EXPLORE THE REGULATORY NETWORK OF AKT SIGNALING IN NEURAL CREST DEVELOPMENT AND CANCER BIOLOGY

by

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A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

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ABSTRACT

DDX3X syndrome is a genetic disease caused by mutations in RNA helicase DDX3X with developmental delay and intellectual disability (ID). As we noticed that many patients also exhibit neural crest (NC) related defects, including craniofacial disorders and congenital heart disease, some of the prevalent birth defects, we hypothesized that DDX3 plays a role in NC development. Using the amphibian model, *Xenopus tropicalis*, we show that DDX3 induces NC formation through AKT activity in a helicase-dependent manner. DDX3 KD reduces AKT activity, leading to the destabilization of β-catenin, a downstream target of AKT and canonical Wnt signaling. The regulation of DDX3 on AKT activity in NC induction is likely mediated by RAC1, whose translation is facilitated by the RNA helicase activity of DDX3. Similar to *DDX3X*, mutations in *RAC1* and *AKT* were also shown to cause ID and NC-related defects in humans, suggesting their function in NC development. We show that depletion of RAC1 or AKT2 impairs expression of NC marker *snai2*, and disrupts normal craniofacial morphogenesis. These results indicate that the function of DDX3 in modulating NC development through AKT activity is conserved between frogs and humans, advancing our understanding of the pathology of the NC-related birth defects caused by mutations in *DDX3X*, *RAC1* and *AKT.*

As an X chromosome-linked genetic disease, DDX3X syndrome affects considerably more female patients but the underpinning mechanisms of this

sexual dimorphism remain largely unclear. It has been speculated that males with inactivating mutations in *DDX3X* suffer from embryonic lethality. However, recent studies suggested that DDX3X depletion might trigger compensatory expression of *DDX3Y*, a close homolog on the Y chromosome. We therefore hypothesized that DDX3X inhibits DDX3Y expression, and show that loss of DDX3X increases the DDX3Y expression through divergent mechanisms in two different human cell lines. In the glioma cell line U87-MG, a novel protein level regulation of DDX3Y by DDX3X was revealed, while in the colon cancer cell line HCT116, the regulation is more predominantly on the mRNA level of DDX3Y. These results provide a potential mechanistic explanation for the compensatory expression of DDX3Y induced by loss of DDX3X.

The disintegrin metalloproteinases (ADAMs) are key regulators of cell signaling by cleaving cell-surface signaling molecules. ADAM9 has been implicated in several human diseases. In particular, ADAM9 is highly expressed in nearly all types of solid tumors, and the expression often correlates with poor prognosis. Understanding the downstream signaling pathways modulated by ADAM9 will provide important insight into its functions in carcinogenesis and tumor progression. Here we apply a systematic and non-biased approach, integrating the transcriptomics and the cellular proteomics analysis, to identify the downstream targets of ADAM9. As we reported previously that AKT is a downstream target of ADAM9, we show that with ADAM9 depletion, the FoxO signaling, one of the major pathways downstream of AKT, is found to be enriched

in the RNA-seq analysis, while the transcription factor FoxO3 is upregulated in protein level. These analyses suggest that FoxO signaling is activated with ADAM9 KD, which is validated by western blotting and RT-qPCR. These results indicate that ADAM9 represses the activation of FoxO signaling, a tumor suppressor pathway, likely through activating AKT, providing a potential mechanism for the oncogenic roles of ADAM9 in various cancer types.

Chapter 1

INTRODUCTION

1.1 The neural crest (NC)

The NC is a group of multipotent cells that is unique to vertebrates, and it arises in the neural plate border (NPB) region between the neural ectoderm and non-neural ectoderm in early embryonic development. During the induction of NC at the gastrula and neurula stages, the subset of NPB progenitors is destined to form the NC. After neural tube closure, the NC cells undergo epithelial-mesenchymal transition and delamination to acquire migratory ability. The migratory NC subsequently migrates away from the NPB region to diverse locations, where they differentiate into a large range of cell lineages including craniofacial cartilage and bone, peripheral neurons and glia, smooth muscle, and melanocytes (Gilbert, 2000; Bronner & LeDouarin, 2012; Mayor & Theveneau, 2013). The appearance of the NC was key to the evolution of vertebrates as many of the NC derived structures are defining features of the vertebrate clade (Gans & Northcutt, 1983; Meulemans & Bronner-Fraser, 2004; Martik & Bronner, 2021).

The derivatives of NC cells are classified into four subpopulations along the anterior-posterior axis, including the cranial, vagal, trunk, and sacral crest

(Gilbert, 2000; Mayor & Theveneau, 2013; Martik & Bronner, 2017). The cranial crest gives rise to most of the cranial cartilage and bone, cranial nerves and ganglia. The vagal and sacral crest both contribute to the ganglia in the enteric nervous system. A subset of the anterior vagal crest comprises the cardiac crest, which gives rise to structures within the heart including part of the outflow tract, the valves, and the arteries (George et al., 2020). The trunk crest gives rise to the dorsal root ganglia of the sympathetic nervous system. Besides, all four populations comprise a subset of cells differentiated into melanocytes that give us pigmentations on the skin. Due to its developmental plasticity, biologists nicknamed it the “fourth germ layer” and made it one of the most studied progenitor populations (Gilbert, 2000; Bronner & LeDouarin, 2012).

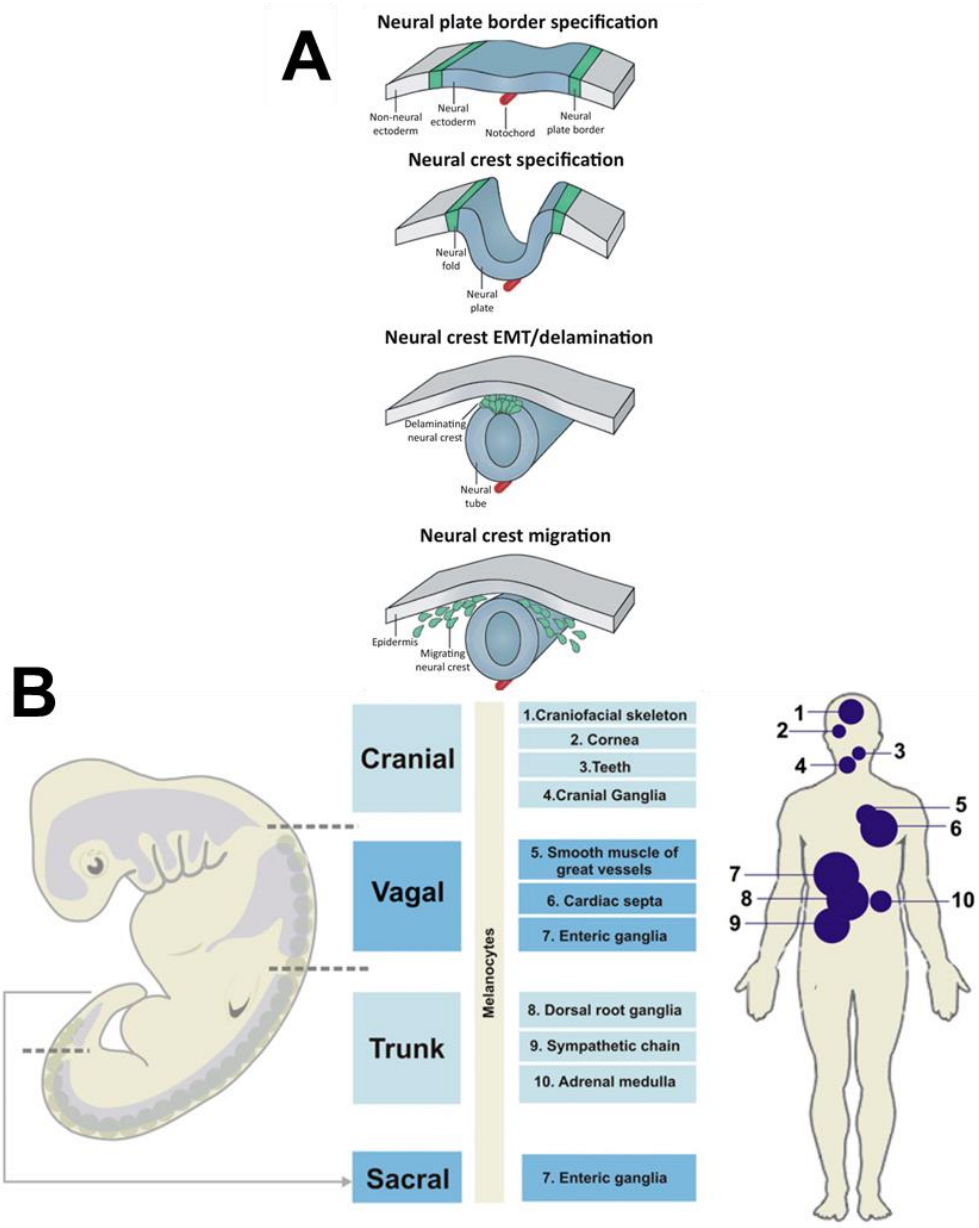

Figure 1. Neural crest formation and derivatives.

(A) The NC forms by a series of important regulatory events: induction at the neural plate border, specification, delamination from the neural tube, migration throughout the embryo, and differentiation into many derivatives. Adapted from (Martik & Bronner, 2017). (B) Contributions of different NC cell (NCC) populations to adult tissues and organs. Adapted from (Simões-Costa & Bronner, 2013)

Apart from being a great subject for developmental biologists, studying the NC also has clinical significance as abnormal induction, migration or differentiation of the NC cells leads to a broad spectrum of birth defects collectively called neurocristopathies (NCPs), which affect an appreciable percentage of newborns (Vega-Lopez et al., 2018; Watt & Trainor, 2014). Craniofacial defect is one of the symptoms associated with cranial crest malformation. For example, Treacher Collins Syndrome (TCS) is a genetic disorder with patients exhibiting underdevelopment of the zygomatic complex, jaws, and cleft palate (Watt & Trainor, 2014; T. S. Sato et al., 2019; Sanchez et al., 2020). Another example is CHARGE syndrome (coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, and ear abnormalities) with kids having asymmetric facial palsy caused by cranial nerve abnormalities, while most of them also exhibit congenital heart defects linked to disrupted cardiac crest development (Watt & Trainor, 2014; Okuno et al., 2017). In addition, two of the metastatic cancer types, melanoma and neuroblastoma, are also examples of NCPs attributable to reestablishing the proliferative and migratory ability of NC cells in the melanocyte and sympathoadrenal lineage, respectively (Maguire et al., 2015). Broadening our knowledge of NC development and its molecular mechanism will help us understand the pathogenesis of NCPs and aid in development of better treatment strategies (Pilon, 2021).

1.2 Xenopus tropicalis

Xenopus tropicalis, also known as the western clawed frogs, is one of the amphibian model organisms that has been widely used by developmental biologists to study vertebrate embryo development (Wallingford et al., 2010). It is a fully aquatic species with easy and low-cost animal husbandry. Their eggs are large (~0.8 mm in diameter), produced in huge numbers synchronically, and get fertilized and then develop externally, which makes their embryos easy to manipulate at very early stage of embryonic development and suitable for high-throughput biomedical studies (Fisher et al., 2023). Ovulation can be induced in an artificially controlled manner through injecting human chorionic gonadotropin (hCG) into the female frogs (del Viso & Khokha, 2012; Lane et al., 2022). Back in the 1930s, *Xenopus* was used in the hospitals for urine-based pregnancy tests as hCG is produced by the placenta and eventually gets passed into the urine (Elkan, 1938). Hormone injected male frog, if placed together with the injected female frog, will be induced for natural mating ~5 hours after injection (Lane & Khokha, 2022). After fertilization, the embryos develop rapidly, having neural crest induced in ~9 hours, growing to tadpoles which have full sets of functional organs and tissues including the head cartilage with NC contribution in 4-5 days, and growing to adult frogs within 6 months. After obtaining embryos, genetic manipulations are easy to achieve through injecting a range of materials, including morpholino, plasmid DNA, messenger RNA, small guide RNA and Cas proteins, into the whole embryo or a specific blastomere (Nakayama et al., 2013;

Guille & Grainger, 2022). Later stage embryos or tadpoles are then examined to see if the manipulation has had effects on the development of a certain group of cells or tissue, e.g., the neural crest population in our lab.

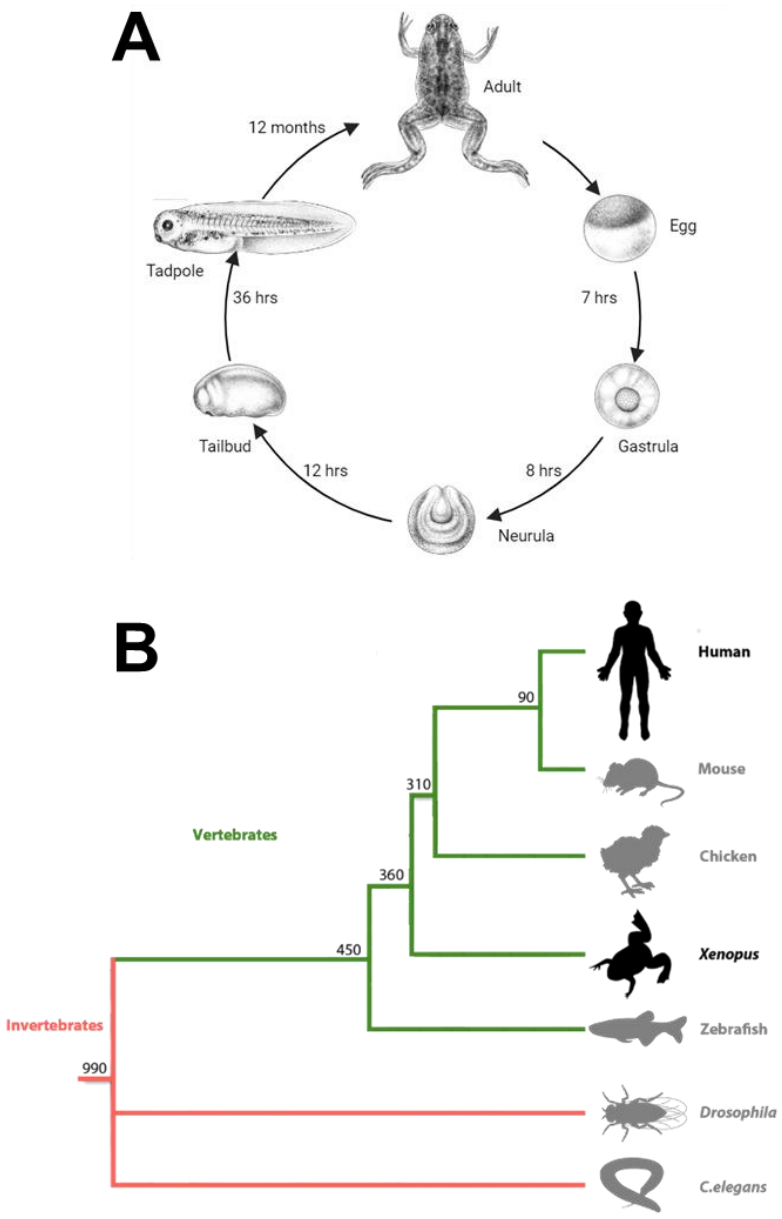

Figure 2. *Xenopus* life cycle and evolutionary relationships.

(A) The life cycle of Xenopus. Embryo sketches by (Zahn et al., 2022).(B) Phylogenetic tree showing the main animal models commonly used in biomedical research and their evolutionary relationships. The divergence time, in millions of years (Mya), is based on multiple gene divergence and protein divergence studies. Adapted from (Wheeler & Brändli, 2009).

Non-mammalian vertebrates including *Xenopus* (frog), *Danio rerio* (zebrafish), and *Gallus gallus* (chicken), were used initially to study the GRNs regulating NC development via loss-of-function (LOF) experiments (Simões-Costa & Bronner, 2015). They are more simplified models for NC studies comparing to mammalian models, as inhibition of NC regulators identified with non-mammalian models in mice oftentimes didn't cause NC related defects, especially the early-stage induction phenotypes (Barriga et al., 2015), with mechanism unknown but we speculated that might be due to higher levels of functional redundancy. Compared to other aquatic models, *Xenopus* has a closer evolutionary relationship with humans and has been estimated to share 79% of the human disease associated genes identified (Nenni et al., 2019). Besides, our genes of interest also share high protein sequence identity between frogs and humans. Compared to the other *Xenopus* species that are also commonly used in biology labs, *Xenopus laevis*, which is allo-tetraploid, the diploid model *Xenopus tropicalis* is easier to manipulate genetically (Hellsten et al., 2010).

1.3 Wnt/β-catenin signaling

Underlying the development of the NC is the gene regulatory networks (GRNs) that comprise sequential, separate, and spatiotemporal regulatory modules, each including a set of signaling molecules and transcription factors to trigger downstream effectors that confer cells multipotency or migratory ability. Three of the major signaling pathways, including Bone Morphogenetic Protein (BMP), Fibroblast growth factor (FGF) and Wnt signaling, work jointly to regulate NC

development (Meulemans & Bronner-Fraser, 2004; Simões-Costa & Bronner, 2015; Martik & Bronner, 2017).

Our lab focuses on Wnt signaling and more specifically canonical Wnt signaling, also called the Wnt/β-catenin pathway which has been extensively studied for its function in early embryonic development and tumor progression (Wodarz & Nusse, 1998; Clevers, 2006; Steinhart & Angers, 2018). Wnt ligands are secreted protein growth factors that transduce signals into nearby cells or sometimes the same cell through cell surface receptors. With the absence of Wnt ligand, cytoplasmic β-catenin gets phosphorylated via interacting with the destruction complex consisting of Axin, adenomatosis polyposis coli (APC), glycogen synthase kinase 3 (GSK3), and casein kinase 1 (CK1). Subsequently, the phosphorylated β-catenin is ubiquitinated and targeted for proteasomal degradation. While upon activation, Wnt ligand binds to the transmembrane receptor Frizzled and its co-receptors, low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) on the cell surface. Through interaction with the activated receptors, scaffolding protein Dishevelled (Dvl) recruits the destruction complex to the cell membrane, resulting in accumulation of non-phosphorylated active β-catenin in the cytosol. Then β-catenin is translocated into the nucleus where it functions as the coactivator of T cell factors and lymphoid enhancer-binding factor (TCF/LEF) transcription factors, promoting expression of Wnt target genes (Wodarz & Nusse, 1998; Albrecht et al., 2021; Rim et al., 2022).

