anti-HA (Sigma-Aldrich H9658), mouse anti-FLAG (Sigma-Aldrich H9658), and rabbit anti-Calnexin (Enzo Life Sciences ADI-SPA-860). The rabbit anti-ADAM13 and anti-ADAM19 antibodies were generated previously (Alfandari et al., 1997; Neuner et al., 2009). Commercial secondary antibodies that were used include HRP-conjugated rabbit anti-mouse and goat anti-rabbit (Sigma-Aldrich), as well as Alexa Fluor 488 donkey anti-

mouse and Alexa Fluor 594 donkey anti-rabbit (Jackson Laboratories). For loading control, unconjugated mouse anti- β 1-integrin (DSHB 8C8) or anti-GAPDH (Millipore MAB374), or HRP-conjugated mouse anti- β -actin (Sigma-Aldrich A5316) were used.

Animals and embryo manipulation

Wild-type *X. tropicalis* and *X. laevis* adults were purchased from NASCO. The transgenic *X. tropicalis* Wnt reporter frogs were a courtesy of Dr. Kris Vleminckx, and the transgenic *X. tropicalis* snail2-eGFP line was generated as described elsewhere (Li et al., manuscript in preparation). Morpholino oligos were designed and synthesized by Gene Tools. Embryo preparation, injection and culturing were carried out as described (Wei et al., 2010b). Briefly, embryos were obtained by natural mating, and injections were carried out to target specific blastomeres (Huang et al., 1998) using a PLI-100A microinjector (Harvard Apparatus). Alexa Fluor 555 or 488 dextran (Invitrogen) was co-injected as a lineage tracer. Embryos were cultured in 0.1x MBS to desired stages, and injected embryos were sorted according to the injected side using a Zeiss Axiozoom.v16 epifluorescence microscope. Embryo lysates were prepared as described below for cell lysates. For embryo dissociation, *X. tropicalis* embryos were injected in both blastomeres at 2-cell stage and cultured to stage ~12.5. The embryos were placed in 1.5 mL centrifuge tubes with 0.1x MBS and 100 μ M MG132 (or DMSO as control). The embryos were then dissociated using a 28 gauge 1 ml syringe (Exel) and incubated in 0.1x MBS and 100 μ M MG132 or DMSO at 23°C for 5 hours.

Cell culture and transfection

HEK293T cells were purchased from ATCC, and cultured in DMEM (ATCC) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C with 5% CO₂. XTC cells (Pudney et al., 1973) were cultured in L-15 medium (GE Life Sciences) supplemented with 10% FBS and 1 mM sodium pyruvate at 23°C. Cells were transfected using Lipofectamine 3000 (Invitrogen) at 50-70% confluency, and allowed to grow for another 24-48 hr. For MG132 treatment, cells were cultured in the presence of 40 μ M MG132 for 24 hr before being harvested. To prepare whole-cell lysates, cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM Na₄P₂O₇, 1 mM PMSF, and 5 mM EDTA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and 10 mM o-phenanthroline, and centrifuged for 20 min at 12,000 g at 4°C. Supernatants were processed for SDS-PAGE and blotted with corresponding antibodies.

In situ hybridization and immunocytochemistry

Embryos were fixed at desired stages, and in situ hybridization was carried out as described (Sive et al., 2000). For immunocytochemistry, cells were cultured and transfected on glass coverslips (Fisher Scientific), and fixed with 1:1 methanol/acetone at -20°C for 10 min. The cells were then rehydrated with 1x PBS for 10 min, blocked with 1% bovine serum albumin (Sigma-Aldrich) for 1 hr at room temperature, and incubated with the primary antibody at 4°C overnight. After being washed with PBS containing 0.5% Tween-20, the cells were subsequently incubated with Alexa Fluor- conjugated secondary antibody for 1 hr at room temperature, and labeled with DAPI (Sigma-Aldrich) to identify cell nuclei. Images were taken with a Zeiss 780 multiphoton confocal

microscope.

Western blot and Immuno-Precipitation

Detection of Western blot was carried out using HRP-conjugated secondary antibodies and chemiluminescence substrates (GE Healthcare). Membranes were stripped and reblotted for β -actin, β 1-integrin or GAPDH as a loading control. For IP, cells were lysed with 20 mM Tris-HCl, pH 7.6, 167 mM NaCl, 1% NP-40, and protease inhibitor cocktail (Sigma-Aldrich) at 4°C for 30 min. The lysates were then centrifuged for 20 min at 12,000 g at 4°C. Supernatants were collected and incubated with 2-10 μ g of antibody with rotation at 4°C overnight. Protein G PLUS-Agarose beads (Santa Cruz) were added to the mixture and rotated for 1 hr at 4°C. Beads were then washed 5 times with ice-cold PBS. The bound proteins were dissociated by incubating with SDS-PAGE loading buffer at 50°C for 10 min and then 100°C for 10 min, and subsequently subjected to Western blot analysis.

Phenotype Scoring and Statistics

For craniofacial phenotypes, injected snail2-eGFP embryos were allowed to develop to stage ~46 and scored for defects in head cartilage structures. For eye phenotypes, injected wild-type embryos were allowed to develop to stage ~38 and scored for defects in eye structures. Images of head cartilages (eGFP) or eyes (bright field) were taken with a Zeiss Axiozoom.v16 epifluorescence microscope. The percentage of normal and reduced embryo phenotypes were calculated and averaged for multiple independent experiments. Fisher's exact tests were performed for phenotype comparisons.

Acknowledgements

We thank Dr. Kris Vleminckx for providing the transgenic Wnt reporter frogs, and Dr. Carole Labonne for the FLAG-ubiquitin construct. This work was supported by the National Institute of Health (GM114105, GM094793, DE022813, DE016289, and 1S10RR027273-01), and March of Dimes Foundation (1-FY10-399).

Competing interests

The authors declare no competing interest.

Tables

Table I. Prediction of ubiquitination sites in ADAMs

Protein			C-terminal	Sequence	Score
Zebrafish ADAM13	935	PLIVPAVFR	944	3.7	
		K			
<i>X.trop</i> ADAM13	902	AAAAAFLQR	911	5.3	
		K			
<i>Anolis</i> ADAM13	956	VQTSSFPPK	965	4.5	
		K			
Chick ADAM13	938	VQSTSFPK	947	4.4	
		K			
Opossum ADAM13	934	PGRGSLTLK	943	4.8	
		K			
Mouse ADAM12	894	PRPSHNAYI	903	4.3	
		K			
Mouse ADAM6	745	RSWQEQAKD	754	4.6	
		K			

The C-terminal 10 residues of ADAMs from indicated species are aligned, and the lysine (K) residue at or near the C-terminus is shown in red. Scores indicate the probabilities of this lysine residue being ubiquitinated, as predicted by the BDM-PUB algorithm (<http://bdmpub.biocuckoo.org>). Scores above threshold (0.3 or higher) suggest high probability of ubiquitination.

Figures and legends

Figure 2. KD of ADAM13 or 19 inhibits Wnt signaling and NC specification but not NPB formation. A. cDNA sequences of adams 12 and 19 showing the translation start site (red) and targets of MOs (colored boxes). B. KD of ADAM13 or 19 inhibits head cartilage formation. Transgenic *X. tropicalis* *snail2-eGFP* embryos were injected in one

anterodorsal (D1) blastomere at 8-cell stage with the indicated MO (1.5 ng each), cultured to stage ~46 and imaged for eGFP expression. One representative embryo of each injected group (ventral view) is shown in upper panels, and results of 5 independent experiments are graphed in the lower panel (error bars represent standard deviations). ***: $P < 0.001$. C, D. Effects of ADAM13 or 19 KD on NC and NPB marker expression. Wild-type embryos were injected in one blastomere at 2-cell stage with the indicated MO (6 ng each) and cultured to stage ~12.5. Embryos were processed for in situ hybridization for the indicated genes. E. KD of ADAM13 or 19 inhibits endogenous Wnt signaling. Transgenic Wnt reporter embryos were injected as in C, D, cultured to stage ~12.5 and imaged for eGFP expression. White arrowheads point to the Wnt signal at the NPB. Embryos in c-e are shown in dorsal view, with anterior at the top. Red asterisks denote the injected side. CT, control MO; n, number of embryos scored; N, number of independent experiments performed.

Figure 3. ADAMs 13 and 19 function in NC specification through different mechanisms. Wild-type embryos were injected in one blastomere at 2-cell stage with the indicated MO (6 ng each) and mRNA (100 pg each), cultured to stage ~12.5, and processed for in situ hybridization for the indicated genes. A. Effects of ectopically expressed ADAM13 and 19 variants on NC markers. B. Both wild-type ADAM19 and the protease-dead E/A mutant rescue the NC specification defects caused by ADAM19 KD. Rescue constructs were generated as described in Materials and methods. All embryos are shown in dorsal view, with anterior at the top and a red asterisk denoting the injected side. U, uninjected; WT, wild-type.

Figure 4. ADAM19 controls ADAM13 protein levels in vivo. A. Effects of various MOs on exogenously expressed ADAMs 13 and 19. Embryos were injected in one blastomere at 2-cell stage with the indicated RNA (100 pg each) and MO (6 ng each), and cultured to stage ~19. Whole-embryo lysates were processed for Western blot (WB) for the C-terminal myc₆ tag on the ADAM constructs. B. Effects of various MOs on endogenous ADAM13. One-cell stage embryos were injected with the indicated MO (12 ng each) and cultured to stage ~12.5. Embryo lysates were processed for Western blot for endogenous ADAM13. C. Upregulation of exogenous ADAM13 by co-expressed ADAM19. Embryos were injected with the indicated transcripts (100 pg each; the encoded ADAM13 was myc₆-tagged and ADAM19 was HA-tagged) and cultured as in A, and lysates were processed for Western blot with an anti-myc antibody. Single and

double arrowheads indicate the pro- and mature forms of ADAMs, respectively.

Figure 5. ADAM19 co-localizes and interacts with ADAM13. A, B. Co-localization of ADAMs 13 and 19 in the ER. XTC cells were transfected with 1 μ g of plasmid encoding ADAM19, and immunocytochemistry for ADAM19 (red), Calnexin (green in A) and ADAM13 (green in B) as well as DAPI labeling for nuclei (blue) were carried out as described in Materials and methods. Arrowheads point to lamellipodia. C, D. Association of ADAM19 with ADAM13. HEK293T cells were transfected with plasmids (0.5 μ g each) encoding ADAM19 and untagged ADAM13 (C) or different variants of myc₆-tagged ADAM13 (D). IP and Western blot were carried out with the indicated antibodies. Single and double arrowheads indicate the pro- and mature forms of ADAMs, respectively. CR,

cysteine-rich domain; D, disintegrin domain; DC, disintegrin and cysteine-rich domains;
FL, full-length.

Figure 6. The cytoplasmic K911 residue is critical for ubiquitin-proteasome-mediated ADAM13 turnover and regulation by ADAM19. A, F. Responses of different variants of ADAM13 to ADAM19 KD. Embryos were injected in one blastomere at 2-cell stage with the indicated RNA (100 pg each) and MO (6 ng each), and cultured to stage ~19. Whole-embryo lysates were processed for Western blot for the C-terminal myc₆ tag on the ADAM constructs. A shorter exposure was performed for the ΔC mutant in A. B, E. Ubiquitination of wild-type ADAM13 and the K911R mutant. HEK293T cells were transfected to express the FLAG-tagged ubiquitin with and without HA-tagged ADAM13 (B), or myc₆-tagged wild-type ADAM13 or the K911R mutant (E). IP and Western blot were carried out with the indicated antibodies. C. MG132 protects ADAM13 and rescues the effect of ADAM19 KD on ADAM13. Embryos were injected, dissociated, and cultured in the absence or presence of MG132 as described in Materials and methods. Cell lysates were processed for Western blot. D. XTC cells were transfected with 0.5 μg of plasmids encoding myc₆-tagged wild-type ADAM13 or the K911R mutant.

Immunocytochemistry for myc (green) and DAPI labeling for nuclei (blue) were carried out as described in Materials and methods, and images taken in green and blue channels were merged. Single and double arrowheads indicate the pro- and mature forms of ADAM13, respectively, and asterisk denotes the autocleavage product. UB, ubiquitin.

Figure 7. The stabilized ADAM13 mutants can rescue the NC specification defects caused by ADAM19 KD and induce ectopic *snail2* in the ventral ectoderm. A, B. Rescue of the reduced *sox9* expression caused by ADAM19 KD (A) or ADAM13 KD (B) by various transcripts. Embryos were injected in one blastomere at 2-cell stage with the indicated RNA (100 pg each) and MO (6 ng each), cultured to stage ~12.5, and processed for in situ hybridization for *sox9*. Embryos are shown in dorsal view, with anterior at the top and a red asterisk denoting the injected side. C. Induction of ectopic *snail2* by different variants of ADAM13. Embryos were injected in one posterior-ventral

(V2) blastomere at 8-cell stage with the indicated RNA (50 pg each) and MO (1.5 ng each), cultured to stage ~12.5, and processed for in situ hybridization for *snail2*. Only the injected side is shown, with anterior at the top and dorsal to the left. Arrows point to the ectopic *snail2* expression. D. A model for the regulation of ADAM13 stability and NC specification by ADAM19. Crescents denote the metalloproteinase domains of ADAMs. See Discussion for explanation.

Figure 8. Developmental expression of *adam19* in *X. tropicalis* embryos. Embryos were fixed at the indicated stages, and in situ hybridization was carried out for *adam19*. A. Vegetal view with dorsal at the top. Arrow denotes the dorsal blastopore lip. B. A bisected embryo shown with dorsal at the top and anterior to the left.

Figure 9. KD of ADAM13 or 19 inhibits eye field induction in *X. tropicalis* embryos. A. Eye phenotypes caused by ADAM13 or 19 KD. Embryos were injected in one anterodorsal (D1) blastomere at 8-cell stage with the indicated MO (1.5 ng each), cultured to stage ~38, and the injected side was imaged. One representative embryo of each injected group is shown in upper panels, and results of 3 independent experiments are graphed in the lower panel (error bars represent standard deviations). ***: $P < 0.001$. B, C. Effects of ADAM13 or 19 KD on the expression of eye field markers. Embryos were injected in one blastomere at 2-cell stage with the indicated MO (6 ng each), cultured to stage ~12.5, and processed for in situ hybridization for the indicated genes. Embryos in B, C are shown in dorsal view, with posterior at the top and a red asterisk denoting the injected side. CT, control MO; n, number of embryos scored; N, number of independent experiments performed.

Figure 10. ADAM13 inhibits the autocleavage of ADAM19. Embryos were injected in one blastomere at 2-cell stage with the indicated RNA (100 pg each) and MO (6 ng each), and cultured to stage ~19. Whole-embryo lysates were processed for Western blot for the C-terminal myc₆ tag on the ADAM19 construct. Single and double arrowheads indicate the pro- and mature forms of ADAM19, respectively, and asterisk denotes the autocleavage product.

Figure 11. Effects of exogenous ubiquitin on the functions of different ADAM13 variants in *X. tropicalis* embryos. Embryos were injected in one blastomere at 2-cell stage with the indicated mRNA (100 pg each) and the plasmid encoding FLAG-tagged ubiquitin (UB; 2.5 pg). The injected embryos were cultured to stage ~12.5, and processed for in situ hybridization for *sox9*. All embryos are shown in dorsal view, with anterior at the top and a red asterisk denoting the injected side.

Figure 12. The stabilized ADAM13 mutants rescue the NC specification defects caused by ADAM19 KD. *X. tropicalis* embryos were injected in one blastomere at 2-cell stage with MO 19-1 (6 ng) and the indicated mRNA (100 pg each) encoding myc₆-tagged (A) or untagged (B) variants of ADAM13. The injected embryos were cultured to stage ~12.5, and processed for in situ hybridization for the indicated genes. All embryos are shown in dorsal view, with anterior at the top and a red asterisk denoting the injected side. Arrows point to the ectopic *snail2* expression.

Figure 13. The stabilized ADAM13 mutants rescue the eye field defects caused by ADAM19 KD. *X. tropicalis* embryos were injected in one blastomere at 2-cell stage with the indicated mRNA (100 pg each) and MO 19-1 (6 ng). The injected embryos were cultured to stage ~12.5, and processed for in situ hybridization for the indicated genes. All embryos are shown in dorsal view, with posterior at the top and a red asterisk denoting the injected side.

Chapter 3: The RNA helicase DDX3 induces neural crest through the AKT-GSK3 β axis

The RNA helicase DDX3 induces neural crest through the AKT-GSK3 β axis

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Keywords: DDX3; RNA helicase; Neural crest (NC); neurocristopathy; neural plate border (NPB); Akt; GSK3 β ; Wnt signaling; *Xenopus*

Abstract

Mutations in the RNA helicase DDX3 have emerged as a frequent cause of intellectual disability in humans. Because many patients carrying DDX3 mutations have additional defects in craniofacial structures and other tissues that derive from the neural crest (NC), we hypothesize that DDX3 is also important for NC development. Using *Xenopus tropicalis* as a model, we show that DDX3 is required for normal NC induction and craniofacial morphogenesis by regulating AKT kinase activity. Depletion of DDX3 decreases AKT activity and AKT-dependent inhibitory phosphorylation of GSK3 β , leading to reduced levels of β -catenin and Snai1, two GSK3 β substrates that are critical for NC induction. DDX3 function in regulating these downstream signaling events during NC induction is mediated by RAC1, a small GTPase whose translation depends on the RNA helicase activity of DDX3. These results suggest an evolutionarily conserved role of DDX3 in NC development through the AKT-GSK3 β axis, and provide a potential mechanism for the NC-related birth defects displayed by patients harboring mutations in DDX3 and its downstream effectors in this signaling cascade.

Introduction

Neural crest (NC) cells are multipotent stem cells that are induced to form at the neural plate border (the border of neural plate and epidermis; NPB) during vertebrate gastrulation. After the induction and subsequent maintenance, NC cells migrate out of the closing neural tube to specific destinations, where they differentiate into various types of cells and contribute to many tissues, such as the craniofacial structures, dental tissues, the peripheral nervous system, pigment cells, and cardiac tissues (Bronner and Simões-Costa, 2016; Simões-Costa and Bronner, 2015; Stuhlmiller and Garcia-Castro, 2012). In humans, impaired NC development can lead to birth defects that are collectively called neurocristopathies, including craniofacial disorders, congenital heart diseases, and pigment defects (Dubey and Saint-Jeannet, 2017; Vega-Lopez et al., 2018).