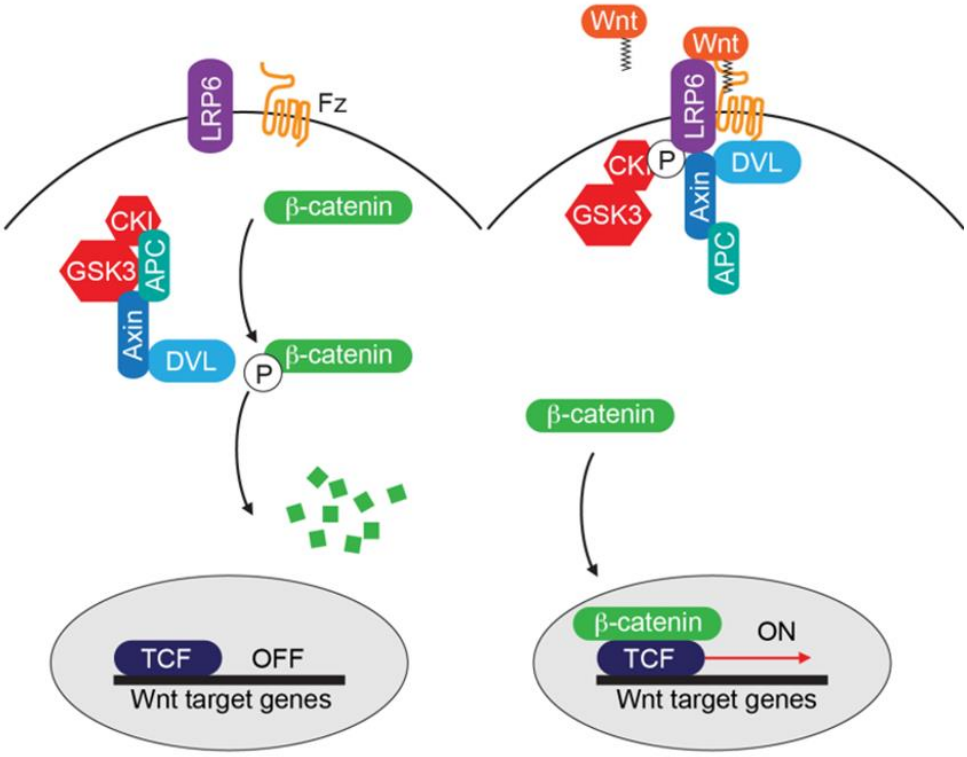

Figure 3. The canonical Wnt signaling pathway.
The binding of Wnt ligands to the receptors Frizzled (Fz) and LRP6 protect β-catenin from degradation. β-catenin in turn interacts with members of the TCF/LEF-1 family of transcription factors to co-activate target gene transcription. Adapted from (Sawa & Korswagen, 2013)

Many studies conducted in various vertebrate model organisms have demonstrated the function of Wnt/β-catenin signaling in inducing NC (Elkouby & Frank, 2010). In the *Xenopus* embryos, overexpression of the canonical Wnt ligand Wnt1 or Wnt3a, or components of Wnt signaling such as Frizzled, LRP6 or β-catenin enhances NC formation, while antagonizing Wnt/β-catenin pathway

through overexpressing dominant-negative LRP6 or TCF inhibits NC formation (Saint-Jeannet et al., 1997; LaBonne & Bronner-Fraser, 1998; Tamai et al., 2000; Deardorff et al., 2001). In humans, canonical Wnt signaling is also required for the induction of NC from embryonic stem cells or induced pluripotent stem cells, indicating that its function in NC induction is conserved during vertebrate evolution (Menendez et al., 2011; Mica et al., 2013; Leung et al., 2016; Gomez et al., 2019).

1.4 Transgenic frogs facilitating NC studies

A bunch of transcription factors are activated by multiple signaling pathways upon NC formation, including paired box gene 3 (Pax3), Zinc finger of the cerebellum 1 (Zic1) and homeobox protein Msx1, which subsequently induce the expression of Zinc-finger protein Snai2, Forkhead box D3 (FoxD3) and SRY-box 9 (Sox9) to specify NC cell fate (Stuhlmiller & García-Castro, 2012; Simões-Costa & Bronner, 2015; Bronner & Simões-Costa, 2016). Snai2, previously often called Slug, is a transcription factor that is expressed specifically in the early NC precursors and is required for NC induction in *Xenopus* (Shi et al., 2011). The canonical Wnt signaling promotes *snai2* expression during NC induction, as artificial activation of Wnt signaling causes ectopic expression of *snai2*, while blocking Wnt signaling inhibits *snai2* expression and prevents NC induction (J. Wu et al., 2003). The direct regulation of *snai2* expression by canonical Wnt signaling is further confirmed by the fact that the *snai2* gene enhancer contains an evolutionarily conserved LEF/TCF binding site that is responsive to canonical

Wnt signaling (Vallin et al., 2001). After NC induction, *snai2* continues to be expressed in the pre-migratory and migrating NC as it facilitates EMT and migration of the crest cells via inhibiting E-cadherin expression through transcriptional repression (J. Li et al., 2019). Subsequently, differentiating crest cells have *snai2* re-expressed, which was demonstrated by our lab previously, with the functions largely understudied.

To facilitate NC studies in *Xenopus*, our lab generated a transgenic frog line using this most used NC marker gene. Applying the I-SceI meganuclease-mediated transgenic approach, we made this transgenic line that expresses eGFP driven by the *cis*-regulatory elements of *X. tropicalis snai2* gene, labeling the spatiotemporal expression of *snai2*. In the *snai2:eGFP* transgenic embryos, specific fluorescence patterns were observed in the pre-migratory NC populations and subsequently the migrating crest streams. Later in the tadpoles, eGFP labels multiple structures with NC contributions including the head cartilage and several cranial nerves (J. Li et al., 2019). This transgenic line is a very useful tool to identify NC regulators as dysregulated induction, migration or differentiation caused by LOF experiments can be visualized directly in the live embryos.

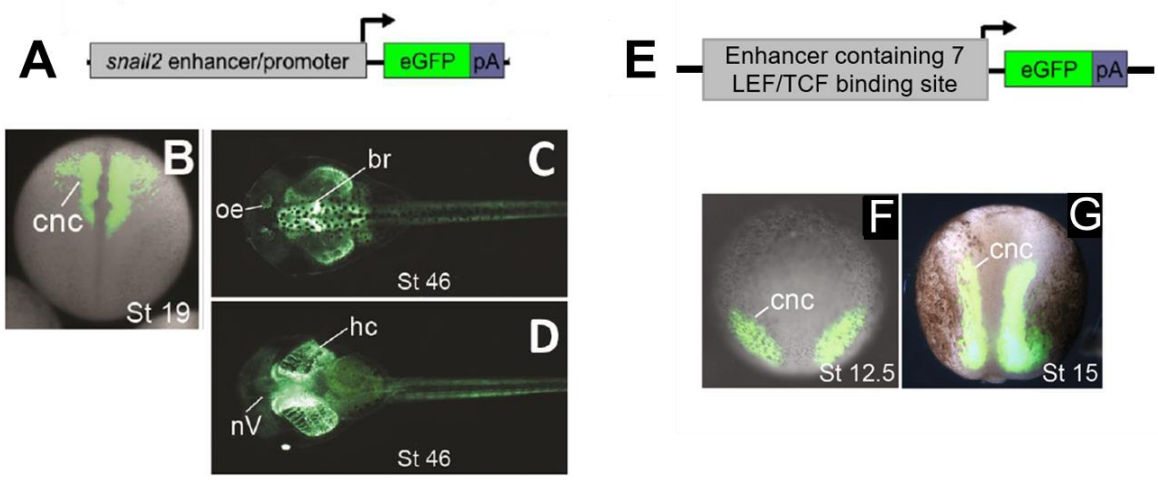

Figure 4.Transgenic *X. tropicalis* lines: *snai2:eGFP* and Wnt reporter line. (A) Schematic representation of eGFP expression driven by enhancer containing LEF/TCF binding site. Expression of eGFP is detectable in pre-migratory CNC (B) and structures with NC contributions (C&D). (E) Schematic representation of eGFP expression driven by enhancer containing LEF/TCF binding site. Expression of eGFP is detectable in NPB region upon NC induction (F) and in the pre-migratory CNC (G). Adapted from (J. Li et al., 2019)

Our lab has also acquired transgenic Wnt reporter *X. tropicalis* from Dr. Kris Vleminckx (Flanders Institute for Biotechnology, Belgium). The reporter construct consists of a synthetic promoter containing eight copies of the binding sequence for TCF/LEF factors upstream of the reporter gene EGFP to reveal the spatiotemporal activation of Wnt/β-catenin pathway (Tran et al., 2010). Using this transgenic line, we have shown that Wnt activity is high at NPB region during NC induction and persists during NC migration and differentiation, consistent with previously reported functions of Wnt signaling in those steps. With this handy

tool, I was able to demonstrate if my genes of interest regulate NC development through the Wnt/β-catenin pathway.

1.5 AKT signaling pathway

The AKT signaling pathway, or phosphoinositide 3-kinase (PI3K)/AKT signaling is a signal transduction pathway that promotes cell survival, growth, and metabolism in response to extracellular stimulus. As the most commonly activated pathway in cancers, this signaling pathway has been intensively studied in the past 30 years since it was discovered (Manning & Toker, 2017; Hoxhaj & Manning, 2020). Initial stimulation of receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs) by extracellular growth factors or cytokines, recruits PI3K to the plasma membrane and leads to its activation. Subsequently, activated PI3K phosphorylates lipids on the membrane, predominantly phosphatidylinositol (4,5)-bisphosphate (PIP2), to produce phosphatidylinositol (3,4,5)-trisphosphate (PIP3). The Ser and Thr kinase AKT, also known as protein kinase B, in cytosolic inactive form, is translocated to the membrane via direct binding with PIP3, which causes AKT conformational changes leading to the exposure of its phosphorylation sites. Phosphorylation of AKT involves mainly two steps, a priming phosphorylation of Thr308 by pyruvate dehydrogenase kinase 1 (PDK1), followed by phosphorylation of Ser473 by mammalian target of rapamycin (mTOR) Complex 2 (mTORC2), to be fully activated. AKT activity could be attenuated through dephosphorylation of PIP3 by Phosphatase and Tensin homolog (PTEN) or the dephosphorylation of itself by

Protein Phosphatase 2A (PP2A) and PH domain and Leucine rich repeat Protein Phosphatases (PHLPP). Activated AKT phosphorylates a range of downstream substrates, three of which are multifunctional and well-established including glycogen synthase kinase 3 (GSK3), Forkhead box protein O (FoxO) and mTOR Complex 1 (mTORC1), to mediate downstream functions (Fig. 5) (Manning & Toker, 2017; Hoxhaj & Manning, 2020).

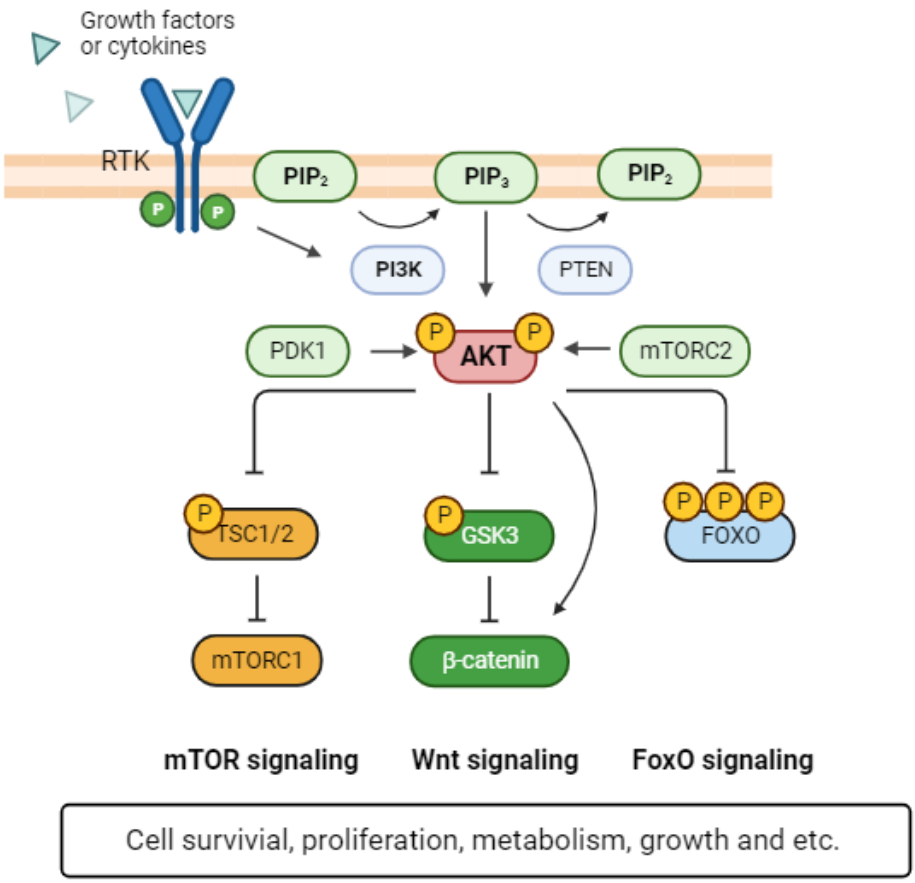

Figure 5. AKT signaling pathway.

Upon stimulation, active PI3K phosphorylates membrane bound PIP2 to PIP3, which then recruits AKT to the membrane where it is activated through phosphorylation of T308 and S473 by PDK1 and mTORC2, respectively. Activated AKT inhibits FOXO, GSK3 and TSC2 via phosphorylation to regulate cellular functions. Prepared with Biorender.

While GSK3 is a core component of the β-catenin destruction complex in canonical Wnt signaling, it also participates in AKT signaling and is the first identified AKT substrate. GSK3 is generally active without simulation, and it functions to inhibit downstream targets through phosphorylation which, in most

cases, tags them for ubiquitination and proteasomal degradation. The AKT-mediated phosphorylation of GSK3 inhibits the GSK3-mediated phosphorylation of the targeted proteins so that they get stabilized. As β-catenin is one of the targeted proteins, this is one way that AKT signaling and canonical Wnt signaling crosstalk. The FoxO transcription factors, regulating expression of genes involved in apoptosis, cell cycle and glucose metabolism, are also direct substrates of AKT. Upon AKT-mediated phosphorylation, FoxO gets exported from the nucleus to the cytosol. This process is facilitated by 14-3-3 phospho-binding proteins, so that FoxO function in regulating transcription will be repressed. Last but not least, the multifunctional node of AKT signaling, mTORC1, is indirectly activated by AKT through tuberous sclerosis complex (TSC) and Ras-related GTPase Ras Homolog Enriched in Brain (RHEB). Generally, TSC promotes the conversion of RHEB from GTP bound form to GDP bound to inhibit its activation of mTORC1. Inactivation of TSC via AKT-mediated phosphorylation of its component TCS2, retains RHEB in GTP bounded form thus facilitates mTORC1 activation and then promotes the downstream effects including protein synthesis (Manning & Toker, 2017; Hoxhaj & Manning, 2020).

The function of PI3K in NC induction is controversial. Blockage of PI3K by inhibitor treatment of *Xenopus* embryos after gastrulation stage downregulates *snai2* expression at NC induction stage (Pegoraro et al., 2015). On the contrary, overexpression of a constitutively active PI3K subunit reduces expression of NC marker genes Sox9 and FoxD3, using reprogrammed neural crest cell mass that

is induced from *Xenopus* embryo animal pole explants by neural plate boarder factors Pax3 and Zic1 (Geary & LaBonne, 2018). As PI3K and AKT can also act independently of each other (Faes & Dormond, 2015), there is some consistent evidence showing that upregulation of AKT activity results in overgrowth of NC derivatives. *De novo* gain-of-function mutations in *AKT* or loss-of-function (LOF) mutations in *PTEN,* which promote AKT activation, cause overgrowth of brain and craniofacial structures in humans (Rivière et al., 2012; Akgumus et al., 2017). Mice with *Pten* deletion also show excessive growth of craniofacial structures and other NC-derived tissues (Yang et al., 2018), which might associate with increased AKT activity. However, the mechanisms underlying the possible roles of AKT in NC induction remain largely unknown.

1.6 DDX3X

DDX3X is an ATP-dependent RNA helicase belonging to the DEAD box protein family characterized by the conserved Asp-Glu-Ala-Asp motif. As an RNA helicase that uses energy from ATP hydrolysis to rearrange RNA secondary structure or RNA-protein interactions, DDX3X is able to regulate nearly all stages of RNA metabolism ranging from transcription, splicing, export, translation, and degradation (Mo et al., 2021; Gadek et al., 2023). The helicase core of DDX3X, responsible for ATP binding and hydrolysis, and RNA binding, contains two conserved RecA-like s flanked by N-terminus and C-terminus, which gives DDX3X a unique feature distinguishing it from other DEAD-box RNA helicases. Another characteristic of DDX3X resides in the intrinsically disordered regions on

the extended N-terminus and C-terminus outside of its helicase core, which contains nuclear export signal (NES) and protein binding sites associating with eukaryotic translation initiation factors eIF4 and eIF3, nuclear export receptor TAP (transporter associated with antigen processing 1), etc (Lai et al., 2008; Lee et al., 2008; Soto-Rifo et al., 2012).

DDX3X is an X chromosome linked gene which partially escapes X chromosome inactivation (Godfrey et al., 2020; Zito et al., 2023). There is a Y-linked homolog in humans, *DDX3Y*, which shares high protein sequence identity (92%) with DDX3X. Originally it has been reported that they have distinct expression patterns and functions, as DDX3X is expressed ubiquitously and accounts for most of DDX3 functions in somatic tissues, whereas DDX3Y is translated specifically in the testes and acts as a key spermatogenic factor (Ditton et al., 2004; Foresta et al., 2000). However, whether DDX3Y has functions beyond that is still debatable, which I will explain more in the next section. In mice, it is even more complicated as there is an additional, functional, and autosomal homolog of *DDX3* besides *DDX3X* and *DDX3Y, D1Pas1/PL10*, that has been shown to substitute the function of DDX3Y in spermatogenesis (Chang & Liu, 2010; Matsumura et al., 2019). In contrast, *X. tropicalis* has only one autosomal *ddx3* homolog, sharing 91% identity to human DDX3X in protein sequence, which makes it a more simplified model for functional studies of DDX3X. Moreover, the *X. tropicalis ddx3* mRNA is expressed ubiquitously throughout early development, similar to human *DDX3X* (Cruciat et al., 2013).

DDX3X in RNA metabolism

One of the most studied functions of DDX3X is its regulation of protein synthesis via translation, more specifically translation initiation. In eukaryotes, translation initiation is the recruitment of the ribosome and its associated factors to the start codon of a mRNA, using mostly the cap-dependent mechanism, in which the ribosome binds at the 5' cap initially, or the cap-independent mechanism without binding at the 5' cap but through interaction with the internal ribosome entry site (IRES). The cap-dependent initiation is facilitated by the cap binding protein complex eIF4F which contains eIF4E and eIF4G. Whereas eIF4E directly binds to the 5’ cap on the mRNA, eIF4G is the adaptor protein that links eIF4E with the ribosome-associated factor (Jackson et al., 2010). DDX3X functions to regulate both the cap-dependent and -independent translation initiation. It has been reported that in liver cancer, DDX3X represses global protein synthesis through disrupting the interaction between eIF4E-eIF4G via competing with eIF4G for binding to eIF4E (Shih et al., 2008). On the contrary, in melanoma cells, DDX3X is required for cap-dependent translation to promote global protein synthesis (Phung et al., 2019). Moreover, it has also been shown that DDX3X facilitates the translation initiation of selected mRNAs with highly structured 5′UTRs through directly binding to the secondary structure to facilitate unwinding and assist in pre-initiation scanning. Transcripts encoding Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell cycle regulator cyclin E1 are among the selected mRNAs that have been shown to have DDX3X bind to the 5′UTRs of its mRNA

that is responsible for the translational regulation (Lai et al., 2010; H.-H. Chen et al., 2015, 2016). On the other hand, DDX3X promotes cap-independent translation initiation mediated by IRES of viral RNAs as well as some cellular transcripts including cellular tumor antigen p53 and microphthalmia-associated transcription factor (MITF) (W.-J. Chen et al., 2017; Phung et al., 2019; Han et al., 2020).

Besides regulating translation, another function of DDX3X that is more relevant to this dissertation resides in its regulation of transcription. In cancer biology, it has been characterized that DDX3X interacts with transcription factor specific factor 1 (Sp1) to promote its binding to the promoter region of its targeted genes, including cyclin-dependent kinase inhibitor P21, Kirsten rat sarcoma virus (KRAS) and E3 ubiquitin-protein ligase mouse double minute 2 homolog (MDM2) (Chao et al., 2006; D.-W. Wu et al., 2014, 2016). In breast cancer, the oncogenic role of DDX3X is mediated by repressing E-cadherin transcription through interaction with its promoter region (Botlagunta et al., 2008). DDX3X is also found to be present in the stress granules (SGs) , which are phase-separated, membrane-less assemblies composed of RNA and protein that are formed in the cytosol under stress conditions (Protter & Parker, 2016), whose functions remain largely understudied. DDX3X has been shown to be vital for the cell fate determination between pro-survival SGs assembly and programmed cell death caused by activation of the inflammasome (Samir et al., 2019). DDX3X mutations

associated with medulloblastoma have been shown to induce SGs formation and impair global translation (Valentin-Vega et al., 2016).

DDX3X-associated diseases

DDX3X mutations were identified in various cancer types including brain tumors, such as medulloblastoma and meningioma, and blood cancer types including T-cell acute lymphoblastic leukemia (T-ALL), Natural killer/T-cell lymphoma (NKTCL), Aggressive natural killer-cell leukemia (ANKL) and Burkitt lymphoma (BL), as well as neural crest related cancer types, melanoma and neuroblastoma (Mo et al., 2021). The majority of mutations in lymphoma and melanoma are nonsense or frameshift that led to DDX3X LOF which is linked to cancer progression along with poor outcome in clinics (Jiang et al., 2015; Phung et al., 2019). In medulloblastoma, as the second most mutated gene, DDX3X has mostly missense mutations which alter the protein function to promote cell proliferation or support stress granule assembly to help cancer cells adapting to metabolic stress (Northcott et al., 2012; Oh et al., 2016; Patmore et al., 2020; Brown et al., 2021). Besides genetic mutations, the expression level of DDX3X has also been shown to have correlation with the clinical features of a lot of cancer types. In most cases, overexpressed DDX3X is positively correlated with pathological classification and associated with poor patient survival. On the contrary, a few studies have shown that there is low expression level of DDX3X in some lung cancer, colorectal cancer and hepatocellular carcinoma cases, which might have some relation with viral infections (Mo et al., 2021).

From a large-scale study on developmental disorders, *de novo* mutations of DDX3X were identified to be the genetic causes for a neurodevelopmental disorder called DDX3X syndrome, with patients exhibiting developmental delay, intellectual disability (ID) and autism spectrum disorder (ASD), while some of them have neural crest defects including dysmorphic facial features, congenital heart disease, melanoma and neuroblastoma (Johnson-Kerner et al., 1993; Deciphering Developmental Disorders Study, 2015; Snijders Blok et al., 2015; Lennox et al., 2020). Unlike the cancer associated DDX3X mutations that emerge later in patients' lifetimes, DDX3X syndrome related mutations are germline mutations that affect early embryonic development, which occurs approximately 1 in 50000 live births. The mutations are composed of approximately half nonsense and half missense, suggesting that haploinsufficiency or protein function alteration of DDX3X may contribute to developmental defects (Gadek et al., 2023). While complete depletion of DDX3X is embryonic lethal, conditional knockout of both alleles in the nervous system of female mice results in impaired neurogenesis and cortical development which phenocopied DDX3X syndrome patients with microcephaly (Hoye et al., 2022). Our lab has shown that DDX3 KD in *Xenopus* embryos inhibits neural crest formation, which would be rescued by human DDX3X, indicating its function in promoting neural crest development (Perfetto et al., 2021). Most of the missense mutations reside in the two helicase domains, and a few have been studied for their roles in modulating canonical Wnt signaling or impairing target transcript

translation (Snijders Blok et al., 2015; Lennox et al., 2020). As there are no established treatments specific for DDX3X syndrome yet, functional studies on DDX3X and characterization of its downstream targets will aid in developing therapeutic strategies such as restoration or reactivation of DDX3X-related RNA metabolism.

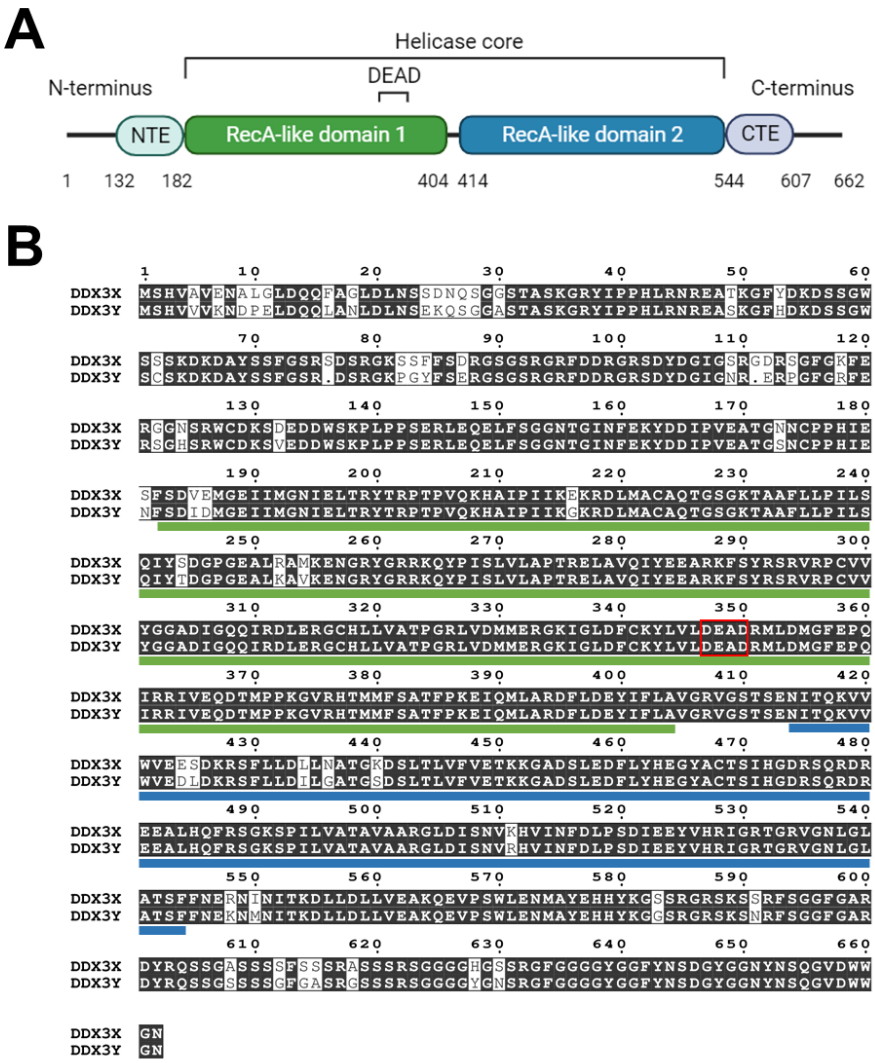

Figure 6. DDX3 protein structures and sequence alignment

(A) The structure of DDX3. DDX3 can be divided into three regions, the helicase core, which consists of two conserved RecA-like domains flanked by the other two regions, the N-terminus and the C-terminus. NTE, N-terminal extension; CTE, C-terminal extension. Created using BioRender. (B) Sequence alignment of human DDX3X and DDX3Y, with the two helicase domains highlighted with green and blue colored line underneath, respectively, and DEAD box in red colored box. The two proteins share high amino acid sequence identity in the two

helicase domains and the C-terminus, with more divergent sequence on the N-terminus. Sequence alignment performed using ESPript 3.

1.7 DDX3Y

In humans, *DDX3Y*, previously often called DEAD Box Y (DBY), is the one *DDX3* homolog located in the nonrecombining region of the Y chromosome, more specifically in the azoospermia factor a (AZFa) region with deletions linked to a severe testicular pathology, the Sertoli-cell-only (SCO) syndrome, showing almost no presence of germ cells, while loss of DDX3Y, singularly or in combination with other AZFa genes, has been shown to have major responsibilities for this phenotype (Lahn & Page, 1997, 1999; Foresta et al., 2000).

The expression patterns of DDX3Y in normal human tissues are complicated and remains controversial. At transcript level, a ~5kb transcript is ubiquitously expressed in both somatic tissues and the testis, while a few shorter transcripts around 3kb are only detectable in the testis (Lahn & Page, 1997; Ditton et al., 2004). The ~2kb size difference between the transcripts might be due to alternative polyadenylation on the 3'UTR, which is likely associated with tissue specific extensions on the 5'UTR (Rauschendorf et al., 2011), with mechanisms largely unknown. The numerous transcript variants resulted from alternative transcription start sites, splicing, and polyadenylation added multiple layers of complications for DDX3Y related research (Gueler et al., 2012; Jaroszynski et al.,

2011; Rauschendorf et al., 2011). At protein level, DDX3Y is detectable in the testis but not in normal human brain or kidney samples (Ditton et al., 2004; Gueler et al., 2012), which might be insufficient to draw the conclusion that DDX3Y is specifically expressed only in the male reproductive system. Due to the high protein sequence identity shared by DDX3X and DDX3Y, the absence of commercially available DDX3Y antibodies with good specificity might be another inducement for the slow progress in relevant research. Translational repression on DDX3Y was proposed to be responsible for the protein level dimorphism between the pre-meiotic germ cells and post-meiotic germ cells, with the former having DDX3Y protein while the latter having none, likely depending on upstream ATG triplets in the 5'UTR (Jaroszynski et al., 2011). The repressed tissue-specific expression of DDX3Y might associate with the extra-long 3'UTR, however, the mechanism of how the longer 3'UTR links to complete absence of the protein without a dramatic change in transcript stability remain largely understudied.

DDX3Y doesn't seem to have vital functions in somatic tissues as the SCO syndrome patients with complete deletion of DDX3Y didn't suffer from other noticeable symptoms besides the testicular pathology. Consequently, researchers rarely studied its functions beyond spermatogenesis. Recently, as more studies on DDX3X-associated diseases were carried out and the sex bias linked to it brings DDX3Y back to our sight. In patients with DDX3X syndrome, there are many more affected females than males with the widely accepted explanation that males with inactivating mutations in DDX3X suffer embryonic

lethality, although the experimental evidence is limited. In mice, conditional KO of both copies of DDX3X in females impairs neurodevelopment leading to profound microcephaly, while cKO of the single copy in the males does not cause significant changes in cortical size (Patmore et al., 2020; Hoye et al., 2022). The authors also suggested that there is an upregulation of DDX3Y at the transcript level, implying the possibility of compensation from DDX3Y in rescuing the brain defects. The role of DDX3Y in regulating neurogenesis was confirmed by another study, as knockdown of DDX3Y in human neural progenitor cells impairs neuronal differentiation (Vakilian et al., 2015). Even if there is one more autosomal homolog in mice that might also help attenuate the phenotypes, the potential compensation from DDX3Y cannot be neglected. On the contrary, in Burkitt lymphoma, DDX3X mutations were more frequent in male patients (Gong et al., 2021). The study suggested that the ectopic expression of DDX3Y compensates for the loss of DDX3X to restore protein synthesis in the developing tumors, and that could explain the sex bias in this tumor type, while what triggers DDX3Y aberrant expression remains unknown. In the same year, the functional redundancy shared by DDX3X and DDX3Y in regulating protein synthesis was confirmed by another group (Venkataramanan et al., 2021). Besides the common features shared by the two proteins grounded in their highly conserved helicase cores, their divergent N-terminus assigns them different characteristics in other aspects of RNA metabolism, e.g. stress granule assembly (Shen et al., 2022). Last but not least, DDX3Y has been reported by three independent

research groups to be linked to human heart failure although no mechanistic study has been conducted (Isensee et al., 2008; Fan & Hu, 2022; Xu et al., 2023). In summary, more characterization of DDX3Y beyond fertility is necessary and will aid in better understanding the sex bias in DDX3X associated human diseases.

1.8 ADAM9

The disintegrin metalloproteinases (ADAMs) are a family of transmembrane metalloproteinases that cleave cell-surface proteins and extracellular matrix, to mediate their functions in regulating cell signaling, including Notch and epidermal growth factor receptor (EGFR) signaling (Lichtenthaler et al., 2018). ADAMs are type I transmembrane proteins that consist of a pro-domain, metalloprotease domain, disintegrin domain and cysteine-rich domain on the extracellular side (Wei, 2013). In general, the pro-domain binds to the metalloprotease domain to inhibit its protease activity and its removal is required for protease activation. In the metalloprotease domain, most of the ADAMs are characterized by a conserved zinc-binding motif in the active site that can cleave the ectodomain of cell-surface proteins (Lichtenthaler et al., 2018; Wei, 2013). Besides the extracellular domains, ADAMs also contain a transmembrane domain to anchor ADAMs to the plasma membrane, and a cytoplasmic domain, which is involved in intrinsic signaling activity (Giebeler & Zigrino, 2016).

ADAM9, originally known as metalloprotease disintegrin cysteine-rich protein 9 (MDC9), was first identified in both mice and humans in 1996 (Weskamp et al., 1996). ADAM9 expression is detected in various tissues ranging from the nervous system, vascular smooth muscle, lung, colon, kidney, reproductive system, and secretary organs, indicative of a wide range of biological functions (Chou et al., 2020). As a sheddase, ADAM9 can cleave numerous substrates that can be categorized into three groups including cytokines (and related receptors), growth factors (and related receptors), and the other molecules, which relate to biological processes including inflammation, adhesion, angiogenesis, migration and proliferation (Chou et al., 2020). Despite the wide distribution of ADAM9-expressing tissues and its wide range of biological functions mediated by various substrates, loss of ADAM9 in mice did not show apparent developmental defects, possibly due to compensation from other ADAMs (Weskamp et al., 2002; P. Zhang et al., 2016). However, loss of ADAM9 in older mice or ADAM9 null mutations in humans results in retinal degeneration, leading to cone-rod dystrophy in human patients, which is an inherited progressive retinal dystrophy (Parry et al., 2009).

Besides visual impairment, ADAM9 has been implicated in other human diseases including Alzheimer's Disease (AD). As the most prevalent neurodegenerative disease, AD is the most common cause of dementia, characterized by a progressive decline of cognitive ability that affects memory, thinking and behavior (Weller & Budson, 2018). One of the hallmarks of AD pathology is the plaque

formation caused by abnormal accumulation of amyloid β (Aβ) from amyloidogenic processing of the amyloid β precursor protein (APP) (Grimm et al., 2013). As a type I integral transmembrane protein, APP contains a large ectodomain, a transmembrane domain harboring the Aβ region, and a short cytoplasmic domain. In normal physiological conditions, APPs are cleaved predominantly via the non-amyloidogenic pathway by α-secretases within the Aβ region, preventing the release of intact Aβ. However, in AD patients, APPs are first cleaved by the β-secretase in the ectodomain. The remaining stub is then further cleaved by γ-secretase, resulting in the release of Aβ to the extracellular matrix (Chow et al., 2010; Kandalepas & Vassar, 2012). Despite that ADAM10 was reported to be the major α-secretase for APP non-amyloidogenic processing, ADAM9 also has α-secretase activity for APP (Koike et al., 1999; Fahrenholz et al., 2000; Asai et al., 2003). On the contrary, inhibition of ADAM9 activity via treatment with ADAM9 pro-domain, in SY5Y neuroblastoma cells with APP overexpression, reduced Aβ production. As ADAM10 is also a substrate of ADAM9, this contradicting effect is likely caused by that ADAM9 cleaves the ectodomain of ADAM10 resulting in the inhibition of ADAM10 proteolytic activity (Moss et al., 2011).