Nearly all of our knowledge on the induction of NC was obtained from studies using *Xenopus* and other non-mammalian vertebrates such as chicks, due to technical difficulties that have been encountered in mice (Barriga et al., 2015; Betters et al., 2018). These studies have uncovered a two-stage process for NC induction. First, the interplay of multiple signaling pathways activates the expression of transcription factors such as Pax3, Zic1 and Msx1 (the “NPB specifiers”), which induce NPB formation.

These NPB specifiers subsequently induce other transcription factors, including Snai2, FoxD3 and Sox9 (the “NC specifiers”), to specify the NC fate within the NPB (Bronner and Simões-Costa, 2016; Simões-Costa and Bronner, 2015; Stuhlmiller and Garcia-

Castro, 2012). A major signaling pathway that induces the NC in non-mammalian vertebrates is canonical Wnt/ β -catenin signaling (hereinafter referred to as “Wnt signaling”), which is required for both the initial formation of the NPB and the subsequent specification of the NC (Li et al., 2018; Prasad et al., 2019). Similarly, the induction of NC from human pluripotent stem cells also requires the activation of Wnt signaling (Gomez et al., 2019; Leung et al., 2016; Menendez et al., 2011; Mica et al., 2013), indicating that Wnt function in NC induction is conserved during vertebrate evolution.

The DEAD/H box RNA helicase DDX3 plays important roles in many cellular processes including cell cycle regulation, differentiation, survival, and apoptosis, primarily by regulating ATP-dependent RNA metabolism, such as RNA splicing, nuclear export, and translation (He et al., 2018; Heerma van Voss et al., 2017; Sharma and Jankowsky, 2014). DDX3 contains a helicase ATP-binding domain and a helicase C-terminal domain, both of which are evolutionarily conserved and required for its RNA helicase activity. There are two functional DDX3 homologs in humans, DDX3X and DDX3Y, which are localized on the X and Y chromosome, respectively. Although the two gene products share high sequence similarity, they have distinct expression patterns and functions. DDX3Y is translated specifically in the testes and is necessary for male fertility, whereas DDX3X is expressed ubiquitously and accounts for most, if not all, DDX3 functions in somatic tissues (Kotov et al., 2017; Snijders Blok et al., 2015). In rodents, there is at least one additional homolog of DDX3 that is actively expressed. The tissue specificity of DDX3X and DDX3Y is also different in rodents and primates,

suggesting that these genes may have evolved differently in the two mammalian orders (Chang and Liu, 2010; Matsumura et al., 2019). In contrast, *Xenopus tropicalis* has only one *ddx3* homolog, which is localized on an autosome, and the encoded protein is 91% identical to human DDX3X. Similar to human DDX3X, the *X. tropicalis* *ddx3* mRNA is expressed ubiquitously throughout early development (Cruciat et al., 2013). Thus, *X. tropicalis* may be a suitable model to study DDX3 function in somatic tissue development.

Recently, de novo mutations in DDX3X were reported to be a common cause of intellectual disability in humans (<https://ddx3x.org>) (Dikow et al., 2017; Kellaris et al., 2018; Lennox et al.; Nicola et al., 2019; Snijders Blok et al., 2015; Wang et al., 2018). These mutations were found primarily in females, and there are strong indications that some of them lead to embryonic lethality in males (Nicola et al., 2019; Snijders Blok et al., 2015). Most of these mutations were predicted to result in decreased DDX3X activity, and the defects in female patients may be caused by haploinsufficiency or dominant-negative effects of the mutants; partial X inactivation of the DDX3X gene may also contribute to the phenotypes (Garieri et al., 2018; Lennox et al.; Snijders Blok et al., 2015). Patients with DDX3X mutations display various defects in central nervous system (CNS) development, including intellectual disability, autism spectrum disorder, microcephaly, and corpus callosum hypoplasia. Most patients also have craniofacial disorders, ranging from mild dysmorphic facial features to severe oral-facial clefts (Dikow et al., 2017; Kellaris et al., 2018; Lennox et al.; Nicola et al., 2019; Snijders Blok et al., 2015; Wang et al., 2018). Other common symptoms include congenital heart

diseases, pigment defects, and hearing/visual impairment (Snijders Blok et al., 2015; Wang et al., 2018); a recent report also suggests that these patients have high rates of neuroblastoma, a rare NC-derived childhood tumor (Lennox et al.). Because this spectrum of non-CNS symptoms is typically caused by impaired NC development (Dubey and Saint-Jeannet, 2017; Vega-Lopez et al., 2018), we hypothesize that DDX3X mutations can lead to not only CNS defects but also neurocristopathies.

In this study, we investigated the roles of DDX3 in NC development using *X. tropicalis* as a model. Knockdown (KD) of DDX3 in *X. tropicalis* embryos caused inhibited NC induction and hypoplasia in craniofacial cartilage, phenotypes that could be rescued by wild-type human DDX3X but not an RNA helicase-dead mutant. In both *X. tropicalis* embryos and human cells, DDX3 KD reduced endogenous AKT activity and Wnt signaling. The latter could be attributed to the decrease in AKT-mediated GSK3 β phosphorylation and inhibition, which resulted in enhanced degradation of β -catenin. In addition, Snai1, another GSK3 β substrate, was also reduced post-transcriptionally. The defects in NC induction caused by DDX3 KD could be rescued by a constitutively active form of AKT, or by co-expression of β -catenin and Snai1. Finally, DDX3 KD led to decreased levels of RAC1, a direct downstream target of DDX3 and upstream activator of AKT, and exogenous RAC1 also rescued the NC induction phenotypes caused by DDX3 KD in *X. tropicalis* embryos. Collectively, our data suggest a critical role of DDX3 in regulating the AKT-GSK3 β signaling axis through RAC1, and provide a mechanistic explanation for the similar neurocristopathies that have been observed in patients with

mutations in DDX3X, RAC1, or AKT-related genes.

Results

DDX3 RNA helicase activity is required for normal NC induction and craniofacial morphogenesis in X. tropicalis embryos

To understand the potential roles of DDX3 in NC development and craniofacial morphogenesis, we used a new transgenic *X. tropicalis* line expressing enhanced green fluorescent protein (eGFP) driven by the *snai2* promoter/enhancer. The strong eGFP expression in the differentiating cranial NC lineage allows direct observation of craniofacial cartilage development in live *snai2:egfp* embryos (Li et al. 2019). Injection of a well-characterized translation-blocking DDX3 morpholino (MO), which targets the 5'- untranslated region (5'-UTR)(Cruciat et al., 2013), led to hypoplasia of craniofacial cartilage structures on the injected side, compared to the uninjected side (Fig. 14A). The same phenotype was obtained with a second MO that targets a different site (MO DDX3-2), confirming that this phenotype is specific for DDX3 KD (Fig. 14A). These results suggest that, similar to human DDX3X, *Xenopus* DDX3 is also important for craniofacial morphogenesis.

To assess the effects of DDX3 KD on early NC development, we examined the expression of NPB and NC specifiers at the end of gastrulation (stage ~12.5), when the induction of cranial NC is nearly complete. KD of DDX3 reduced the expression of *snai2* and *sox9*, two NC specifiers, indicating that NC induction is inhibited. Interestingly,

different NPB specifiers responded differently to DDX3 KD: while both *pax3* and *msx1* were downregulated, *zic1* expression remained largely unchanged and was slightly expanded in some embryos (Fig. 14B, 18C). The expansion of *zic1* was probably secondary to the reduction in PAX3, as KD of PAX3 causes a similar effect (Sato et al., 2005). Consistent with these results, injection of a plasmid encoding DDX3 expanded the expression of *snai2* and *msx1* (Fig. 21). Because loss of either Pax3 or Msx1 can block subsequent NC specification (Monsoro-Burq et al., 2005), we conclude that DDX3 depletion inhibits NC induction by interfering with proper NPB formation, although we cannot rule out the possibility that DDX3 also regulates NC specification directly.

Importantly, co-injection of human DDX3X mRNA restored the expression of all markers to normal levels (Fig. 14B, 18C). These results further confirm the specificity of the MO, and indicate that DDX3 function in NC induction is conserved between frogs and humans.