In addition, ADAM9 has been reported to play critical roles in tumor proliferation, metastasis and angiogenesis. Overexpression of ADAM9 was observed in various cancer types including brain cancer, lung cancer, pancreatic cancer, breast cancer and prostate cancer, and was usually associated with poor

prognosis and tumor aggressiveness (Chou et al., 2020). In breast cancer, ADAM9 promotes cancer progression and is responsible for therapy resistance in patients, while siRNA-mediated silencing of ADAM9 inhibits tumor cell invasion and metastasis (Moelans et al., 2010; Micocci et al., 2013, 2016). Consistently, as ADAM9 can be post-transcriptionally repressed by microRNAs, overexpression of its regulatory microRNAs inhibits breast cancer cell proliferation and metastasis, mediated by suppression of ADAM9 (C.-Z. Wang et al., 2015; C. Zhang et al., 2015; Qin et al., 2017). In lung cancer, ADAM9 overexpression was reported to be associated with its invasion and brain metastases, mediated by cleavage and activation of key transmembrane proteins, as well as through promoting vascular remodeling by regulation of angiogenesis (Lin et al., 2014, 2017). Besides, our lab has shown that ADAM9 mRNA is upregulated in human colon cancer samples, while siRNA-mediated knockdown of ADAM9 in cultured colon cancer cell lines inhibits cell migration and invasion (Chandrasekera et al., 2022).

Last but not least, ADAM9 has been reported to be involved in Coronavirus Disease 2019 (COVID-19), a contagious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Loss of ADAM9 reduces SARS-CoV-2 replication in an engineered human lung cancer cell line (Gordon, Hiatt, et al., 2020; Gordon, Jang, et al., 2020). Meanwhile, ADAM9 was found to be upregulated in COVID-19 patients with more critical symptoms and was identified to be the driver of disease severity (Carapito et al., 2021), with the

mechanism unknown. Understanding the mechanisms of action for ADAM9, including its substrate spectra and roles in cell signaling, will provide important insight into the functions of this versatile metalloproteinase in human health.

Chapter 2

DDX3 REGULATES NC DEVELOPMENT THROUGH THE RAC1-AKT- β-CATENIN AXIS

2.1 Introduction

NC cells are a group of multipotent cells that contribute to various tissues including the craniofacial structures, the peripheral nervous system, cardiac structures and pigment cells (Bronner & LeDouarin, 2012; George et al., 2020). Disrupted development of the NC leads to a broad spectrum of birth defects collectively called NCPs, including craniofacial disorders, congenital heart diseases as well as NC-derived tumors (Watt & Trainor, 2014; Vega-Lopez et al., 2018). Canonical Wnt signaling is one of the major signaling pathways that is critical for NC induction in both non-mammalian and mammalian vertebrates (Elkouby & Frank, 2010).

De novo mutations of DDX3X are common causes of ID in newborns (Snijders Blok et al., 2015; Lennox et al., 2020). Patients carrying DDX3X mutation show defects in development of the central nervous system (CNS) (Johnson-Kerner et al., 1993). Consistently, LOF studies indicated that DDX3X plays a critical role in CNS development in mice (Lennox et al., 2020; Hoye et al., 2022). Besides the CNS defects, we noticed that most of the patients also exhibit craniofacial defects, while some individuals have heart defects and NC-derived tumors,

suggesting impaired NC development (Johnson-Kerner et al., 1993; Snijders Blok et al., 2015). So we hypothesized that DDX3X is required for normal NC development. As human DDX3X shares a high protein identity with *X. tropicalis* DDX3, here we investigated the roles of DDX3 in NC development using our frogs as the model. Previously, DDX3 has been shown to promote canonical Wnt signaling through stimulating the casein kinase CK1 using a mechanism independent of its RNA helicase activity (Cruciat et al., 2013). As canonical Wnt signaling plays a vital role in NC induction, we further tested whether the functions of DDX3 in NC is mediated by downstream canonical Wnt signaling. Instead of the helicase-independent mechanism, we showed here that DDX3 promotes canonical Wnt signaling using a novel helicase-dependent mechanism. Besides, since RAC1 and AKT has been reported to be direct and indirect downstream targets of DDX3, respectively (H.-H. Chen et al., 2015, 2016; D.-W. Wu et al., 2016), we also proposed that they are functional mediators of DDX3 in inducing NC.

What's interesting is the strikingly similar phenotypes caused by mutations affecting RAC1 and AKT activity in humans. Similar to DDX3X, *de novo* mutations in *RAC1* were also shown recently to cause ID and ASD in humans, while mutations in *AKT* that are associated with neurodevelopmental disorders and NCPs, are usually gain-of-function mutations that result in overgrowth of the brain and NC-derived tissues (Rivière et al., 2012; Akgumus et al., 2017; Reijnders et al., 2017). Here, we investigated their functions in regulating NC

development and whether it's mediated by downstream canonical Wnt signaling. This work can hopefully expand our understanding of NC regulatory network and help explain the NC-related defects in patients with *DDX3X*, *RAC1* or *AKT* mutations.

2.2 Experimental Methods

X. tropicalis embryo manipulation

Wild-type *X. tropicalis* adult frogs were purchased from NASCO and Xenopus 1 Corp. The transgenic *snai2*:eGFP line was generated as described previously (J. Li et al., 2019) and the Wnt reporter frogs were acquired from Dr. Kris Vleminckx (Tran et al., 2010). Embryos were obtained by natural mating and injected as described (Ogino et al., 2006; Lane & Khokha, 2022). Alexa Fluor 555 (Invitrogen) was co-injected as a linage tracer. Morpholino antisense oligos (MO) were designed and generated by Gene Tools, including DDX3 (5'-CCGTGATTGATGCTT-3'), DDX3-2 (5'-TTTCCACGGCCACATGACTCATAAC-3') RAC1 (5'-GACCACACATTTAATGGCCTGCATG-3') and AKT2 (5'-ATCACCATCACTTCATTCATGTTGG -3'). To KD DDX3, individual embryo at 2-cell stage was injected with 6ng of MOs on one side. To KD RAC1 or AKT2, individual embryo at 8-cell stage embryos were injected with 1.5ng of indicated MOs at one anterior-dorsal blastomere. The small guide-RNAs (sgRNAs) for CRISPR/Cas9 mediated KO were generated as described (Nakayama et al., 2013). Protospacers were cloned separately into the pUC57-T7-gRNA scaffold

vector, including *rac1*-g1 (5'-GGCATTAAATGTGTGGTCGT-3'), *rac1*-g2 (5'-GGTATGGGATACAGCCGGAC-3'), *akt2*-g1 (5'-GGACCAGGTGGCCGAAGATG -3'), and *akt2*-g2 (5'-GGCCAAAGGGCGCCCCAAAG -3'). *In vitro* transcription was carried out to generate sgRNAs using the TranscriptAid T7 high yield transcription kit (Thermo Scientific #K0441). To KO indicated genes, individual embryo at 4-cell stage was injected with 300 pg of indicated sgRNA and 1 ng Cas9 protein (PNA Bio) at one dorsal blastomere. Injected embryos were cultured to desired stages and then imaged directly or processed for *in situ* hybridization (ISH), western blotting or RT-qPCR.

Reagents

The cDNAs for human DDX3X (BC011819), *RAC1* (BC050687) and *Xenopus gbx2* (6993680) were purchased from GE-Dharmacon and subcloned into pCS2+ expression vector with a C-terminal in-frame HA tag. The AAA mutant (S382A/T384A) of DDX3X were generated by mutagenesis using primers CCACACTATGATGTTTGCTGCTGCTTTTCCTAAGGAAATAC (forward) and GTATTTCCTTAGGAAAAGCAGCAGCAAACATCATAGTGTGG (reverse). Constructs encoding constitutively active (caAKT, 10841) and dominant-negative AKT (dnAKT, 9031) were purchased from Addgene. AKT Inhibitor IV (124011) were purchased from Sigma-Aldrich. Antibodies used for western blotting include rabbit anti-DDX3 (Abcam ab151965, 1:1000) , rabbit anti-active-β-catenin (Cell Signaling Technology 8814, 1:1000), rabbit anti-β-Catenin (Sigma C2206, 1:1000), rabbit anti-pS473-AKT (Cell Signaling Technology 9271, 1:1000), rabbit

anti-AKT1 (Aviva Systems Biology AVARP06008_P050, 1:1000), mouse anti-RAC1 (Abcam ab33186, 1:1000), HRP-conjugated mouse anti-β-actin (Cell Signaling Technology 8H10D10, 1:5,000), HRP-linked horse anti-mouse IgG (Cell Signaling Technology 7076, 1:10,000) and HRP-linked goat anti-rabbit IgG (Cell Signaling Technology 7074, 1:10,000).

In situ hybridization

ISH digoxigenin-UTP labeled probes for *snai2, pax3, zic1, msx1* and *sox9* were obtained from previous studies (Wei et al., 2010; J. Li et al., 2018). *In vitro* transcription was conducted to generate ISH probe for *gbx2* as described previously (Wei et al., 2010). Embryos were collected at NC induction stage 12.5 and processed for whole-mount ISH as described (Sive et al., 2000). Bright-field images of the embryos were taken with a Zeiss Axio Zoom V16 fluorescence microscope and an AxioCam camera.

Western blotting

Embryos or cells were lysed on ice in RIPA lysis buffer (Fisher Scientific 89901) with Halt protease inhibitor cocktail (Thermal Fisher 78429) and Halt phosphatase inhibitor cocktail (Thermal Fisher 78420) added, and processed for western blotting as described ((J. Li et al., 2018). Blots were detected with HRP-conjugated antibodies and Clarity Western ECL substrate (Bio-Rad #1705061) using a Bio-Rad ChemiDoc touch imager.

RT-qPCR

Total RNA was extracted from collected embryos using RNeasy Mini Kit (Qiagen 74104) and then reverse-transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad 170-8891). Using qMax Green qPCR Mix (Accuris ACC-PR2000-L-100), quantitative PCR (qPCR) was carried out on Quant Studio 6 Flex Real-Time PCR system (Applied Biosystems). The cycling conditions include an initial denaturation step at 95°C (2 min), 40 cycles of 5 sec at 95°C and 30 sec at 60°C. The primer sets used was shown in table 1.

Table 1. qPCR primer sets for Chapter 1

RAC1 1st Forward	CTTGGAATGCTCTGCCCTTA
RAC1 1st Reverse	TCTCTTTCGCTTCTTGACTGG
RAC1 2ed Forward	ACAGGAGGACTACGACAGATTA
RAC1 2ed Reverse	GGTACCACTTAGCACGAACAT
GAPDH Forward	GTGACACTCATTCCTCCATCTT
GAPDH Reverse	GCTGTAGCCACACTCGTTAT

Statistics

For craniofacial morphology, embryos were cultured to stage 46 and scored for craniofacial defects compared with the un-injected side of the same embryos. For ISH, embryos were scored for expression reduction of indicated genes. Statistics

was done with chi-squared tests comparing the proportion of embryos with detectable defects in craniofacial structures, or with expression reduction of indicated genes. For western blotting and RT-qPCR, statistics was all done with unpaired student t test.

2.3 Results

DDX3 is required for NC development in *X. tropicalis* embryos.

To evaluate the potential functions of DDX3 in NC development, we KD DDX3 on one side of the embryos in *snai2*:eGFP transgenic line. KD of DDX3 was achieved by injection of a morpholino antisense oligo (MO), which targets the 5' UTR of *ddx3* mRNA to block translation initiation (Cruciat et al., 2013). Loss of DDX3 disrupted craniofacial morphology, especially the head cartilage structures which are smaller and less organized on the injected side compared with the un-injected side (Fig. 7A). To confirm that the phenotype is caused specifically by DDX3 KD, we applied another translation-blocking MO targeting *ddx3* mRNA and observed similar craniofacial defects (Fig. 7A). Taken together, we showed that DDX3 is critical for the development of *X. tropicalis* NC.

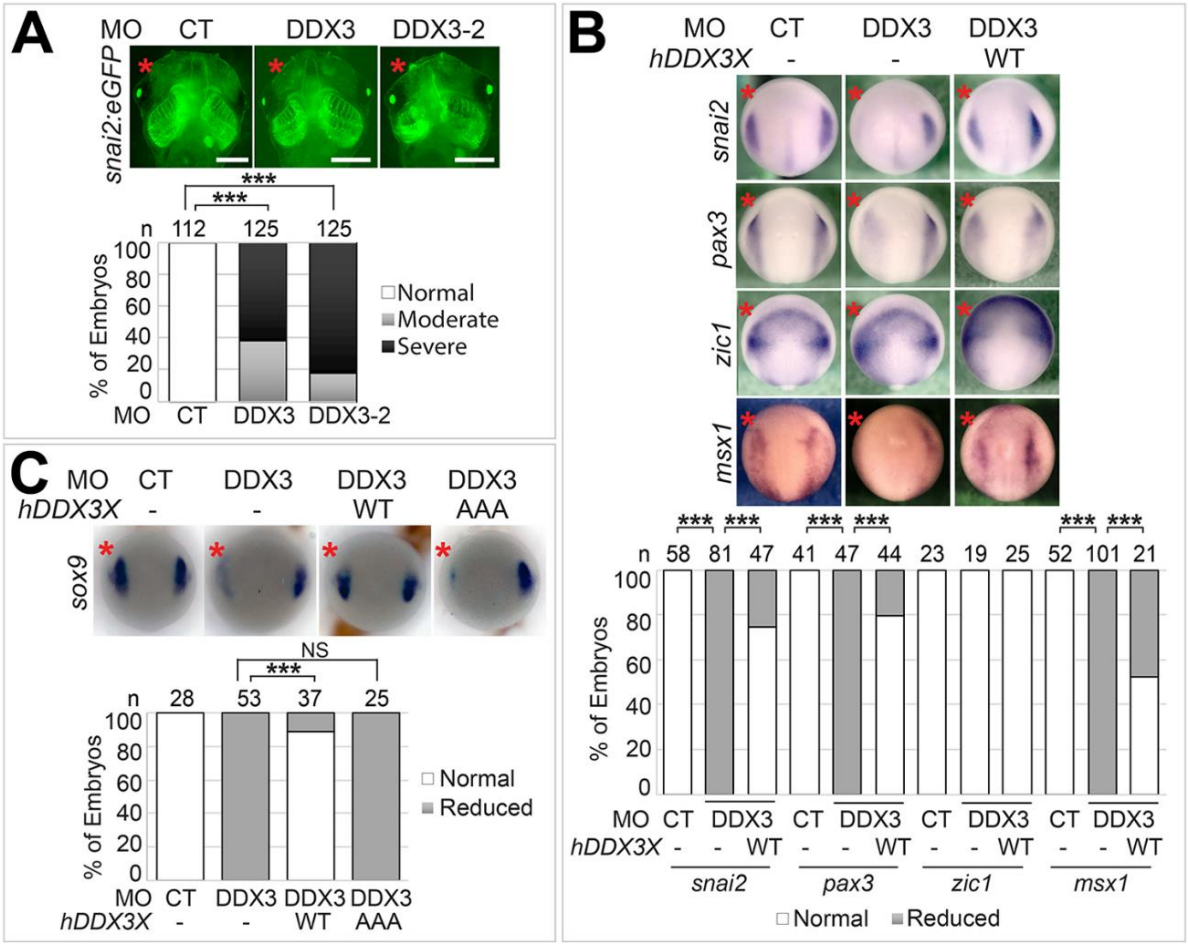

Figure 7. DDX3 is required for craniofacial morphogenesis through promoting NC induction.

(A) 1.5 ng of indicated MO was injected into an anterior-dorsal blastomere of 8-cell stage embryos in *snai2:*eGFP transgenic line. Embryos were then incubated for 5 days to tadpole stage 46 and imaged from ventral view with anterior on top. Scale bars: 500 μm. (B, C) 6ng of DDX3 MO and 200 ng of mRNA encoding WT or AAA mutant of human DDX3X were injected into one blastomere at two-cell stage. Embryos were incubated for 8 hours to stage 12.5, processed for ISH with indicated probes, and then imaged from the dorsal view with anterior on top. Red asterisks indicate the injected side. n, number of embryos. ***P<0.001; NS, not significant (chi-squared tests).

Craniofacial defects could be caused by disrupted early induction, migration or differentiation of NC. We started by examining the early NC induction with embryos at stage 12.5 when the blastopore just closed. Loss of DDX3 downregulated the expression of NPB marker genes, *pax3* and *msx1*, NC marker genes, *snai2* and *sox9*, suggesting inhibition of NC induction (Fig. 7B, C). What's interesting is that one of the NPB marker genes, *zic1*, stayed unchanged or even slightly expanded with DDX3 KD (Fig. 7B), which phenocopied PAX3 KD (T. Sato et al., 2005). As a result, we speculated that this phenotype is likely a secondary effect of PAX3 reduction. Even though *zic1* expression is not reduced, NC induction would be strongly inhibited with either depletion of *pax3* or *msx1*. So, we concluded that DDX3 is required for NC induction in *X. tropicalis* embryos. Expression reduction of all four marker genes could be rescued when we co-injected human *DDX3X* mRNA, which confirmed that the expression reduction of NC marker genes is specifically caused by loss of DDX3. Additionally, it indicated that *Xenopus* DDX3 and human DDX3X are functionally conserved.

The pathogenic mutations carried by DDX3X syndrome patients include approximately half missense mutations and half nonsense mutations. For the missense mutations, almost all of them reside in the two helicase cores while a few of them have been reported to have their helicase activity impaired (Lennox et al., 2020). These findings implied that the role of DDX3 in regulating NC development is dependent on its RNA helicase activity. To test this, we generated an AAA mutant of human DDX3X (S382A/T384A) with abolished helicase activity

and this mutant cannot restore *sox9* expression with DDX3 KD at the NC induction stage (Fig. 7C), which suggested DDX3 promotes NC induction in an RNA helicase activity-dependent manner.

DDX3 induces NC by regulating Wnt/ β-catenin and AKT activity.

Previously, DDX3 has been reported to regulate Wnt/β-catenin signaling (Cruciat et al., 2013), which is critical for NC induction. Consistently, it has been shown that Pax3, Msx1, Snai2 and Sox9 are downstream targets of the canonical Wnt signaling but Zic1 is not (Hong & Saint-Jeannet, 2007; B. Li et al., 2009; de Crozé et al., 2011), implying that the different responses of marker genes with loss of DDX3 are potentially caused by loss of canonical Wnt signaling. To test this more straightforwardly, we KD DDX3 in the Wnt reporter transgenic embryos and observed reduced eGFP expression at the NPB region on the injected side compared with the un-injected side, suggesting that loss of DDX3 inhibits Wnt activity (Fig. 8A). To further confirm the regulation on Wnt target genes, we probe for *gbx2*, a transcription factor and a direct transcriptional target of Wnt/ β-catenin signaling. DDX3 KD reduced expression of *gbx2* at stage 12 that can be restored by WT human DDX3X but not the AAA mutant (Fig. 8B), which confirmed the specificity and also indicated that the regulation of Wnt activity by DDX3 is dependent on its RNA helicase activity. In addition, KD of DDX3 in whole embryos downregulated the protein level of both total and active (unphosphorylated) β-catenin (Fig. 8E).