DDX3 can regulate β -catenin levels through an RNA helicase-dependent mechanism

DDX3 is known to be a key regulator of canonical Wnt/ β -catenin signaling (Cruciat et al., 2013), and the differential responses of NPB specifiers to DDX3 KD (Fig. 14B, 18C) are consistent with previous reports showing that Wnt signaling induces the expression of *pax3* and *msx1* but not *zic1* (de Croze et al., 2011; Hong and Saint-Jeannet, 2007; Li et al., 2009). To investigate directly the roles of DDX3 in Wnt signaling during NC induction, we took advantage of a transgenic Wnt reporter line (Tran et al., 2010). Using

this reporter line, we showed recently that there is strong endogenous Wnt signaling at the NPB during NC induction (Li et al., 2018). As expected, this activity was inhibited by KD of DDX3 (Fig. 15A). The transcription factor GBX2 is a direct Wnt target that mediates β -catenin-induced pax3 and msx1 expression during *Xenopus* NC induction (Li et al., 2009). Similarly, when human ES and iPS cells are induced to differentiate into NC cells, gbx2 is expressed prior to the NPB specifiers such as pax3 and msx1 in a β -catenin-dependent manner (Leung et al., 2016). In embryos injected with the DDX3 MO (“morphants”), there was a reduction of gbx2 at stage ~12 that can be rescued by the mRNA encoding wild-type human DDX3X (Fig. 15B). Conversely, ectopic DDX3 expanded the expression of gbx2 (Fig. 21), further supporting an important role for DDX3 in Wnt signaling in NC induction.

Activation of the Wnt signaling pathway starts with binding of the soluble Wnt ligands to cell-surface Frizzled receptors and LRP5/6 co-receptors, which leads to inhibition of glycogen synthase kinase 3 beta ($GSK3\beta$ -mediated β -catenin phosphorylation and degradation. Consequently, β -catenin accumulates and enters the nucleus, where it interacts with LEF/TCF family transcription factors to control target gene expression (Loh et al., 2016; Nusse and Clevers, 2017). DDX3 has been shown to regulate Wnt signaling by stimulating the casein kinase CK1 ϵ to phosphorylate the adaptor protein Dishevelled, resulting in enhanced β -catenin nuclear import. This mechanism is independent of the RNA helicase activity of DDX3, as mutants with the ATP-binding domain and part of the helicase C-terminal domain deleted can still activate Wnt

signaling (Cruciat et al., 2013). However, the S382A/T384A (“AAA”) mutant of human DDX3X, which cannot unwind RNA but retains the ATPase activity (Chao et al., 2006), failed to rescue gbx2 expression in DDX3 morphants (Fig. 15B). We also detected decreased β -catenin levels in DDX3 morphants (Fig. 16A), which cannot be explained by the helicase-independent mechanism. Further, in TOP/FOPFLASH luciferase reporter assays, the DDX3 inhibitor RK-33 caused a strong inhibition of Wnt signaling in HEK293T cells, which was mimicked by a DDX3X siRNA (Fig. 16B, C). Importantly, both DDX3X siRNA and RK-33 decreased total β -catenin levels in HEK293T cells (Fig. 16D and data not shown), consistent with what we observed in vivo in DDX3 morphants (Fig. 16A) but not the published helicase-independent mechanism (Cruciat et al., 2013). Together these results strongly indicate that DDX3 regulates β -catenin levels and Wnt signaling in NC induction through its RNA helicase activity.

Depletion of DDX3 leads to reduced AKT activity and AKT-dependent GSK3 β phosphorylation

We next investigated the mechanisms through which DDX3 upregulates β -catenin levels and downstream signaling. The levels of β -catenin are mainly controlled by GSK3 β -mediated phosphorylation, and GSK3 β activity can be regulated by upstream Wnt ligand-receptor interaction or other signal inputs. Among the latter, the serine/threonine kinase AKT can inhibit GSK3 β activity via direct phosphorylation of the Ser9 residue (Cross et al., 1995; Fukumoto et al., 2001; Naito et al., 2005; Sharma et al.,

2002). Mutations that cause gain of AKT activity in humans and mice lead to overgrowth phenotypes in the CNS and NC (Akgumus et al., 2017; Butler et al., 2005; Rivière et al., 2012), which are opposite to those caused by loss of DDX3X function (Fig. 14). In addition, phosphatidylinositol 3-kinase (PI3K), a kinase that activates AKT, regulates NC induction in *Xenopus laevis*, a species closely related to *X. tropicalis*, although the exact roles of PI3K in NC induction remain controversial (Geary and LaBonne, 2018; Pegoraro et al., 2015). We therefore asked if DDX3 KD interferes with the AKT-GSK3 β signaling axis. AKT is activated through two consecutive phosphorylation events, the first occurring at Thr308 and the second at Ser473 (Manning and Toker, 2017). In HEK293T cells, siRNA-mediated KD of DDX3X inhibited both phosphorylation events without affecting total AKT levels (Fig. 16D). Similarly, MO-mediated DDX3 KD also blocked Ser473 phosphorylation (Fig. 16A); our pThr308 antibody did not pick up the endogenous pThr308 signal in *X. tropicalis* embryos.

Consistent with the reduced AKT activity, the phosphorylation of GSK3 β at Ser9, but not the total GSK3 β levels, was also reduced upon DDX3X KD or treatment with RK-33 in HEK293T cells (Fig. 16E, F). Because phosphorylation at Ser9 inhibits GSK3 β activity, these results provide an explanation for the decrease in β -catenin level when DDX3 activity was reduced (Fig. 16A, D). Besides β -catenin, the transcription factor Snai1 is also phosphorylated by GSK3 β and subsequently targeted to degradation (Yook et al., 2005; Zhou et al., 2004). Ectopically expressed Snai1 was clearly reduced when DDX3X was knocked down in *X. tropicalis* embryos or HEK293T cells (Fig. 16G,

H), indicating that DDX3X upregulates Snai1 protein levels post- transcriptionally. Together, our data suggest that the RNA helicase activity of DDX3 is required for AKT activation and downstream GSK3 β inhibition in *Xenopus* embryos and human cells.

AKT is a key regulator of Wnt/ β -catenin signaling and NC induction

The function of PI3K-AKT signaling in NC induction is controversial. While treatment of gastrula-stage *X. laevis* embryos with a PI3K inhibitor reduces the expression of NC markers, a constitutively active form of PI3K can also reduce NC markers induced by Pax3 and Zic1 in isolated *X. laevis* animal caps, which are thought to contain pluripotent stem cells (Buitrago-Delgado et al., 2015; Geary and LaBonne, 2018; Pegoraro et al., 2015). However, PI3K has AKT-independent functions (Faes and Dormond, 2015), and the mechanisms underlying the possible roles of AKT in NC induction remains largely unknown. To address these questions, we first determined the patterns of NPB and NC markers in embryos treated with AKT inhibitor IV (AKTi), which blocks AKT activity downstream of PI3K. Because inhibition of AKT activity at earlier stages caused gastrulation defects, we cultured the embryos in AKTi from stage ~10 to ~12.5. This transient inhibition of AKT activity reduced the expression of NC specifiers snai2 and sox9 as well as the NPB specifier msx1, but not the other NPB specifier zic1 (Fig. 17A). These effects are similar to those caused by DDX3 KD (Fig. 14B). As discussed above, msx1 but not zic1 is induced by Wnt signaling, and AKT can stabilize β -catenin by directly inhibiting GSK3 β activity. We therefore hypothesized that AKT is required for activating the Wnt signaling pathway, one of the most important signaling pathways in

NC induction. To test this hypothesis directly, we treated the Wnt reporter embryos with AKTi, and indeed observed a reduction in the endogenous Wnt activity at the NPB (Fig. 17B). A similar reduction was obtained with the injection of an mRNA encoding a dominant-negative AKT mutant (dnAKT; Fig. 17C), confirming that AKT is a key regulator of Wnt signaling during NC induction.

DDX3 induces the NC by regulating AKT and the GSK3 β substrates β -catenin and Snai1

To establish the relationship between DDX3 and its downstream targets in NC induction, we carried out a series of rescue experiments. AKT is recruited to the plasma membrane, where it is activated, through the interaction of its pleckstrin homology (PH) domain and the phosphorylated lipid products of PI3K (Manning and Toker, 2017). Replacement of the PH domain by a myristoylation signal results in a constitutively active form of AKT1 (caAKT) that functions independently of PI3K (Kohn et al., 1996). Co-injection of the caAKT mRNA rescued the reduction in the NC specifier *snai2* and Wnt signaling at the NPB caused by DDX3 MO (Fig. 18A, B), confirming the roles of AKT in mediating DDX3 function in Wnt signaling and NC induction. We have shown that KD of DDX3 reduced the inhibitory phosphorylation of GSK3 β at Ser9, and have identified β -catenin and Snai1 as two GSK3 β substrates whose levels are regulated by DDX3 (Fig. 17A, D-G). Because both of these GSK3 β substrates are essential for NC induction in *Xenopus* (Aybar et al., 2003; LaBonne and Bronner-Fraser, 1998), we tested if they are downstream effectors of DDX3 in NC induction. Ectopic expression of either β -catenin or Snai1 could partially rescue the NPB specifier *msx1* and the NC

specifier *sox9*; a combination of both GSK3 β substrates resulted in a nearly complete rescue (Fig. 18C). Based on these results, we conclude that DDX3 functions in NC induction by regulating AKT as well as β -catenin and Snai1.