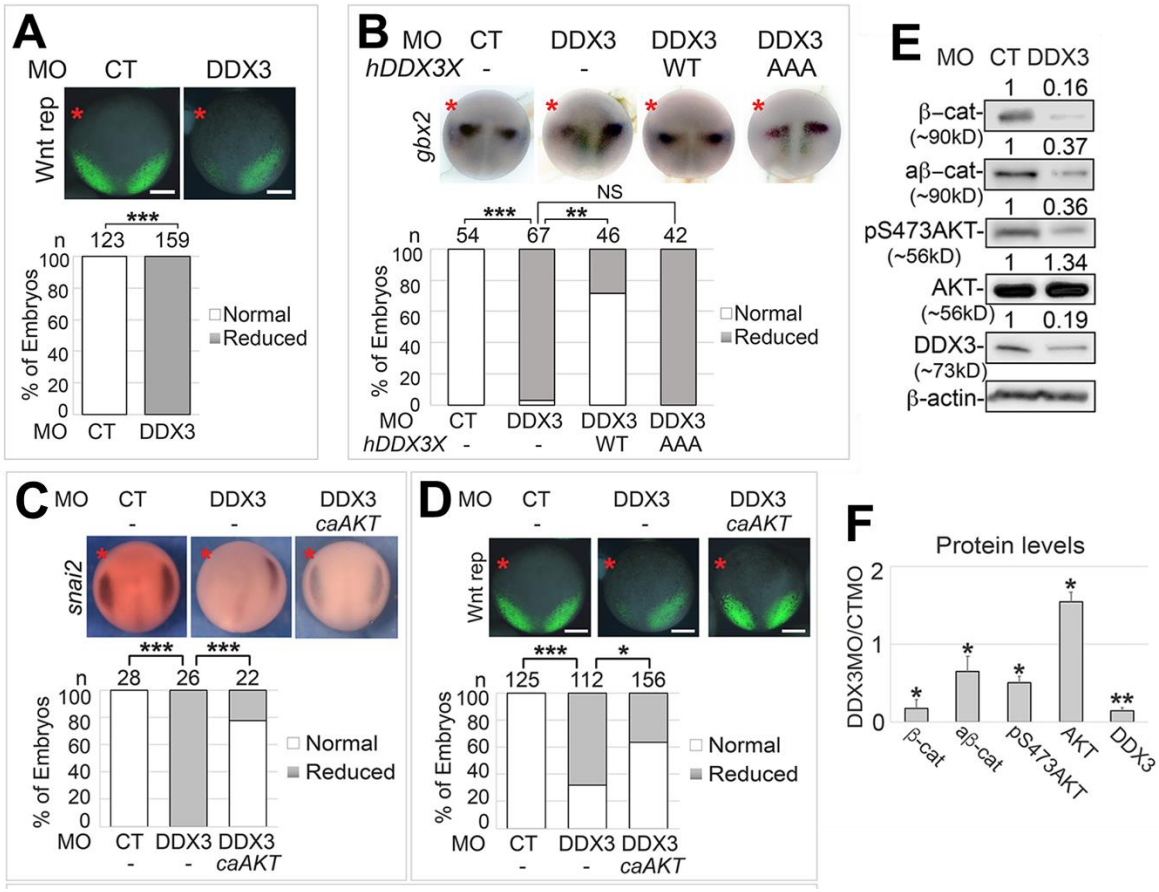

Figure 8. DDX3 induces NC by promoting Wnt/ β-catenin and AKT activity. 6 ng of indicated MO and 200 pg of indicated mRNA were injected into one blastomere at 2-cell stage embryos in Wnt reporter (A, D) or WT (B, C) line. Injected embryos were cultured to stage 12.5 (A, C, D) or stage 12 (B) and then imaged directly for green fluorescence (A, D) or processed for ISH with indicated probes. Embryos were imaged from the dorsal view with anterior on top. Scale bars: 250 μm. n, number of embryos. **P<0.01; ***P<0.001; NS, not significant (chi-squared tests). (E) 12 ng of indicated MO was injected into 1-cell stage embryos which were then cultured to stage 12.5, followed by processing for western blot analyses with indicated antibodies. (F) Quantification of band intensity from three biological triplicates, with target protein normalized against β-

actin. Fold change was shown in mean +/- SEM. *P<0.05; **P<0.01 (unpaired t-tests).

As a core component of the β-catenin destruction complex in canonical Wnt signaling, GSK3 destabilizes β-catenin through phosphorylation. Besides, GSK3 can be directly phosphorylated by active AKT resulting in inhibition of GSK3 and stabilization of β-catenin. To test if AKT activity is regulated by DDX3, we showed that DDX3 KD downregulated the protein level of Ser473 phosphorylation of AKT but not total AKT (Fig. 8E), suggesting that DDX3 promotes AKT activation. Next, to assess whether the role of DDX3 in NC is mediated by downstream AKT, we restored AKT activation by injecting mRNA encoding a constitutively active AKT in embryos with DDX3 KD. As expected, we observed restoration of *snai2* expression and Wnt activity at the NC induction stage (Fig. 8C, D).

The direct downstream target of DDX3 in regulating NC is probably RAC1

As an RNA helicase, DDX3 can facilitate mRNA translation initiation through unwinding 5' UTR structures. As a result, DDX3 promotes AKT activation very likely in an indirect manner. One of the direct translational targets of DDX3 is RAC1, which mediates the functions of DDX3 in cancer biology and neurogenesis (H.-H. Chen et al., 2015, 2016). RAC1, as a small Rho-GTPase, has been reported to facilitate AKT activation through interaction with PAK (Higuchi et al., 2008). Based on these findings, we hypothesized that RAC1 is the direct downstream target of DDX3 in inducing NC. KD of DDX3 in embryos

significantly downregulated RAC1 at the protein level (80%), but there was only a 20% reduction at the mRNA level using one primer pair for RT-qPCR, while no significant reduction when using a different primer pair (Fig. 9A, B). These results suggested that the regulation of RAC1 by DDX3 is predominantly at the protein level, consistent with the translational regulation we proposed. Lastly, we generated a mRNA encoding *X. tropicalis* RAC1 without its highly structured 5' UTR. This *rac1* mRNA rescued the disrupted craniofacial structures caused by loss of DDX3 (Fig. 9C), indicating that RAC1 is the downstream mediator of DDX3 in promoting NC development.

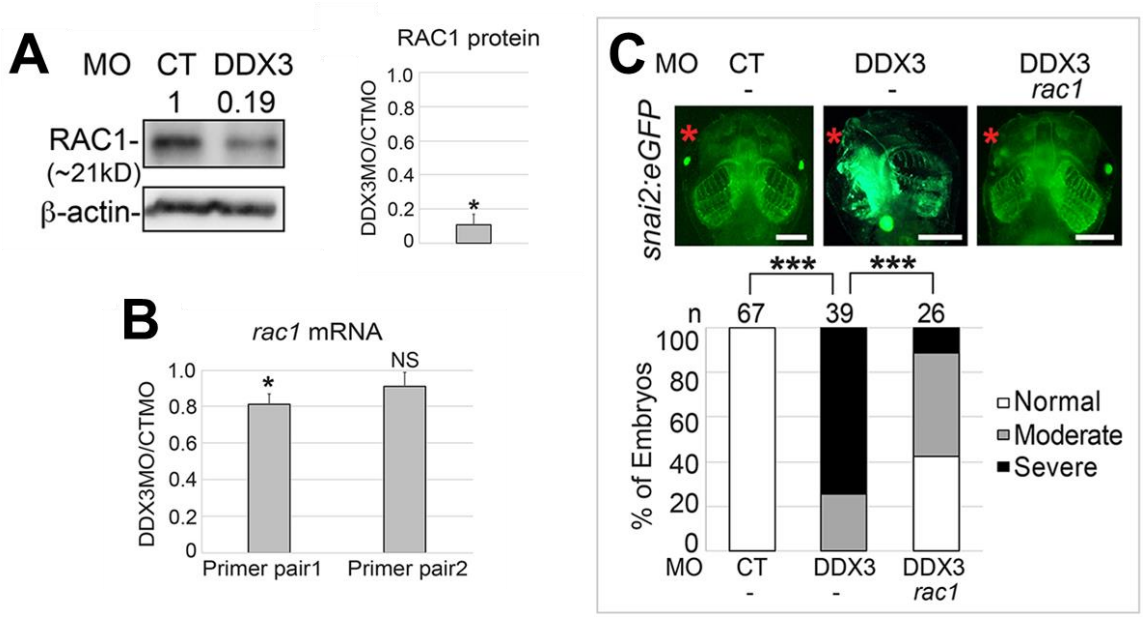

Figure 9. RAC1 is downstream of DDX3 in craniofacial morphogenesis. (A) Embryo lysates collected from Fig. 8E were rerun and probed for RAC1. Quantification of band intensity from three biological triplicates was shown on the right, with RAC1 normalized against β-actin. Fold change was shown in mean +/- SEM. (B) The same batches of injected embryos in A were processed for RT-qPCR with primer pairs targeting *rac1* and *gapdh* mRNA. Results were *rac1* mRNA normalized against *gapdh* mRNA. Fold change was shown in mean +/- SEM. *P<0.05; NS, not significant (unpaired student t-tests). (C) 1.5 ng of indicated MO and 100pg of *rac1* mRNA were injected into an anterior-dorsal blastomere of 8-cell stage embryos in *snai2:*eGFP transgenic line. Embryos were then incubated for 5 days to tadpole stage 46 and imaged from ventral view with anterior on top. Scale bars: 500 μm. n, number of embryos. ***P<0.001 (chi-squared tests).

AKT activation is required for NC induction and canonical Wnt signaling.

Mutations in AKT that are associated with neurodevelopmental disorders and NCPs, are usually gain-of-function mutations that result in the overgrowth of brain and NC-derived tissues (Akgumus et al., 2017; Rivière et al., 2012). In addition, CNS and NC-related overgrowth phenotypes in *Pten* heterozygous KO mice can be rescued by genetic reduction of β-catenin (Y. Chen et al., 2015), suggesting the regulation of AKT on NC is very likely mediated by canonical Wnt signaling. However, how AKT functions directly in NC induction remains largely unknown. To characterize the mechanisms underlying the possible roles of AKT in NC induction, we treated the embryos with AKT inhibitor IV, which inhibits AKT phosphorylation by blocking a kinase downstream of PI3K. As AKT activity is required for gastrulation, we had the embryos treated with the inhibitor from stage 10 to 12.5 to avoid early developmental defects. Inhibition of AKT activity downregulated expression of *msx1*, *snai2* and *sox9*, suggesting that AKT activation is required for NC induction. Similar to DDX3 KD, *zic1* expression was not significantly affected with AKT inhibitor treatment, implying that AKT promotes NC induction through canonical Wnt signaling. To test this directly, we treated the Wnt reporter transgenic embryos with AKT inhibitor and observed downregulation of eGFP expression at the NPB region compared with DMSO control treatment. What's more, we observed similar eGFP downregulation when injected a dominant-negative mutant of AKT (dnAKT) into the Wnt transgenic embryos at

NC induction stage. So we concluded that AKT activation promotes canonical Wnt signaling and NC induction.

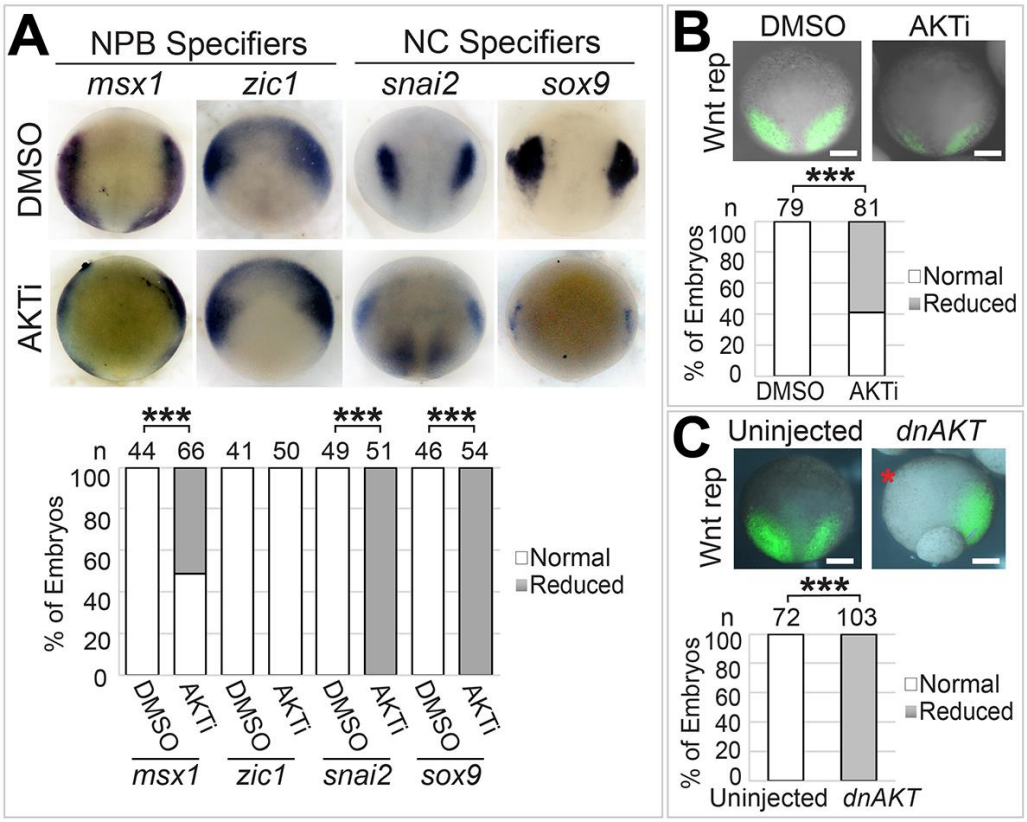

Figure 10. Inhibition of AKT activity blocks NC induction and Wnt signaling. DMSO or 20 μM AKT inhibitor (AKTi) was used to treat WT (A) or Wnt reporter transgenic (B) embryos from stage 10 to stage 12.5. Embryos were collected at stage 12.5 and then imaged directly (B) or processed for ISH with indicated probes (A). (C) 50 pg of mRNA encoding dnAKT was injected into one blastomere at 2-cell stage embryos in Wnt reporter line. Embryos were cultured for 8 hours until stage 12.5 and then imaged from dorsal view with anterior on top. Red asterisks represent the injected side. n, number of embryos. ***P<0.001 (chi-squared tests). Scale bars: 250 μm.

RAC1 and AKT2 are both required for NC development in *X. tropicalis.*

There are both loss- and gain-of-function mutations of RAC1 which lead to microcephaly and macrocephaly, respectively, and both types are associated with craniofacial disorders and other possible NC-related abnormalities including congenital heart disease (Fig. 11A) (Rivière et al., 2012; Akgumus et al., 2017; Reijnders et al., 2017), suggesting the important role of RAC1 in NC development. Consistently, conditional *Rac1* deletion causes severe craniofacial and cardiovascular defects in mice (Migeotte et al., 2011). In *Xenopus* embryos, injection of an mRNA encoding dominant-negative RAC1 downregulates *snai2* expression, while a constitutively active RAC1 upregulates *snai2* expression (Broders-Bondon et al., 2007). What's more, the *X. tropicalis* RAC1 protein is 100% identical to its human and mouse orthologs. We have obtained an MO for RAC1 and injected it into *snai2:eGFP* transgenic embryos. Consistent with the observed lethality in *Rac1* deleted mice, knockdown of Rac1 resulted in severe early developmental impairments including gastrulation defects and neural tube closure defects, even when targeted only to one anterior-dorsal blastomere of an 8-cell stage embryo to minimize the side effects on whole embryo development. Different from mammalian embryo development that cell fate determination happens later on, *Xenopus* embryos have the cell fate determined as early as the second cleavage and the one anterior-dorsal blastomere at 8-cell stage contributes to the brain, spinal cord, somite, NC, etc (Moody, 1987; Heasman,

2006). As an independent validation of phenotype specificity, we have designed two guide RNAs (gRNAs) for *rac1*, and injected gRNAs with the CAS9 protein to achieve an efficient mosaic knockout (KO) of *rac1*. Compared with MO-mediated knockdown which becomes effective immediately after injection, CRISPR/CAS9 mediated gene editing won't have the functional inactivation until after maternal-zygotic transition around stage 8, when the embryos have the maternal transcripts and proteins degraded and start having zygotic transcription and translation (Vastenhouw et al., 2019). Thus, theoretically, the injected embryos will have normal RAC1 before stage 8 with unaffected embryo development and then have RAC1 depleted afterward. Even though, we observed similar developmental defects as MO-mediated RAC1 KD and only one embryo survived to the late tadpole stage with smaller head cartilage and brain on the injected side (Fig. 11E), which phenocopied human patients with RAC1 LOF mutations. With both experiment strategies, loss of RAC1 reduces eGFP expression in *snai2*:eGFP transgenic embryos at stage 23 (Fig. 11B, C), suggesting that it's required for expression of endogenous *snai2*, which is very likely mediated by canonical Wnt signaling, as loss of RAC1 inhibits Wnt signaling in Wnt reporter embryos at NC induction stage (Fig. 11D).

A

Genes/mutations	General development	Brain	Dental/Craniofacial	Other possible NC-related
RAC1/ loss- and gain-of-function	ID, ASD, developmental delay	Microcephaly (loss-of-function); macrocephaly (gain-of-function)	Anomalies in forehead, ears, nose, philtrum and lips; high palate; dental deficiencies	Congenital heart diseases, pigment defects, hearing/visual impairment
AKT1, *AKT3*/ gain-of-function	ID, ASD, developmental delay	Macrocephaly (gain-of-function	Anomalies in forehead, ears and nose; high palate	Pigment defects

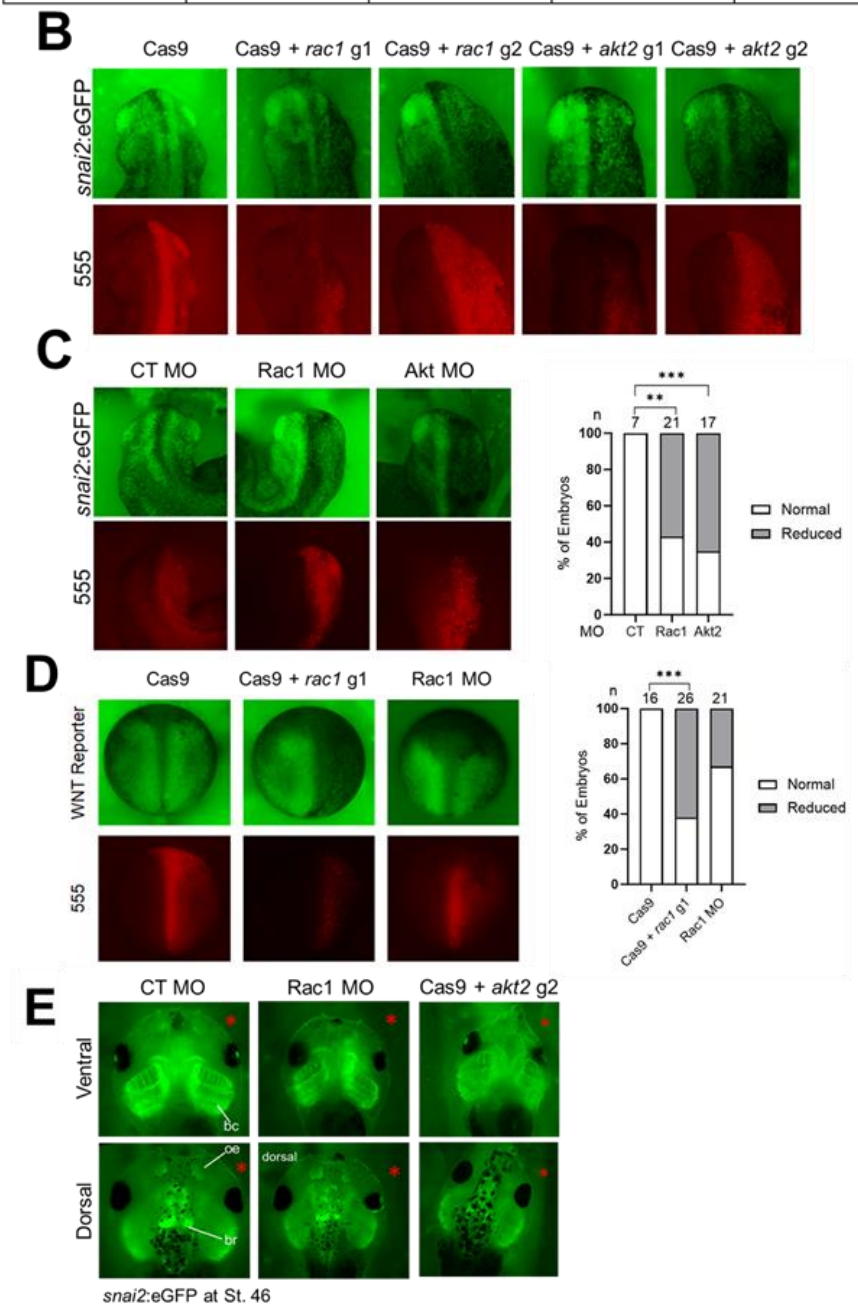

Figure 11. RAC1 and AKT2 are required for NC development in *X. tropicalis*. (A) Birth defects caused by mutations affecting RAC1 or AKT in humans (Rivière et al., 2012; Akgumus et al., 2017; Reijnders et al., 2017). ID, Intellectual Disability; ASD, Autism Spectrum Disorder. (B) One anterior blastomere of 4-cell stage snai2:eGFP embryos was injected with Cas9 protein (1ng) and indicated gRNAs (300pg). Dextran Alexa Fluor® 555 was co-injected as a lineage tracer. Embryos were cultured to stage 23 and imaged for eGFP expression and tracer dye. (C) One anterior-dorsal blastomere of 8-cell stage *snai2*:eGFP embryos was injected with the indicated MO (1.5 ng). Embryos were cultured to stage ~23 and imaged for eGFP expression and tracer dye; (D) In the Wnt reporter line, Cas9 protein (1ng) and rac1 gRNA (300pg) were co-injected in one anterior blastomere of 4-cell stage embryos, or Rac1 MO (1.5ng) was injected in one anterior-dorsal blastomere of 8-cell stage embryos. Injected embryos were cultured to ~stage 13 and imaged for eGFP expression and tracer dye. (E) The same batch of embryos from (B,C) was cultured to stage 46 with very few survived and imaged for eGFP expression which is detectable in branchial cartilage (bc), the olfactory epithelium (oe), and the brain (br). Embryos are imaged in dorsal (B, C, D) or ventral (E) view with anterior on top; CT, control; n, number of embryos. **P<0.01, ***P<0.001 (chi-squared tests).

We have shown in the previous section that AKT activity is required for NC induction, which is very likely mediated by canonical Wnt signaling, whereas the specific member(s) of the AKT family required for NC induction is not identified. In most vertebrates other than amphibians, there are three AKT family members, and at least two of them have been shown to play important roles in NC development in humans (Hoxhaj & Manning, 2020). Our bioinformatics analyses indicate that the *X. tropicalis* genome encodes only two AKT family members,

AKT1 and 2. The expression of *akt1* is not detectable at mid- to late gastrula stages, when NC is induced, and is limited to the inner neural plate at early neurula stages in *Xenopus* embryos (Z. Zhang et al., 2017). By contrast, *akt2* is abundantly expressed during NC induction and is localized to the NB at early neurula stages (Wen et al., 2010; Z. Zhang et al., 2017). Moreover, *X. tropicalis* AKT2 shares high sequence homology with all three human AKTs (81%, 87%, and 77% identical to human AKT1-3, respectively), suggesting that it is likely the primary homolog that is responsible for inducing the NC. Thus, *X. tropicalis* may serve as a simplified model for studying AKT function in NC induction. We have obtained MO and gRNAs for AKT2 and injected them into *snai2*:eGFP transgenic embryos which both showed reduced eGFP expression at stage 23 (Fig. 11B,C). Similar to embryos with loss of Rac1, the injected embryos suffer severe developmental defects with very few embryos surviving to later tadpole stage, while the survived ones have reduced head cartilages and impaired anterior craniofacial structures on both sides, suggesting craniofacial morphogenesis defects caused by loss of AKT can probably penetrate to the un-injected side (Fig 11E). However, the few stage-46 tadpoles are not representative of the phenotypes due to limited sample numbers and the phenotypical pre-selection along development, as only the tadpoles with relatively mild defects survived.

Further mechanism characterization hasn't been conducted by the time of this dissertation due to the catastrophic loss of *Xenopus* colonies caused by artificial incidents. After being granted permission for colony rebuilding from NIH in early

2023, we were able to resume *Xenopus*-related research very recently and hopefully can continue working on this project.

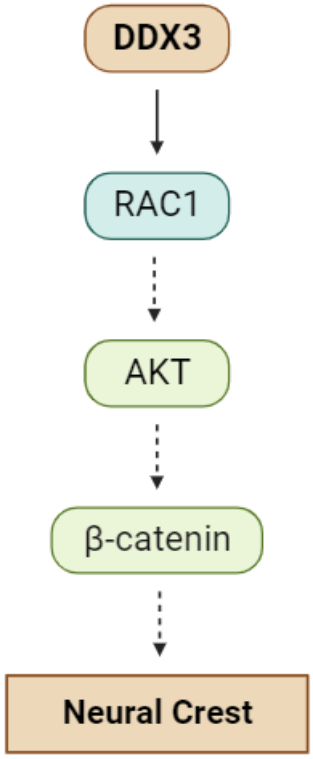

Figure 12. Diagram for the signaling cascade downstream of DDX3 in regulating NC development.
Arrows with solid lines represent potential direct regulation, while the dashed line represents possibly indirect regulation. Prepared with Biorender.

2.4 Discussion

First identified from a large-scale study on developmental disorders in 2015, *de novo* mutations of DDX3X associated with intellectual disabilities have quickly attracted a great deal of attention from neuroscience biologists (Deciphering

Developmental Disorders Study, 2015; Snijders Blok et al., 2015; X. Wang et al., 2018; Lennox et al., 2020; Hoye et al., 2022), who studied mostly on the roles of DDX3X in regulating neurogenesis in the central nervous system. As a research group that studies the NC, we noticed that most DDX3X syndrome patients also exhibit NC-related deficiencies. Based on this observation, we hypothesized that DDX3X plays a role in NC development which was tested and validated using the *X. tropicalis* model. We also proposed that DDX3 promotes NC induction through the Rac1-AKT- β-catenin axis (Fig. 12), which is dependent on its RNA helicase activity.

Whether the regulation of Wnt/β-catenin signaling by DDX3 is dependent on its RNA helicase activity is controversial. As (Cruciat et al., 2013) suggested a helicase-independent mechanism for the activation of Wnt/β-catenin signaling through CK1, pathogenic mutations of DDX3X with impaired helicase activity have been shown to have decreased ability to activate Wnt/β-catenin signaling in the zebrafish model (Snijders Blok et al., 2015). We showed that WT human DDX3X can restore the expression downregulation of both the NC marker gene and the Wnt target gene caused by DDX3 KD, but did not observe sufficient rescue when we used the helicase dead mutant of human DDX3X, suggesting an RNA helicase-dependent regulatory mechanism.

As DDX3X is required to promote the translation initiation of selected mRNAs with highly structured 5’ UTR and *rac1* mRNA is one of them which has been shown to have DDX3X binding on its 5’ UTR (H.-H. Chen et al., 2015, 2016), we

showed the DDX3 KD in *X. tropicalis* embryos downregulates RAC1 protein but not mRNA, and co-injection of *rac1* mRNA without its 5' UTR can rescue the craniofacial defects caused by loss of DDX3. Both observations suggested that the function of DDX3 in NC induction is mediated by RAC1. Mutations in *RAC1* cause NC defects in humans, and *rac1* mRNA is expressed in the NC cells in *Xenopus* embryos (Lucas et al., 2002; Reijnders et al., 2017). RAC1 is implicated in NC induction, as dominant-negative RAC1 inhibits while constitutively-active RAC1 promotes *snai2* expression during the induction of NC in *Xenopus* embryos (Broders-Bondon et al., 2007), strongly implying its regulation on NC development. To address this more directly and specifically, we applied LOF experimental approaches and observed eGFP expression reduction and craniofacial defects in the *snai2:eGFP* transgenic embryos with loss of RAC1. What needs to be noted is that RAC1 has essential functions in gastrulation (Sugihara et al., 1998; Migeotte et al., 2011), which could potentially explain the severe developmental defects we observed in embryos with loss of RAC1. Based on our current results, we cannot jump to the conclusion that the NC defects we observed with loss of RAC1 are only restricted to the NC lineage, without considering the possibility of secondary effects caused by gastrulation defects. Additional validation of the proper formation of the three germ layers during gastrulation needs to be conducted to confirm that the phenotype caused by loss of RAC1 is a bona fide NC effect.

Previous studies showed that AKT activation is required for NC development in zebrafish (Ciarlo et al., 2017), while aberrant upregulation of AKT activity results in overgrowth of NC derivatives in humans and mice development (Rivière et al., 2012; Akgumus et al., 2017; Yang et al., 2018), suggesting the role of AKT in promoting NC. Consistently, we showed that inhibition of AKT activity by pharmaceutical drug treatment or overexpression of dominant-negative AKT inhibits NC induction and endogenous Wnt signaling. In *X. tropicalis*, we show that one member of the AKT family, AKT2 is required for NC development as loss of AKT2 mediated by MO KD or CRISPR KO results in reduced eGFP expression in the *snai2:eGFP* transgenic embryos and craniofacial defects. Although Geary and LaBonne has shown that aberrant activation of PI3K inhibits NC lineage differentiation using pluripotent cells isolated from the animal pole of the blastula (Geary & LaBonne, 2018), which is not strictly considered as *in vivo* study, we have also found that ectopic overexpression of DDX3X can reduce the expression of NC markers (data not shown), suggesting that hyper-activation of AKT might also inhibit NC formation. Last but not least, similar to RAC1 morphants, the embryos with loss of AKT2 suffered from severe early embryonic developmental defects, which is not surprising as altering AKT signaling in zebrafish impairs cell movement and gastrulation (Finkielsztein & Kelly, 2009; Yeh et al., 2011). Again, we need to conduct additional validation for proper formation of the three germ layers during gastrulation with the loss of AKT2.

Chapter 3

DDX3X SUPPRESSES DDX3Y EXPRESSION THROUGH DIVERGENT MECHANISMS IN VARIOUS CELL LINES

3.1 Introduction

As mentioned above, DDX3X syndrome is a genetic disease caused by *de novo* mutations of the DDX3X gene that link to neurodevelopmental disorders and NC-related defects. Although 39 missense mutations were identified among 52 individuals by 2020 (Lennox et al., 2020) with the number still growing (*DDX3X Foundation*, 2023), limited characterization of individual missense mutations has been performed. There is a compelling need for the characterization of individual mutations, which will help better understand the pathology of individual patients. As our lab has previously demonstrated that DDX3 induces NC by promoting AKT activity and Wnt/ β-catenin signaling, how individual missense variants alter the signaling cascades downstream of DDX3X and how that relates to the pathology of NC impairments remains unclear. Therefore, we hypothesized that pathogenic mutants associated with DDX3X syndrome disrupt NC development through AKT activity and Wnt/β-catenin signaling. Ultimately, we want to generate CRISPR/CAS9 mediated knock-in (KI) of the variants in cultured human cells to model the molecular variation in patients and test our hypothesis. We started with trying to KO DDX3X in human cells mediated by CRISPR/CAS9 gene editing and planned to employ the KO cell line in screening the missense mutants for

corresponding variations on downstream signaling pathways via exogenous expression. We encountered a sea of troubles with DDX3X KO and thought we got a complete KO clone for a short moment but ended with obtaining two mutants without complete depletion of DDX3X. This unfortunate outcome is not very surprising as prediction using DepMap/CRISPR suggested that DDX3X is an essential gene in a lot of cell lines including the one we selected (*DDX3X DepMap Gene Summary*, 2023).

When we were picking cell lines to manipulate in 2021, our knowledge of DDX3Y was still limited to that it only functions in the male reproductive system, and choosing a male-derived cell line would leave us only one copy of DDX3X to KO which is easier to achieve. Our project diverged in a completely distinct path since then as a profit from the misfortune, cause DDX3Y might be critical for the sex dimorphism of DDX3X associated diseases. In the neurodevelopment disorders linked with *de novo* DDX3X mutations, there are many more female patients identified than male, with the mechanism unknown. Since male patient has only one copy of DDX3X, we first speculated that LOF mutation in DDX3X might leads to embryonic lethality, as mice with DDX3X KO cannot survive. However, very recently, it has been reported the DDX3X and DDX3Y share functionally redundancy in regulating translation of targeted mRNAs (Venkataramanan et al., 2021). What's more, although the female mice with cKO of both DDX3X copies have much smaller cortex than normal, male mice with cKO of the only one copy of DDX3X have normal sized cortex, unexpectedly

(Hoye et al., 2022). With the embryonic lethality theory being challenged, we leant towards an alternative explanation that DDX3Y might compensate for the loss of DDX3X resulting in rescued phenotypes. First identified in lymphoma cell lines, the compensatory expression of DDX3Y could be triggered by loss of DDX3X and contribute to lymphomagenesis (Gong et al., 2021). Here we further investigated the mechanisms of how loss of DDX3X induces DDX3Y expression in various human cell lines.

3.2 Experiment Methods

Reagents

Full-length cDNA clones for human *DDX3Y* (Accession: BC034942) were purchased from GE-Dharmacon. The cDNA for human DDX3Y was subcloned into a pCS2+ expression vector with an in-frame HA tag. Pharmacological inhibition of transcription was performed with Actinomycin D (Sigma-Aldrich A1410). Antibodies that were used in this study include mouse anti-DDX3X (Santa Cruz sc-81247, 1:1000), rabbit anti-DDX3X (Bethyl Laboratories A300-474A, 1:1000), rabbit anti-DDX3X (Aviva OAAB01241, 1:1000), rabbit anti-HA (Cell signaling C29F4, 1:2000), rabbit anti-GFP (Invitrogen A-11122,1:1000) and custom-made rabbit DDX3Y antibody acquired from Dr. Nicholas Grigoropoulos (1:2000) (Gong et al., 2021).

Cell culture and transfection

U87-MG cells (ATCC) were cultured in MEM (Corning) supplemented with 10% fetal bovine serum (FBS Gibco) at 37°C with 5% CO2. HCT-116 cells (ATCC) were cultured in McCoy's 5A (ATCC) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C with 5% CO2. Cells were transfected with 10nM DDX3X, DDX3Y or control siRNA (Dharmacon J-006874-06-0002, L-011904-01-0005, and D-001810-01-05) at 50% confluency using Lipofectamine RNAiMAX (Invitrogen), or with 40ng/ml plasmids at 70% confluency, using Lipofectamine 3000 (Invitrogen). For experiments that used both siRNA and plasmid, siRNA was transfected first, the media were changed and the plasmid was transfected on the following day.

CRISPR/CAS9 mediated gene editing

Small guide RNAs (sgRNAs) were designed to target exon 5 or exon 1 of the DDX3X gene using CRISPRscan. Protospacers DDX3X-g1(5'-CGTGGACGGAGTGATTACGA-3') and DDX3X-g2 (5'-TGGCAGTGGAAAATGCGCTC-3') were cloned into a pSpCas9(BB)-2A-GFP/PX458 expression vector (Addgene 48138) using a scarless golden gate cloning strategy as described (Ran et al., 2013). Plasmid was transfected in U87-MG cells at 500ng/ml for 48 hours followed by Fluorescence-activated Cell Sorting (FACS) to sort GFP-expressing cells and plated them individually into 96 well plates. Clones were cultured in MEM supplemented with 20% FBS and expanded for 2-3 months to collect enough samples for sequential procedures. Genomic DNA was isolated and then PCR amplified using primers for exon 5,

TTGTGAGCTGTGTGCCGATT (forward) and TCTGTGTCTTAAAAAGTCAAGCAAA (reverse), and for exon 1, GATCTCGAGAACTCCGAGGC (forward) and GACAATACAGCGGGCCGAG (reverse), followed by PCR cloning (NEB E1202S) and sanger sequencing. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and then reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) with DNase I (Qiagen) treatment. With the cDNA as the template, PCR amplification was performed using primers for g1, CCAGCAAAGGGCGCTATATTC (forward) and CTTTTGCACTGGAGTTGGGC (reverse) and for g2, CTTCGCGGTGGAACAAACAC (forward) and CAGTTGTTGCCTGTTGCCTC (reverse), followed again by PCR cloning and sanger sequencing.