RAC1 is an immediate downstream effector of DDX3 in NC induction

We next asked how DDX3 activates AKT. DDX3 can promote translational initiation of mRNAs with long and structured 5'-UTR through its RNA helicase activity, a function that is conserved from yeast to humans (Guenther et al., 2018; Sen et al., 2015). One of these mRNAs encodes RAC1, which can mediate DDX3 function in other physiological processes in mammals (Chen et al., 2015; Chen et al., 2016; Ku et al., 2018). Notably, RAC1 can activate AKT through its downstream effector p21-activated kinase (PAK) (Higuchi et al., 2008). In *Xenopus* embryos, a dominant-negative mutant of RAC1 reduces, whereas a constitutively active mutant expands, the expression of NC specifiers (Broders-Bondon et al., 2007). We therefore examined if RAC1 functions downstream of DDX3. To do this, we obtained an *X. tropicalis* *rac1* cDNA with a 257-nucleotide 5'-UTR. Although the 5'-UTR of *X. tropicalis* *rac1* did not show any significant sequence similarity to that of human RAC1, a prediction using the Mfold algorithm (<http://unafold.rna.albany.edu/?q=mfold/download-mfold>) suggests that it also contains strong secondary structures (Fig. 22). Thus, the mechanism of translational regulation of RAC1 may be conserved. As shown in Fig. 19A-C, blocking DDX3 activity by RK-33 in HEK293T cells, or KD of DDX3 in either *X. tropicalis* embryos or HEK293T cells, resulted in a decrease in endogenous RAC1 protein, indicating that RAC1 may be a target of DDX3 RNA helicase activity in both frogs and humans. Further, an *X. tropicalis*

rac1 mRNA without the 5'-UTR rescued the craniofacial defects caused by DDX3 KD (Fig.19D), suggesting that RAC1 mediates DDX3 function in NC development.

Discussion

First discovered in 2015, mutations in DDX3 have drawn a great deal of research interest as a frequent cause of intellectual disability in humans (Dikow et al., 2017; Kellaris et al., 2018; Lennox et al 2018.; Nicola et al., 2019; Snijders Blok et al., 2015; Wang et al., 2018). Although nearly all published studies are focused on the CNS defects in patients with DDX3 mutations, we noted that the majority of these patients also have abnormalities in craniofacial structures and/or other NC-derived tissues, which have not been addressed previously. We therefore hypothesize that DDX3 plays important roles in NC development, and provide here the first evidence supporting this hypothesis. Based on our data, we propose that the RNA helicase activity of DDX3 is required for efficient translation of RAC1, which activates AKT to phosphorylate and inhibit GSK3 β . The inhibition of GSK3 β leads to stabilization of β -catenin and Snai1, two proteins essential for NC induction (Fig. 18E).

While DDX3 has been shown to activate Wnt signaling through an RNA-helicase independent mechanism, several lines of evidence suggest the existence of an additional helicase-dependent mechanism. Nearly all missense DDX3X mutations associated with human birth defects affect residues within the two RNA helicase domains, and assays with zebrafish embryos indicate that most of these mutations

impair DDX3X's ability to activate Wnt signaling (Lennox et al.; Snijders Blok et al., 2015). Further, RK-33, a selective DDX3 RNA helicase inhibitor, reduces Wnt signaling in several cancer cell lines (Bol et al., 2015; Tantravedi et al., 2018). Similarly, RK-33 inhibits Wnt signaling in the non-tumorous HEK293T cells in our reporter assays, and the helicase-dead AAA mutant did not rescue the reduced expression of the Wnt target *gbx2* in DDX3 morphants (unpublished). In addition, we uncovered an effect of DDX3 KD on β -catenin levels, which is consistent with the downregulation of AKT activity (see below) instead of the helicase-independent mechanism through CK1 ϵ , as described previously (Cruciat et al., 2013). Thus, our data suggest that in addition to the helicase-independent mechanism, DDX3 can also regulate Wnt signaling through its RNA helicase activity.

The RNA helicase activity of DDX3 is needed for the efficient translation of certain mRNAs with highly structured 5'-UTR (Guenther et al., 2018; Sen et al., 2015). A known direct target of DDX3 in mammals is RAC1 (Chen et al., 2015; Chen et al., 2016; Ku et al., 2018), which is extremely conserved throughout vertebrate evolution (100% identical between human and *X. tropicalis* orthologs). Interestingly, mutations in RAC1 cause craniofacial disorders and other potential NC and CNS defects, which are highly similar to those caused by DDX3X mutations (Reijnders et al., 2017). In line with these observations, *rac1* is expressed in the NC and CNS in *Xenopus* embryos, and has been implicated in *Xenopus* NC induction (Broders-Bondon et al., 2007; Lucas et al., 2002). KD of DDX3 leads to reduced RAC1 protein levels in *X. tropicalis* embryos, and exogenous RAC1 rescues the craniofacial defects caused by DDX3 KD (Fig. 19C, D),

suggesting that RAC1 mediates DDX3 function in NC induction. RAC1 can activate AKT through its immediate downstream effector PAK (Higuchi et al., 2008). Among the vertebrate PAK family members, four (PAK1-4) contain a CDC42/RAC interaction/binding (CRIB) motif within the N-terminal regulatory domain, which binds and inhibits the C-terminal kinase domain when PAK is inactive. The direct interaction of the CRIB motif with activated RAC1 leads to dissociation of the regulatory domain from the kinase domain and activation of PAK (Zhao and Manser, 2012). The freed kinase domain of PAK1 can serve as a scaffold to bind AKT to facilitate its membrane localization and activation through a kinase-independent mechanism (Higuchi et al., 2008), but a separate study suggests that PAK1 activates AKT by phosphorylating AKT directly at Ser473 (Mao et al., 2008). Hence it would be of interest to test whether PAK also functions downstream of DDX3 in NC induction and, if so, whether this function is dependent on PAK kinase activity.

Previous studies suggest that PI3K, an upstream activator of AKT, can either promote or inhibit NC induction (Geary and LaBonne, 2018; Pegoraro et al., 2015). Here we show that direct inhibition of AKT activity reduces endogenous Wnt signaling at the NPB, leading to altered expression of NPB and NC markers that resembles loss of DDX3 or Wnt signaling (Fig. 16). These data indicate that one mechanism through which AKT functions in NC induction is by regulating Wnt signaling, a major signaling pathway that is critical for NC induction. It should be noted, though, that our results do not contradict with the model that excessive PI3K/AKT activities inhibit NC induction. In fact, we found that high levels of ectopic DDX3 can cause reduction in NPB and NC markers (data not

shown), suggesting that stringently controlled AKT activity is required for proper NC induction.

AKT-mediated phosphorylation of GSK3 β at Ser9 inhibits GSK3 β activity, leading to the stabilization of certain GSK3 β substrates (Cross et al., 1995; Zhou et al., 2004).

However, although some studies show that AKT promotes Wnt signaling, likely by inhibiting GSK3 β and stabilizing β -catenin (Fukumoto et al., 2001; Naito et al., 2005; Sharma et al., 2002), others suggest that AKT and Wnt act on two separate pools of GSK3 β and do not crosstalk with each other (Ding et al., 2000; Ng et al., 2009).

Besides inhibiting GSK3 β , AKT has also been shown to activate Wnt signaling through other mechanisms. For example, PI3K/AKT signaling can upregulate the transcriptionally active β -catenin (containing unphosphorylated Ser37 and Thr41) in HEK293T and other cell lines possibly by inducing the phosphatase PP2A to dephosphorylate these two residues (Persad et al., 2016). Indeed, we detected a reduction in active β -catenin that was more drastic than in total protein (Fig. 17D). Thus, AKT may mediate DDX3-induced Wnt signaling through GSK3 β -dependent and/or -independent mechanisms.

Unlike β -catenin, Snai1, another substrate of GSK3 β , is generally believed to be stabilized upon AKT-mediated phosphorylation of GSK3 β (Zhou et al., 2004). Here we show that KD of DDX3 results in decreased levels of ectopically expressed Snai1

(Unpublished data), and that ectopic Snai1 and β -catenin cooperatively rescue the NC induction defects caused by DDX3 KD in *X. tropicalis* embryos (Unpublished data). These data suggest that DDX3- regulated AKT-GSK3 β signaling axis induces NC through Snai1 in addition to β -catenin. Currently, most protocols for inducing the NC from human pluripotent stem cells include GSK3 β inhibition, which is thought to activate canonical Wnt signaling through β -catenin stabilization (Gomez et al., 2019; Leung et al., 2016; Menendez et al., 2011; Mica et al., 2013). In light of our results, it would be of interest to examine if the stabilization of Snai1 also contributes to the NC induction abilities of GSK3 β inhibitors.

Consistent with our model that DDX3, RAC1, AKT and β -catenin function in the same signaling cascade to regulate NC induction (Fig. 18E), mutations in RAC1, CTNNB1 (encoding β -catenin) and genes that directly control AKT activity (including AKT1, AKT3, PIK3R2, PIK3CA and PTEN) can all lead to craniofacial disorders and other potential neurocristopathies such as pigment defects, in humans (Akgumus et al., 2017; Butler et al., 2005; Kharbanda et al., 2017; Reijnders et al., 2017; Rivière et al., 2012; Tucci et al., 2014). Current efforts are therefore focused on investigating the effects of human mutations in DDX3X and these target genes on downstream signaling and NC induction. In addition, it is worth noting that patients carrying mutations in these genes also display highly similar CNS defects, including intellectual disability, autism spectrum disorder, micro/macrocephaly, and corpus callosum hypo/hyperplasia (Akgumus et al., 2017; Butler et al., 2005; Kharbanda et al., 2017; Reijnders et al., 2017; Rivière et al.,

2012; Snijders Blok et al., 2015; Tucci et al., 2014; Wang et al., 2018). Hence it is tempting to test if the signaling cascade that we propose here (Fig. 18E) is also essential for neurogenesis and CNS development. Our study may lay the foundation for future work to understand the pathophysiology of the birth defects caused by mutations that interfere with this signaling cascade.

Materials and methods

Plasmids and reagents

Constructs for dnAKT (clone 9031), caAKT (clone 10841), FLAG-tagged human β -catenin (clone 16828), and HA-tagged human Snai1 (clone 31697) were purchased from Addgene. Full-length cDNA clones for human DDX3X (Accession: BC011819), human RAC1 (BC050687), and *X. tropicalis* gbx2 (Accession: NM_001011472) were from GE-Dharmacon. Constructs were then subcloned into a pCS2+ vector with either a myc or HA tag. Mutations in DDX3 were generated by PCR. Other *X. tropicalis* expression constructs for x β -catenin and xsnail and for in situ hybridization snail2, sox9, foxd3, zic1, and msx1 were obtained previously (Wei et al. 2010b, Li et al. 2011, Wei et al. 2012). In vitro transcription was carried out as described previously to generate in situ hybridization probes and mRNA transcripts (Sive et al. 2000). Pharmacological inhibition of AKT and DDX3X was performed with AKT Inhibitor IV (Calbiochem 124011) and RK-33 (Selleckchem S8246), respectively. Antibodies that were used in this study include mouse anti-myc (DSHB 9E10 1:500), mouse anti-HA (Sigma-Aldrich H9658, 1:1000), anti-DDX3 (Abcam ab151965), anti-Rac1 (Invitrogen PA5-44839) for blotting,

anti-Rac1 for immunoprecipitation, anti-total- β -catenin (Sigma C2206), anti-active- β -catenin (CST 88145), anti-total-GSK3 β (Santa Cruz sc-53931), anti-piS9-GSK3 β (Invitrogen PA1-4688), anti-Akt1 (Aviva Systems Biology AVARP06008_P050), anti-human-piS473-Akt (CST 4060S), anti-Xenopus-piS473-Akt (CST 9271S), and anti-piT308-Akt. Secondary antibodies that were used include HRP-conjugated rabbit anti-mouse (Sigma-Aldrich A9044, 1:10,000), goat anti-rabbit (Sigma-Aldrich A0545, 1:10,000). Mouse anti- β -actin (Sigma-Aldrich A5316, 1:5000) was used for loading controls.

Animals and embryo manipulation

Wild-type *X. tropicalis* frogs were purchased from NASCO. The transgenic *X. tropicalis* Wnt reporter frogs were courtesy of Dr. Kris Vleminckx (Flanders Institute for Biotechnology, Belgium), and the transgenic *X. tropicalis* snail2:eGFP line was generated as described previously (Li et al.). Methods involving live animals were carried out in accordance with the guidelines and regulations approved and enforced by the Institutional Animal Care and Use Committees at West Virginia University and University of Delaware. Embryos were collected and injected with PLI-100A microinjectors (Harvard Apparatus) as described previously (Li et al., 2018). Morpholinos DDX3 (5'-CCGTGATTGATGCTT-3') and DDX3-2 (5'-TTTCCACGGCCACATGACTCATAAC-3') were designed and generated by Gene Tools. Alexa Fluor 555 (Invitrogen; for direct phenotype observation) or 488 (Invitrogen; for in situ hybridization) was co-injected as a lineage tracer. Embryos were cultured in 0.1x MBS to desired stages, and injected embryos were sorted by the co-injected lineage

tracer using a Zeiss Axiozoom v16 epifluorescence microscope. For western blotting of embryo lysates, embryos were lysed as described (Li et al., 2018).

Cell culture and transfection

HEK293T cells (ATCC) were cultured in DMEM (ATCC) supplemented with 10% fetal bovine serum (Gibco) at 37°C with 5% CO₂, and were authenticated and tested for contamination. The cells were transfected using Lipofectamine 3000 (Invitrogen) with DDX3 or scramble siRNA (GE-Dharmacon) at 50% confluency or with plasmids at 70% confluency. For experiments that used both siRNA and plasmid, siRNA was transfected first, and the media was changed and the plasmid was transfected on the following day. For pharmacological inhibition of AKT, cells were treated with AKT Inhibitor IV (Calbiochem 124011, 10 µM) or equal volume of DMSO (vehicle control) for 24 hours. Cell lysates were prepared as described previously (Li et al., 2018).

In situ hybridization

Embryos were collected at desired stages and fixed in 4% paraformaldehyde (PFA) for 24 hours at 4°C. In situ hybridization was then conducted as described (Sive et al. 2000). Briefly, embryos were bleached, and then fixed with 4% PFA. The embryos were then washed with 1X PTW and then digested in 1X TBS with Protease K for 3 minutes at room temperature. The reaction then was stopped and the embryos were washed with 1X PTW and fixed with 4% PFA for 20 minutes at room temperature. The embryos were then washed again with 1X PTW and then incubated in a 50/50 PTW/hybridization buffer solution for 30 minutes at room temperature. The embryos were then incubated at 65°C in hybridization buffer and then overnight with selected probes at 65°C. The

embryos were then washed with SSC, then 1X MAB, and then incubated with anti-DIG (Roche , 1:5000) overnight at 4°C. The embryos were then washed with 1X MAB and then stained with BM-Purple (Sigma-Aldrich 11442074001). The embryos were then slowly moved in methanol and stored overnight. The next day, the embryos were imaged in 1X PTW and then stored long-term in methanol.

Western blot and Immuno-Precipitation

Detection of Western blot was carried out using HRP-conjugated secondary antibodies and chemiluminescence substrates (GE Healthcare). Membranes were stripped and reblotted for β -actin, β 1-integrin or GAPDH as a loading control. For IP, cells were lysed with 20 mM Tris-HCl, pH 7.6, 167 mM NaCl, 1% NP-40, and protease inhibitor cocktail (Sigma-Aldrich) at 4°C for 30 min. The lysates were then centrifuged for 20 min at 12,000 g at 4°C. Supernatants were collected and incubated with 2-10 μ g of antibody with rotation at 4°C overnight. Protein G PLUS-Agarose beads (Santa Cruz) were added to the mixture and rotated for 1 hr at 4°C. Beads were then washed 5 times with ice-cold PBS. The bound proteins were dissociated by incubating with SDS-PAGE loading buffer at 50°C for 10 min and then 100°C for 10 min, and subsequently subjected to Western blot analysis.

Phenotype Scoring and Statistics

Embryos were scored by comparing the injected side with the uninjected side of the same embryos. The percentage of normal and reduced phenotypes were calculated for injected embryos obtained from multiple independent experiments, and Chi-squared

tests were performed to compare the phenotypes in different treatment groups. For craniofacial phenotypes, injected snail2-eGFP embryos were allowed to develop to stage ~46 and scored for defects in head cartilage structures. Images of head cartilages (eGFP) were taken with a Zeiss Axiozoom.v16 epifluorescence microscope.

Acknowledgements

We thank Dr. Kris Vleminckx for providing the transgenic Wnt reporter frogs. This work was supported by the National Institute of Health (GM114105 and GM104316 to SW).

Competing interests

The authors declare no competing interest.

Figures and Legends

Figure 14. KD of DDX3 inhibits NC induction and craniofacial cartilage formation. A. One anterodorsal (D1) blastomere of 8-cell stage *snai2::egfp* embryos was injected with the indicated MO (1.5 ng each). Embryos were cultured to stage ~46 and imaged for eGFP expression. B. Wild-type embryos were injected in one blastomere at 2-cell stage with the indicated MO (6 ng each) and human DDX3X mRNA (200 pg), cultured to stage ~12.5, and processed for in situ hybridization for the indicated markers. Representative embryos are shown in ventral (A) or dorsal (B) view with anterior at the top, and injected side is denoted with a red star (same below). Phenotypes in craniofacial structure or in situ staining on the injected side are classified as: normal, similar to the uninjected side; moderate, reduced but clearly present; severe, little or no structure/staining (same

below). CT, control; n: number of embryos scored. ***, $P < 0.001$.

Figure 15. KD of DDX3 inhibits Wnt signaling during NC induction. Wnt reporter (A) or wild-type (B) embryos were injected in one blastomere at 2-cell stage with the indicated MO (6 ng each) and mRNA encoding wild-type (WT) human DDX3X or the AAA mutant (200 pg), cultured to stage ~12.5 (A) or ~12 (B), and imaged for eGFP expression (A) or processed for in situ hybridization for *gbx2* (B). Representative embryos are shown in dorsal view with anterior at the top. **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

Figure 16. KD of DDX3 reduces β -catenin, AKT activity and AKT-mediated GSK3 β phosphorylation. A and H. Embryos were injected at one-cell stage with the indicated MO (12 ng each) and mRNA encoding Snai1 (50 pg), and cultured to stage ~12.5. B-G. HEK293T cells were treated with DMSO or 10 μ M RK-33 for 24 hr (B and F), or transfected with the indicated siRNA (200 nM) and a plasmid encoding HA-tagged Snai1 (50 μ g) for 48 hr (C-E). Cell or embryo lysates were processed for western blot analyses using the indicated antibodies (A, D-G), or TOP/FOPFLASH assays (B and C). *, P<0.05; ***, P<0.001.