Western blotting and RT-qPCR analyses

The experimental procedures were described in the method section in Chapter 2. The following primer sets were used for the quantitative PCR: DDX3X, ACTATGCCTCCAAAGGGTGTCC (forward) and AGAGCCAACTCTTCCTACAGCC (reverse); DDX3Y, CGGCAGTAACTGTCCTCCACAT (forward) and CTGGAGTAGGACGAGTATAGCG (reverse); GAPDH, AGGGCTGCTTTTAACTCTGGT (forward) and CCCCACTTGATTTTGGAGGGA (reverse); 18s rRNA, CCCTATCAACTTTCGATGGTAGTCG (forward) and CCAATGGATCCTCGTTAAAGGATTT (reverse). Relative quantity was calculated

by normalizing to the amount of GAPDH mRNA for most of the experiments except for Actinomycin D assay which was normalized to the abundance of 18s rRNA.

3.3 Results

Generation of DDX3X KO human cell line: a tortuous journey with inspiring hints

To generate a DDX3X KO human cell line, we took advantage of the non-homologous end joining (NHEJ) mechanism in CRISPR/CAS9 mediate gene editing via introducing a single guide to the cells without a repair template. After the double-strand cleavage by CAS9 at the targeted locus in the genome, NHEJ-mediated repair will potentially create indel mutations leading to premature stop codon to inactivate the gene. Two sgRNA target sequences were designed for separate experiments and subcloned to PX458, a plasmid containing all components required for CRISPR gene editing, including CAS9 from S. pyogenes with nuclear localization signal (NLS) and 2A-eGFP, and cloning backbone for sgRNA. The plasmid was transfected into U87-MG cells and then sorted for EGFP-positive cell clones (Fig. 13A). Although the sgRNAs have high efficiency in creating idel mutations suggested by initial screening, we only got three clones expanded from three 96-well plates and identified that two of them have DDX3X successfully edited in the genome sequence. For the first clone (clone-1), genotyping results suggested that it has a long insertion on targeted

exon (exon 5) instead of short indel mutations, which resulted in 159 nucleotides deletion on the mRNA sequence corresponding with the entire exon 5, very likely mediated through altered splicing (Fig. 13C). Consistently, western blotting with the cell lysates indicated that the protein is slightly smaller in size compared with WT DDX3X in the parental cells, confirming that this clone has deletion mutant of DDX3X (Fig. 13D). For clone-2, there is 14-nucleotides-deletion on exon 1 leading to the same deletion on the mRNA sequence, resulting in a frameshift and early stop codon with the protein only having 20 amino acids (Fig. 13E). Immunoblotting with a DDX3X specific antibody acquired from Bethyl laboratory showed that the DDX3X band completely disappeared for clone-2, confirming that this clone has DDX3X depleted (Fig. 13F). However, when we used another DDX3X antibody acquired from Santa Cruz, we were able to see a faint band with comparable size as DDX3X protein (Fig. 13F). The difference between the two antibodies is that the former was raised against DDX3X between Met1-Lys50 as the immunogen, while the latter was raised against a longer fragment of the N-terminus with the sequence not disclosed by the company. Further validation with a third antibody raised against the C-terminus of DDX3X confirmed the existence of the band (data not shown). As DDX3Y is probably the only DDX3 homolog that shares high protein sequence identity with DDX3X in humans, with more similar sequence on the C-terminus than the N-terminus, we speculated that the band was compensation from DDX3Y based on what I mentioned previously in the DDX3Y section of Chapter 1, that DDX3Y potentially

compensate for the loss of DDX3X in mice brain or human lymphoma cells (Patmore et al., 2020; Gong et al., 2021; Hoye et al., 2022).

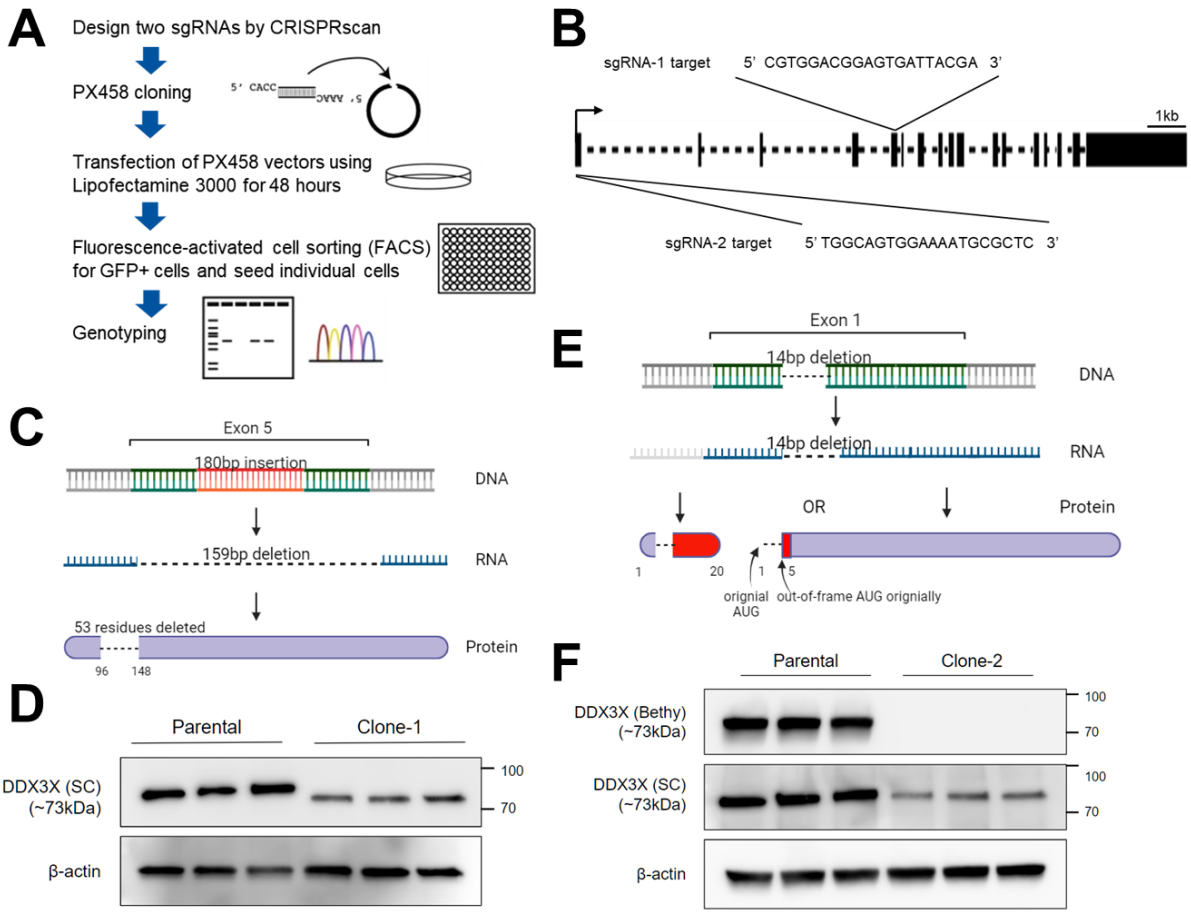

Figure 13. CRISPR/Cas9 mediated gene editing of DDX3X in U87-MG cells. (A) Overview of experimental steps for design, construction, transfection, single cell sorting and validation. (B) Design of sgRNAs targeting *DDX3X*. Exons were shown in solid black squares while introns in dashed lines. (C, E) Schematic representation of the genotyping results for clone-1 and clone-2, respectively. DNA: intron in grey, exon in green, insertion in red, deletion shown with dashed line; RNA: coding sequence in blue, 5' UTR in grey, deletions shown with dashed line; Protein: WT sequence in purple, deletion shown with dashed line, substitution in red. (D, F) Parental U87-MG cells and clone-1 (D), or clone-2 (F) were collected (in biological triplicates) for western blotting, followed by probing with indicated antibodies.

To further validate if the band represents DDX3Y protein, we requested a DDX3Y-specific antibody from our collaborator as no commercially available antibody is specific enough to differentiate between DDX3X and DDX3Y proteins. Meanwhile, we applied an alternative approach to target it with a DDX3Y specific siRNA, but the protein was not downregulated with DDX3Y KD but only when I used a DDX3X specific siRNA, suggesting that it is more likely DDX3X instead of DDX3Y (Fig. 14A) The DDX3Y-specific siRNA has been tested to work fine with successful knockdown of exogenously expressed DDX3Y. After receiving the DDX3Y-specific antibody, we further confirmed that endogenous DDX3Y protein is not detectable even though the cell line was derived from a male patient (Fig. 14B). To characterize the DDX3X expression leakage, we went back to the DNA sequence and searched for open reading frames (ORFs) using an ORF finder. Besides the ORF starts from the same ATG as WT DDX3X, there is a second ORF that starts from an originally out-of-frame ATG which locates 4 nucleotides downstream of the first ATG and 7 nucleotides upstream of the CRISPR-mediated deletion, which would have the sequence downstream of the deletion back in-frame. How the ribosome ignores the first AUG and starts translation from a downstream AUG, especially in different reading frames, remains a big mystery that cannot be explained by our current knowledge of molecular biology. Besides the unknown mechanism, we believe that the translation started from the downstream AUG impaired the translation efficiency possibly due to the presence of an upstream AUG, as the expression level was significantly reduced compared

with the WT DDX3X in parental cells. This unique circumstance will potentially expand our understanding of CRISPR/CAS9 mediate KO using a single guide RNA as well as the limitation of our knowledge in eukaryotic translation.

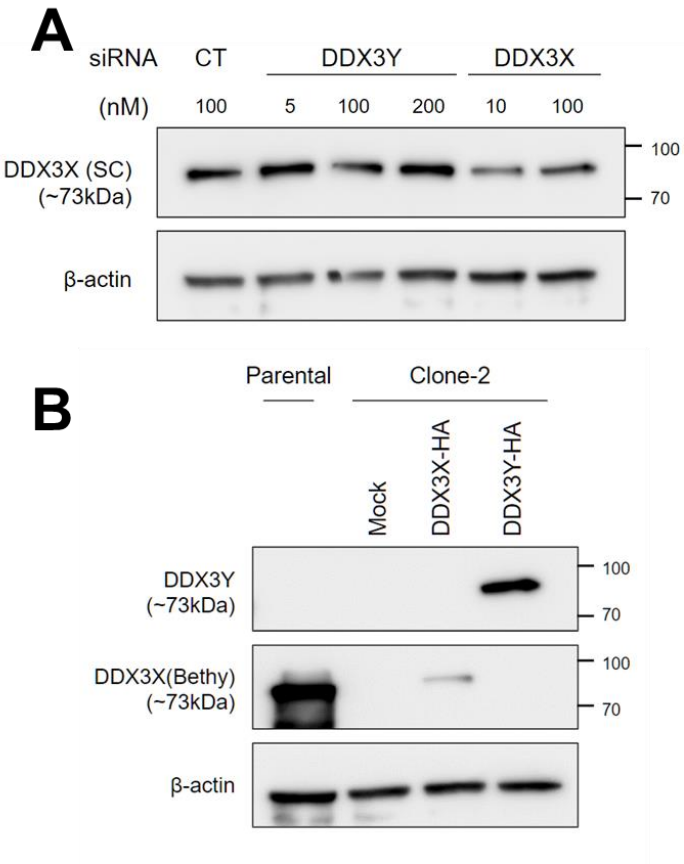

Figure 14. Clone-2 more likey has expression of DDX3X but not DDX3Y. (A) Clone-2 cells were transfected with indicated siRNAs followed by western blotting, probing for indicated antibodies.(B) Untreated parental U87-MG cells, and clone-2 cells that were transfected with either PCS2+ empty vector, PCS2+ harboring HA tagged DDX3X or DDX3Y, were collected for western blotting, followed by probing with indicated antibodies.

Loss of DDX3X promotes DDX3Y expression through protein-level regulation in U87-MG cells

Originally, we picked U87-MG cells to conduct the KO experiment as it is a male-derived cell line with only one X chromosome and a single copy of DDX3X to manipulate. As our knowledge about DDX3Y beyond spermatogenesis has enriched quite a lot in the past two years, even DDX3Y protein expression has been reported to be restricted to the reproductive system in normal human tissues, it might not be the case in abnormal conditions including DDX3X depletion or LOF. As I mentioned in the previous section endogenous expression of DDX3Y was not detected in U87-MG cells. However, we observed increased expression of exogenous DDX3Y in clone-2 compared with parental cells with around 7-fold difference in protein level between the two conditions (Fig. 15A, B). As the minimal amino acid sequence change on the N-terminus (2-11) hasn't been indicated by any study to have critical functions, we believe that DDX3Y expression is more likely promoted by the protein level reduction of DDX3X. To test this, we knockdown DDX3X in parental U87-MG cells by siRNA transfection to only reduce its protein level, and we observed more exogenous DDX3Y expression with DDX3X KD than control (Fig. 15C). Although there was only 2-fold change (Fig. 15D) which is less remarkable compared to what we saw with clone-2, the regulation is at least partially mediated by DDX3X protein level.

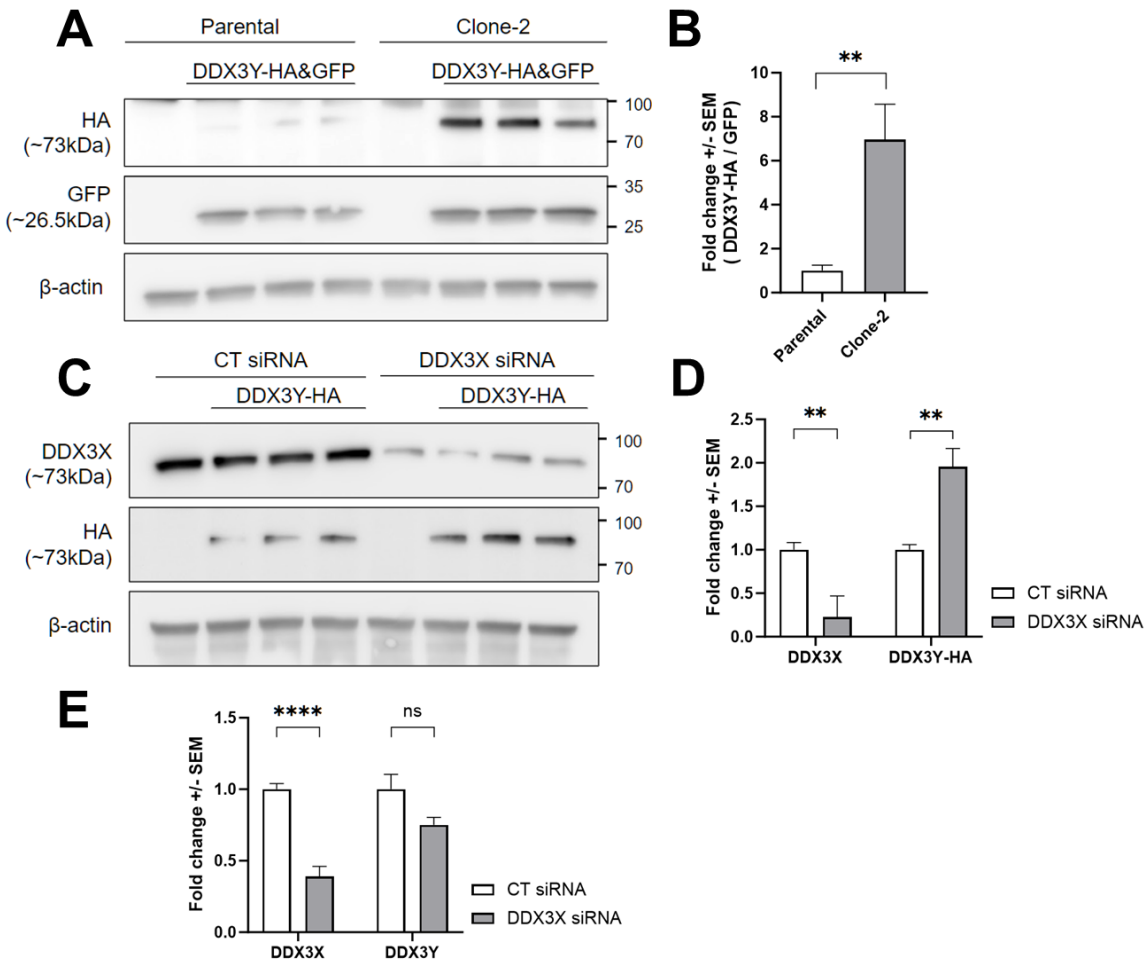

Figure 15. Protein level downregulation of DDX3Y caused by loss of DDX3X in U87-MG cells.

(A) Parental U87-MG cells and clone-2 cells were untreated or transfected with PCS2+ harboring HA tagged DDX3Y and co-transfected with PCS2+ GFP for transfection efficiency control (in biological triplicates), followed by western blotting, probing for indicated antibodies. (B) Density of the target protein was normalized against that of GFP. (C) Parental U87-MG cells were firstly transfected with indicated siRNAs followed by media change and transfection with PCS2+ DDX3Y-HA (in biological triplicates), and then collected for western blotting and probing with indicated antibodies. (D) Density of the target protein was normalized against that of β-actin. (E) The same batch of cells from (C) was processed for RT-qPCR with primers targeting indicated mRNAs. Fold change is

shown in mean +/- SEM; **P<0.01, ****P<0.0001, ns, not significant (unpaired student t-tests).

Our next question to answer is whether the regulation is through the mRNA level or directly on the protein level only. In mouse brains with depletion of DDX3X, upregulation of DDX3Y in mRNA level was observed (Patmore et al., 2020; Hoye et al., 2022), suggesting potential regulation of mRNA. To evaluate the mRNA level change of DDX3Y, we performed RT-qPCR with primers for DDX3Y using the same batch of cells transfected with siRNA followed by exogenous expression of DDX3Y, with the results reflecting both endogenous and exogenous levels of DDX3Y transcripts. Compared with endogenous DDX3Y mRNA detected in untreated U87-MG cells, the combined endogenous and exogenous DDX3Y mRNA in cells with DDX3Y plasmid transfection is about 300-fold more (data not shown). So the results would be more representative of the exogenous DDX3Y. We observed only a minimal decrease in mRNA level and it's not statistically significant (Fig 15E), indicating that DDX3X regulates DDX3Y primarily through post-transcriptional mechanisms in U87-MG cells. In lymphoma cells, DDX3X suppresses DDX3Y translation dependent on its 5'UTR (Gong et al., 2021). However, the expression construct harboring DDX3Y that I transfected into the cells doesn't have 5'UTR or 3'UTR sequence, so if the regulation is mediated by translational control, it is more likely through translation elongation. Alternatively, another possibility is post-translational regulation which needs to be further tested.