Figure 17. AKT activity is required for Wnt signaling and NC induction. A and B. Wild-type(A) or Wnt reporter (B) embryos were treated with 20 μ M AKTi or DMSO from stage ~10 to ~12.5, and processed for in situ hybridization for the indicated markers (A) or imaged for eGFP expression (B). C. Wnt reporter embryos were injected in one blastomere at 2- cell stage with *dnAKT* mRNA (50 pg), cultured to stage ~12.5, and imaged for eGFP expression. Uninj, uninjected. Representative embryos are shown in dorsal view with anterior at the top. ***, P<0.001.

Figure 18. DDX3 induces the NC through AKT, β -catenin, and Snai1. Wild-type (A and C) or Wnt reporter (B) embryos were injected in one blastomere at 2-cell stage with the indicated MO (6 ng each), mRNA (50 pg for caAKT; 200 pg for hDDX3 and snai1), and plasmid (10 pg for β -catenin), cultured to stage ~12.5, and processed for in situ hybridization for the indicated markers (A and C) or imaged for eGFP expression (B). Representative embryos are shown in dorsal view with anterior at the top. *, $P<0.05$; ***, $P<0.001$.

Figure 19. RAC1 is an immediate downstream effector of DDX3 in NC induction. A and B. HEK293T cells were treated (A) or transfected (B) as in Fig. 17B-F. Cell lysates were processed for western blot with the indicated antibodies. C. Wild-type embryos were injected at one-cell stage with the indicated MO, and cultured to stage ~12.5. To obtain a clean signal from the embryo lysates, two RAC1 antibodies were used, one for immunoprecipitation (IP) and the other for western blot (WB) detection. WCL, whole-cell lysate. D. One anterodorsal (D1) blastomere of 8-cell stage *snai2::egfp* embryos was injected with the indicated MO (1.5 ng each) and mRNA (100 pg). Embryos were cultured to stage ~46 and imaged for eGFP expression. Representative embryos are

shown in ventral view with anterior at the top. **, $P < 0.01$. E. A model for the function of DDX3 RNA helicase in regulating downstream signaling during NC induction.

Figure 20. Developmental expression of *ddx3* during *X. tropicalis* embryogenesis. *In situ* hybridization was carried out for *ddx3* with *X. tropicalis* embryos at indicated stages. A-D. Dorsal view with animal pole (A) or anterior (B-D) at the top. A'. Vegetal view of the same embryo in A with dorsal at the top. B'. Transverse section of a stage ~12.5 embryo with dorsal at the top, showing high *ddx3* expression in the ectoderm. E. Side view with anterior to the right and dorsal at the top. Control *in situ* hybridization with sense probe is shown in insets, and arrows in A and A' indicate the dorsal blastopore lip.

Figure 21. Ectopically expressed human DDX3X causes expansion of NPB and NC specifiers. Wild-type *X. tropicalis* embryos were injected in one blastomere at 2-cell stage with 10 pg plasmid encoding *X. tropicalis* DDX3X, cultured to stage ~12.5, and processed for *in situ* hybridization for the indicated markers. Embryos are shown in dorsal view with anterior at the top.

A*X. tropicalis* *rac1* 5'-UTR **B**Human *RAC1* 5'-UTR

Figure 22. Predicted secondary structures of the *X. tropicalis* *rac1* and human *RAC1* 5'-UTRs. Prediction was carried out using the Mfold algorithm (<http://unafold.rna.albany.edu/?q=mfold/download-mfold>).

Chapter 4: Discussion and Future Directions

Chapter 4.1: Overall Thesis Discussion

In further studying ADAM13 and ADAM19 in the neural crest, we were able to elucidate a new mechanism whereby one ADAM binds to another ADAM to protect it from ubiquitination and degradation. This mechanism is necessary to stabilize ADAM13 at the time of neural crest induction that was previously found to be critical for neural crest specification in *Xenopus tropicalis*. Stabilization of ADAM13 by ADAM19 occurs by preventing ubiquitination of the cytoplasmic tail and is independent of the proteolytic activity of ADAM19. This is an important finding, because the ADAMs have been known to form heterodimers since their discovery (Blobel et al., 1992), but the biochemical and physiological consequences of this dimerization had not been elucidated. This finding provides a clear example of ADAMs regulating one another at the protein level and opens up a new area of research in the ADAM field.

To examine the tail of ADAM13 in *X. tropicalis* embryos, we removed it (ADAM13 Δ C) and mutated lysine at the 911th amino acid to an arginine to prevent ubiquitination (ADAM13K911R) and injected them into the *X. tropicalis* embryos. We found that it is ubiquitination of K911 that leads to the degradation of ADAM13, but also unexpectedly found that stabilized ADAM13 was sufficient to cause ectopic expression of *snai2* at the time of neural crest induction. This special relationship between ADAM13 and *snai2* continued when we found that the reduction of neural crest and canonical Wnt signaling in ADAM13 morphants could be rescued with *Snai2*. *Snai2* could also rescue loss of ADAM19.

In 2013, Christof Niehs' lab published a groundbreaking paper in *Science* showing that the RNA helicase DDX3 is necessary for neural plate development in a canonical Wnt signaling manner. After reading the article, we were inspired to test if DDX3 also had a role in neural crest development at this time. What we found was that DDX3 is in the neural crest at the time of induction and that it is necessary for the expression of neural plate border marker and neural crest markers at stage 12.5, but not earlier (unpublished data from our lab). This indicates that DDX3 is likely necessary for controlling a signaling cascade that regulates transcription of both the border and neural crest specifying genes. When examining the profile of genes regulated by DDX3, we observed that the genes known to be controlled by canonical Wnt signaling were down-regulated, notably *gbx2.2* and *snai2*, and those that were known to be independent of canonical Wnt signaling, notably *zic1*, were not affected. We then confirmed that DDX3 is necessary for canonical Wnt signaling by knocking it down in a *X. tropicalis* transgenic canonical Wnt reporter line.

In determining how DDX3 affects canonical Wnt signaling in the neural crest, we examined many components of the pathway by western blot. We found that total and active β -catenin was decreased and that GSK3 β was inhibited by phosphorylation when DDX3 was knocked down. Then in 2015, Wu et al. found that DDX3 affected Akt activity in colorectal cancer. Putting the pieces together, we found that DDX3 also affected Akt in the embryos at the time of neural induction. Then in a series of rescue experiments, we developed a new axis in neural crest induction whereby DDX3 activates Akt signaling through Rac1 and Akt signaling is necessary to induce canonical Wnt signaling. And previously, Sun et al. 2011 found that knockdown of DDX3 reduces the

neural crest transcription factor Snai1 expression at the protein level in breast cancer and DDX3 was positively associated with Snai1 in 31 glioblastoma multiforme patient samples (Sun et al 2011). After confirming that DDX3 is necessary to maintain Snai1 stability in HEK293T cells and embryos at the time of neural crest induction, we had a more complete story and a new mechanism that modulates canonical Wnt signaling: DDX3 allows the translation of Rac1 that activates Akt; the inhibitory phosphorylation of GSK3 β by Akt maintains the protein stability of Snai1 and β -catenin to induce canonical Wnt signaling and neural crest induction.

Chapter 4.2: Xenopus ADAM19 regulates Wnt signaling and neural crest specification by stabilizing ADAM13

Chapter 4.1.1: Discussion

In the ADAM field there are many examples of ADAMs that functionally operate using their non-proteolytic domains (Edwards et al. 2008, Reiss and Saftig 2009). There have also been multiple examples of ADAMs functioning in the neural crest, including several for ADAM13 (Gaultier et al. 2002, Wei et al. 2010a, Christian et al. 2013). In this paper however, we found for the first time that ADAM19 specifies the neural crest and activates canonical Wnt signaling via non-proteolytic protection of ADAM13. This finding opens up an exciting new mechanism to explore in future ADAM research.

Our data suggests that when ADAM19 is present it blocks the K911 residue of ADAM13. A well studied mechanism of activation for ADAMs is through auto-cleavage of the pro-domain, and binding of ADAM13 inhibits the auto-cleavage of ADAM19 (Li et al. 2018). This inhibition of the auto-cleavage seems to only go one way as ADAM13 is fully activated and seems to be able to cleave its known substrates (Wei et al. 2010, Li et al. 2018).

ADAM13 is necessary for multiple stages of neural crest development. During induction, ADAM13 is critical for inhibiting forward ephrin-B signaling, by cleaving the ligands ephrin-B1 and ephrin-B2 (Wei et al. 2010a). Active forward ephrin-B signaling antagonizes canonical Wnt signaling, likely through activation of PP2A and thus inhibiting Akt (unpublished data from our lab). Interestingly, the cytoplasmic tail of ADAM13 is dispensable for neural crest induction but is necessary to act as a transcription regulator during neural crest migration (Cousin et al. 2011, Li et al. 2018). During neural crest migration, the substrate profile of ADAM13 may be different as ADAM13 binds and cleaves fibronectin, pcdh8, pcdh81, and caherin11, and activates EGFR signaling (Alfandari et al. 2001, Gaultier et al. 2002, Khedgikar et al. 2017).

Canonical Wnt signaling has been known to be necessary for neural crest induction for over 20 years (LaBonne and Bronner-Fraser 1998), and has been assumed to be necessary for every module of neural crest development (Lewis et al. 2004). However, in this paper, we show for the first time in vivo evidence that canonical Wnt signaling is necessary for endogenous neural crest specification and not just neural border

specification. This detail was previously hard to tease apart as inhibiting the development of the neural crest early on will also inhibit later neural crest development. Here we found that the neural border could be specified but the neural crest was not specified in a canonical Wnt signaling manner, when ADAM13 or 19 was knocked down. This brings exciting new insight into how the neural crest develops and also brings to light that different regulators are necessary to activate canonical Wnt signaling at different times in development.

Due to ADAM13 affecting both neural crest induction and migration, a limitation of our study was the significance of ADAM13 and ADAM19 in neural crest induction. It is hard to say whether inhibition of neural crest induction by ADAM13 or ADAM19 would cause the same penetrance of later craniofacial abnormalities. I will address this in the future directions section below. Another limitation of our study was the perplexing nature of ADAM13 affecting canonical Wnt signaling at stage 12.5, but not affecting all known transcriptional targets of canonical Wnt signaling. Loss of ADAM13 seems to anteriorize all of the neural ectoderm at 12.5 in a canonical Wnt manner (Wei et al. 2010, Wei et al. 2012a, Wei et al. 2012b, our lab's unpublished data), but neural plate border markers and known canonical Wnt targets *gbx2.2*, *pax3*, and *msx1* were unaffected. And interestingly, loss of ADAM13 can be rescued with canonical Wnt signaling activator *EphB1ΔC* or *Snai2* (Wei et al. 2010, our lab's unpublished data). This may indicate that the neural plate border markers have an alternative mechanism of activation that is possible when ADAM13 is lost.

Chapter 4.1.2: Future Directions

Since this publication, our lab has made many exciting discoveries continuing the research that we published here. One of our first goals in continuing this project was to find a mammalian ortholog of *Xenopus* ADAM13. While ADAM13 is most similar to mammalian ADAM33 in sequence, we have discovered that ADAM9 may be the functional ortholog to ADAM13. Indeed, we have found that overexpression of mouse ADAM9 can rescue loss of ADAM13 in *X. tropicalis* embryos (our lab's unpublished data). We also found that ADAM9 can cleave ephrin-B1 and ephrin-B2 in a similar manner to ADAM13 (our lab's unpublished data). In furthering the mechanism of how ADAM13 affects canonical Wnt signaling, we have found that dominant-negative EphB1 Δ C inhibits canonical Wnt signaling and activates GSK3 β . We have also found that ADAM9 and loss of forward ephrin-B signaling activate Akt and canonical Wnt signaling in a PP2A manner.

Continuing what we have found, I would want to knockdown ADAM9 and observe forward ephrin-B activation by looking at phosphorylation of the receptors by western blot. I would also want to conduct a series of rescue experiments, particularly to test if constitutively active Akt can rescue canonical Wnt signaling when ADAM9 is reduced. This would shed new light on how ephrins affect canonical Wnt signaling and would carry the research that we have found in *Xenopus* to mammals. To further this connection between ADAM13 and ADAM9, I would also like to generate an inducible dominant-negative ADAM9 in mice. I would then inhibit ADAM9 at the time of neural

crest induction and observe later neural crest defects in the mice. Knockout of ADAM9 does not seem to have an affect on the neural crest in mice, but there may be multiple ADAMs that function similarly to ADAM9. An inducible dominant-negative ADAM9 would effectively inhibit many proteases that would cleave ADAM9 substrates. I would like to do a screen for new substrates that are cleaved by ADAM9 and ADAM13. I would do this by conducting MS/MS on the purified condition media of ADAM9 or ADAM13 knockdown cells. This would give a better indication of how much functional overlap the two proteins have, would allow for future research of new substrates, and would enhance our knowledge of the therapeutic value inhibiting ADAM9 would bring to specific patients.

To better understand the relevance of reducing ADAM13 at the time of neural crest induction, I would like to generate an ADAM13 light-caged morpholino. These morpholinos are inhibited until converted by UV light. I would then inhibit ADAM13 at the time of neural crest migration (and when the embryos are better penetrated by UV light) and observe later craniofacial structures. I would then compare the craniofacial abnormality severity and penetrance of embryos that had lost ADAM13 from neural crest induction and migration. A reduction in severity or penetrance would give evidence that ADAM13 in the neural crest at the time of induction is relevant to neural crest development.

Chapter 4.2: DDX3 induces neural crest through activation of an Akt-Wnt signaling axis

Chapter 4.2.1: Discussion

DDX3 has recently been at the forefront of research. With the discovery that mutations of DDX3X are linked with developmental abnormalities in humans, there has been a great interest in this RNA helicase. We had already begun our research into DDX3 in the neural crest, but were intrigued by the findings that inhibiting mutations in DDX3 led to phenotypes associated with aberrant neural crest development. While the published mechanism of how DDX3 affects canonical Wnt signaling notes that the helicase domain of DDX3 is dispensable for activation, most of the inhibiting mutations of DDX3X that were linked to phenotypes associated with abnormal neural crest development are in the helicase core (Lennox et al. 2015, Snijders Block et al. 2015). Indeed, the small molecule selective inhibitor of DDX3, RK33, which prevents its helicase activity, also inhibits canonical Wnt signaling (Bol et al. 2015, Perfetto et al. unpublished). This may indicate that DDX3 operates on canonical Wnt signaling through more than one mechanism.

Interestingly, we found that DDX3 is critical for Akt activity by maintaining Rac1 protein levels, and mutations in human RAC1 also cause phenotypes associated with abnormal neural crest (Reijners et al. 2017). Due to the highly conserved nature of DDX3, Akt, and Rac1 between *Xenopus* and humans, the mechanism we developed in the this paper may have direct clinical relevance.

A recent paper published by Geary and LaBonne 2018 has given evidence that high PI3K and Akt signaling is necessary for neural development but low to moderate PI3K and Akt signaling are necessary for neural crest induction. In our own research, we found that a moderate amount of Akt signaling is needed to induce the neural crest, too much or too little Akt signaling leads to neural crest loss (our lab's unpublished data). Indeed, too much expression of DDX3 can cause a reduction of neural crest.

For the first time we have shown that Akt signaling is necessary to induce the neural crest *in vivo*. We also showed for the first time that Akt is necessary to activate the neural crest at the time of induction and that DDX3 is critical to both of these signaling cascades. Intriguingly, while *rac1* is found in the neural crest, it has not been studied in neural crest induction, and we for the first time have given evidence of Rac1 in the process (Lucas et al. 2002, Broders-Bondon et al. 2007, Perfetto et al. unpublished). Data from our and other labs have opened a new area of research in the neural crest field exploring how Akt operates in neural crest induction.

Due to the ubiquitous nature of DDX3 and DDX3 controlling vital cellular functions like RNA transport and translation, confounding affects in our experiments were a great concern. Aligned with that thinking, it was hard to get DDX3 morphants to survive to craniofacial stages in *Xenopus*. Also, we often observed a phenotype on the uninjected side of the embryo. Rescue experiments were similarly difficult, as too much or too little rescue mRNA resulted in no rescue or a more severe phenotype.

Chapter 4.2.2: Future Directions

The next practical actions would be to explore Rac1 in *Xenopus* neural crest development and to test DDX3 in mouse neural crest development. As stated above, Rac1 has not been explored in neural crest development previously, but mutations in the gene lead to phenotypes typically caused by abnormal neural crest development. To confirm a role of Rac1 in neural crest induction I would want to inject Rac1 morpholino and observe the neural crest induction, craniofacial irregularities, and in vivo canonical Wnt signaling. I would also like to develop Wnt1-cre helicase-dead DDX3 mice (that specifically target the neural crest) and observe craniofacial structures. Again, due to the many housekeeping functions of DDX3, whole embryo knockout may not be the best first experiment to conduct.

Given the recent publication on DDX5 and DDX17 affecting Wnt signaling and DDX5 binding to DDX3, exploring DDX5 and DDX17 in neural crest induction would be fascinating. DDX5 is expressed in the neural crest and DDX17 expression is highly upregulated during neural crest induction. I would like to knockdown DDX5 and DDX17 individually and observe neural crest induction, craniofacial structures and in vivo canonical Wnt signaling at the time of neural crest induction. Due to these helicases possibly being functionally redundant, I would also want to knockdown these proteins together and look for more severe phenotypes.

During our experiments, we noticed that overexpression of *snai1* and β -catenin together induced ectopic expression of the neural border specifier *msx1*. This was not explored

further, but I would like to examine the other neural plate border and neural crest specifying genes to get a more accurate profile. This experiment may indicate that Snai1 and β -catenin are sufficient to reprogram and induce the neural crest. If true, exploring this would open up a new area of research for the neural crest and lead to a much greater understanding of how to induce neural crest cells for therapeutic purposes.

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