Loss of DDX3X promotes DDX3Y expression through mRNA level regulation in HCT-116 cells

As endogenous DDX3Y protein was not detected even though U87-MG cells are derived from a male patient, I screened various male-derived cell lines and observed endogenous DDX3Y expression in HCT-116 cells, which is a colorectal carcinoma cell line, using custom-made DDX3Y antibody. siRNA mediated knockdown of DDX3X in HCT-116 cells upregulated DDX3Y in both protein level and mRNA level, with 1.5-fold and 2-fold change respectively (Fig. 16A, B, C), suggesting a more predominant effect on the mRNA level, which is consistent with the upregulation of DDX3Y mRNA in mice brain with loss of DDX3X (Patmore et al., 2020; Hoye et al., 2022). What remains unknown is at which step of RNA metabolism DDX3X downregulates DDX3Y mRNA level, as DDX3X has been shown to function in almost all aspects of RNA metabolism, including transcription, nuclear export, stability regulation, etc. We started with evaluating if mRNA stability got influenced as it's easier to test than the others. After siRNA transfection for three days, the cells were treated with actinomycin D, a transcription inhibitor that binds DNA at the transcription initiation complex to prevent elongation of the RNA chain (Sobell, 1985). After drug treatment, collection of cells at different times was performed subsequently for RNA isolation, followed by RT-qPCR to visualize the mRNA degradation curve (Fig. 16D). Based on the plot, DDX3Y mRNA got degraded slower in cells transfected with DDX3X siRNA compared to control siRNA transfected, especially within 8-

hour treatment, suggesting that DDX3Y mRNA is more stable with loss of DDX3X (Fig. 16E). Then I repeated the experiment in biological triplicates with only 8-hour treatment of actinomycin D and confirmed the statistical significance (Fig. 1F). Besides, I also conducted the experiments in biological triplicates with other times including 4 and 12 hours which has no significant difference between the two conditions (date not shown). As a result, we were not fully convinced that DDX3X suppresses DDX3Y expression only through destabilizing the stability of its mRNA, because of the insignificance with 4 and 12 hours of drug treatment, and the subtle difference observed with 8 hours of drug treatment. As DDX3X has been reported to regulate transcription through interaction with promoter, we speculated that transcriptional suppression might also contribute to the phenotype, which needs to be tested further.

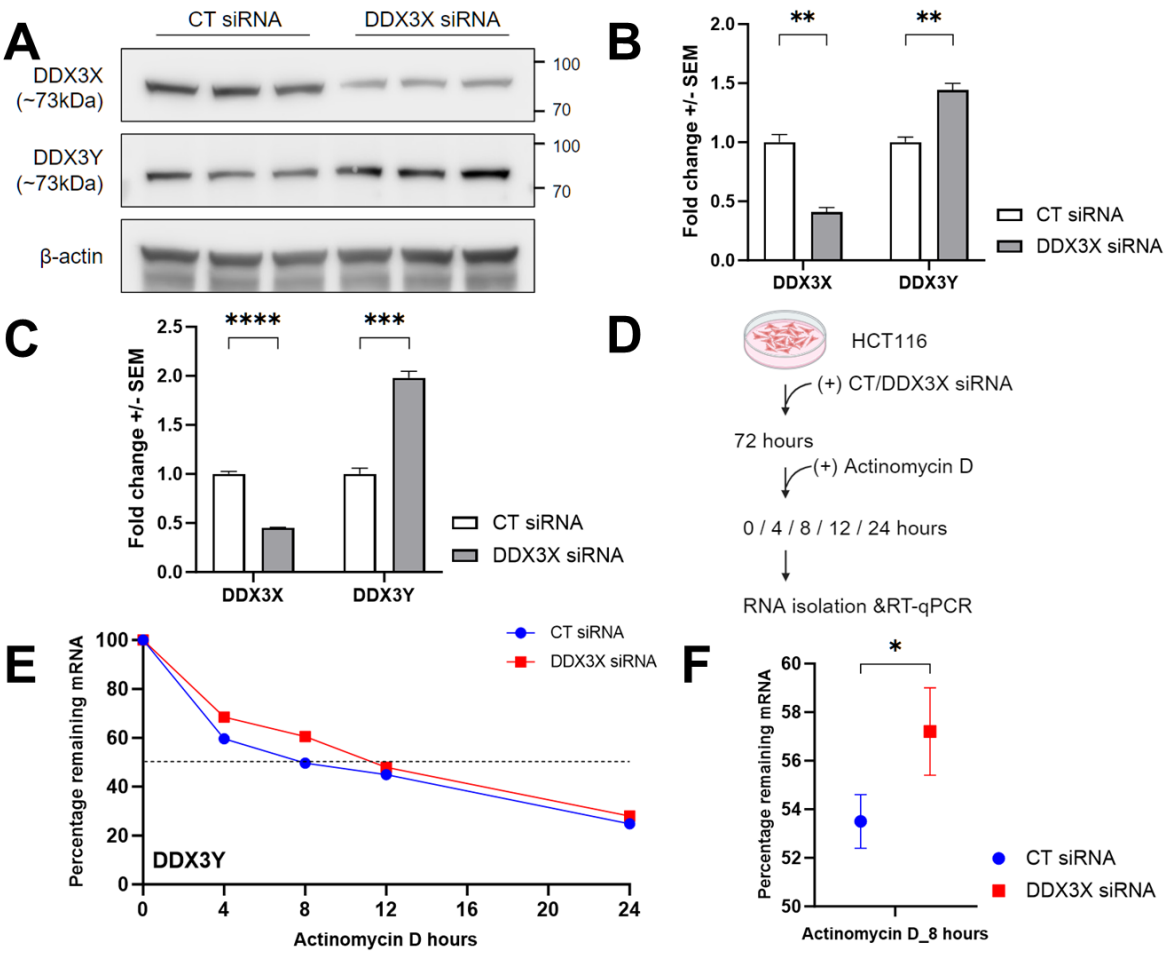

Figure 16. Transcript level downregulation of DDX3Y caused by loss of DDX3X in HCT116 cells.

(A) HCT116 cells were transfected with indicated siRNAs (in biological triplicates), followed by western blotting, probing for indicated antibodies. (B) Density of the target protein was normalized against that of β-actin. (C) The same batch of cells from (A) was processed for RT-qPCR with primers targeting indicated mRNAs. (D) Overview of experimental steps for the mRNA degradation assay with Actinomycin D treatment. (E) Degradation curve of DDX3Y mRNA. Percentage remaining mRNA was calculated by the relative mRNA amount at indicated treatment time divided by that of 0 hour, and then multiplied by 100. (F) Repeat the experimental steps in (D, E) with only 8-hours treatment in biological

triplicates. Results show in +/- SEM. **P<0.01, ***P<0.001, ****P<0.0001 (unpaired student t-tests).

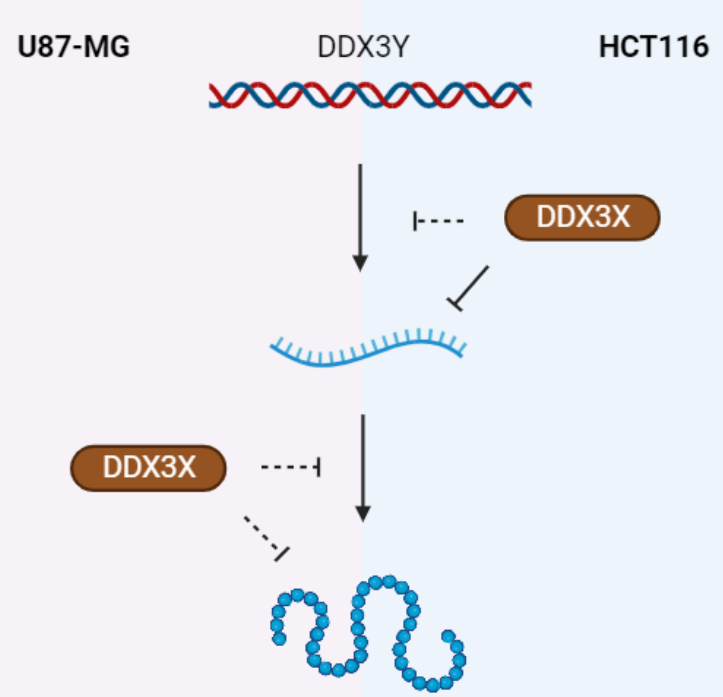

Figure 17. Our model for the divergent mechanisms of DDX3X suppressing DDX3Y expression in separate cell lines.

Prepared with Biorender.

3.4 Discussion

There is clearly a sex difference in the incidence rate of DDX3X-associated diseases, with more females in DDX3X syndrome patients while more males in Burkitt Lymphoma patients harboring DDX3X mutations (Snijders Blok et al., 2015; Gong et al., 2021), and there are no clear explanations of the mechanism so far. As complete depletion of DDX3X is embryonic lethal in mice, the widely

accepted explanation for the sex bias in DDX3X syndrome is that male patients with inactivating mutations in DDX3X suffer embryonic lethality, implying that human DDX3Y cannot functionally compensate DDX3X. However, it has been reported that DDX3X and DDX3Y are functionally redundant in protein synthesis (Venkataramanan et al., 2021). What's surprisingly unexpected is that cKO of the single copy of DDX3X in male mice does not change the cortical size significantly, while cKO of both copies in female mice impairs neurodevelopment leading to profound microcephaly (Hoye et al., 2022). Even through there is one more DDX3 homolog in mice (Chang & Liu, 2010), it's located on the autosome and should not be associated with sex dimorphism in related diseases. The mRNA level of DDX3Y is upregulated in those cKO male mice (Hoye et al., 2022), implying potential compensate from DDX3Y. As a result, we speculated that an alternative explanation for the sex bias in DDX3X syndrome is that the male patients have compensation from DDX3Y and exhibit less severe symptoms so that the carriers won't be identified. This theory is further supported by that loss of DDX3X triggers aberrant expression of DDX3Y in protein level in transformed B cells, and also the fact that DDX3Y protein is barely detectable in normal B cells but shows ectopic expression in various lymphoma cell lines (Gong et al., 2021). Even though the sex difference in Burkitt Lymphoma is the opposite, the compensation from ectopic DDX3Y expression restores the protein synthetic capacity in established lymphoma cells, instead of helping to alleviate the disease severity that we speculated in DDX3X syndrome. Based on this, we

hypothesized that DDX3X inhibits DDX3Y expression. Here, using two cultured human cell lines, U87-MG and HCT116, we showed that loss of DDX3X induces expression of DDX3Y, consistent with the previous findings.

With almost absence of mechanistic studies on how DDX3X suppresses DDX3Y expression, the only hint is that it could be mRNA level regulation as loss of DDX3X upregulates DDX3Y mRNA in mice brains. Although the encoded proteins share 88% identity, mouse DDX3Y mRNA is quite different from human DDX3Y mRNA in both the coding sequence and the non-coding region, especially the 5' UTR. As a result, cultured human cells might be a better system to model the potential mechanism of DDX3Y compensation in humans. Here, in human HCT116 cells, we observed both mRNA and protein level upregulation upon DDX3X KD, with a more predominant change at the mRNA level, mediated at least partially by the regulation of mRNA stability. As the effect on mRNA stability is quite subtle, we thought there might be additional regulatory mechanisms and speculated transcriptional regulation as DDX3X has been shown to modulate transcription through interaction with promoter-binding proteins (Chao et al., 2006; D.-W. Wu et al., 2014, p. 53, 2016). Further characterization of potential transcriptional regulation will be conducted using nascent RNA labeling techniques.

What is more interesting is the novel protein level regulation identified in U87-MG cells, which was first reported by us in this study. Here we showed that DDX3X reduction in both the CRISPR edited clone and DDX3X siRNA transfected cells,

promotes the protein level expression of DDX3Y but not its mRNA level expression, which is validated by western blotting and RT-qPCR, respectively. That suggested a post-transcriptional regulatory mechanism, which could be translational regulation or post-translational regulation. If it's translational regulation, as the construct harboring DDX3Y does not contain any non-coding region of DDX3Y mRNA, it is most likely mediated by regulating translation elongation. If the regulation is somehow post-translational, we will need to determine if the stability of DDX3Y protein is altered upon loss of DDX3X. I have invested quite some time characterizing the protein stability and we have confirmed that DDX3Y protein could be degraded by the proteasome in U87-MG cells, as it is downregulated overtime upon cycloheximide treatment and upregulated upon MG132 treatment (data not shown). As control treatment with DMSO could also protect DDX3Y protein from being degraded which brings the experiment to a standstill, we figured very recently that we could apply alternative solvents like ethanol instead of DMSO and will try this out soon.

Chapter 4

ADAM9 INHIBITS FOXO SIGNALING THROUGH AKT ACTIVITY

4.1 Introduction

Previously, our lab reported that *Xenopus* ADAM13 is required for the activation of canonical Wnt signaling and the induction of NC and eye field, mediated by the forward ephrin-B signaling (Wei et al., 2012, 2010). The Eph receptors are the largest family of receptor tyrosine kinases (RTKs) that interact with ephrin ligands on neighboring cells or the same cell, and they can be divided into two subclasses: EphA receptors, which bind the ephrin-A ligands, and EphB receptors, which bind the ephrin-B ligands, with a few exceptions (Hwang & Daar, 2017; Taylor et al., 2017). The Eph-ephrin interactions can produce bi-directional signals, as the forward signaling involves the activation of Eph receptors by ephrin ligands, while the reverse signaling may happen in the ligand-expressing cell upon the interaction (Hwang & Daar, 2017; Taylor et al., 2017). *Xenopus* ADAM13 cleaves ephrin-B1 and -B2, the two cell-surface ligands for EphB receptors, and this cleavage is required for the activation of canonical Wnt signaling and induction of the NC (Wei et al., 2010). In mammals, the candidate metalloprotease that is responsible for cleaving ephrin-B1 and -B2, and functionally substitutes for *Xenopus* ADAM13 in NC induction, is ADAM9 (Chandrasekera et al., 2022). As forward ephrin signaling can suppress AKT signaling (G. Li et al., 2012; Lisabeth et al., 2013), our lab suggested that

ADAM9 promotes AKT activity through inhibition of forward ephrin-B signaling (Chandrasekera et al., 2022). The downstream pathways of AKT signaling were also altered upon loss of ADAM9, including the canonical Wnt signaling and mTOR signaling (Chandrasekera et al., 2022).

To assess what signaling pathways are potentially regulated by ADAM9, we performed RNA-seq analysis and cellular proteomic analysis with or without ADAM9 knockdown in HCT116 cells to comprehensively understand how ADAM9 regulates intracellular signaling at both the transcriptome and the proteome level. Based on the pathway enrichment analysis, the AKT signaling pathway, together with its downstream signaling nodes mTOR and FoxO, were significantly enriched in the differentially expressed genes (DEGs) in the RNA-seq analysis. Here we investigated how ADAM9 regulates FoxO signaling, a major tumor suppressor pathway, which might contribute to the oncogenic activity of ADAM9.

4.2 Experimental Methods

Reagents

The antibodies used in this study include rabbit anti-ADAM9 (Cell Signaling Technology D64B5, 1:1000), rabbit anti-phospho-AKT(S473) (Cell Signaling Technology D9E, 1:1000), rabbit anti-FoxO3 (Cell Signaling Technology 75D8, 1:1000 for western blotting, 1:200 for immunocytochemistry). Secondary antibodies that were used include HRP-conjugated goat anti-rabbit (Cell signaling 7074, 1:10,000) and horse anti-mouse (Cell signaling 7076, 1:10,000). HRP-

conjugated mouse anti-β-actin (Cell signaling 8H10D10, 1:5000) was used as a loading control.

Cell culture and transfection

HCT-116 cells (ATCC) were cultured in McCoy's 5A (ATCC) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C with 5% CO2. Cells were transfected with 100nM ADAM9 siRNA-1 (AD9-1), ADAM9 siRNA-2 (AD9-2), or control siRNA (SIC) (Cell Signaling Technology 11968, Ambion 4390826, Cell Signaling Technology 6568) at 60% confluency using Lipofectamine RNAiMAX (Invitrogen) for 12 hours before medium change and then cultured for another 48 hours before collection.

Western blotting and RT-qPCR analyses

The experimental procedures were described in the method section in Chapter 2. The following primer sets were used for the quantitative PCR: FoxO3, CGGACAAACGGCTCACTCT (forward) and GGACCCGCATGAATCGACTAT (reverse); MUC13, CCTTCGGTGTGATTATTATGGC (forward) and GCATCTGGCTGTCTCTGGAG (reverse); THBS1, TGTTTGTGCAGGAAGACAGG (forward) and TTGTCAAGGGTGAGGAGGAC (reverse); GDA, GCAACAATTCACACTGACTCATC (forward) and GTGTCACTATGGGCTTCACTC (reverse); GAPDH, TCAAGGCTGAGAACGGGAAG (forward) and CGCCCCACTTGATTTTGGAG

(reverse). Relative quantity was calculated by normalizing to the amount of GAPDH mRNA.

Immunocytochemistry

HCT116 cells were cultured on Poly-L-lysine coated coverslips (Neuvitro) in 24-well plates and transfection with indicated siRNA was performed. Cells were then fixed in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) for 10 min followed by permeabilization with 0.2% Triton X-100 in PBS. After blocking with 1% BSA for 30 min, the coverslips with fixed cells were incubated in diluted FoxO3 antibody (1:100) at 4°C overnight followed by secondary antibody goat anti-rabbit Alexa Fluor 594 (Abcam 150080 1:1000) incubation for 1 h at room temperature. Nuclei were stained by DAPI (1:10000). Imaging was performed with confocal microscopy LSM 880, AxioObserver, and objective Plan-Apochromat 20x. Nuclear FoxO3 intensity was calculated by measuring the fluorescent intensity of the FoxO3 signal within DAPI stained area using ImageJ software.

4.3 Results

FoxO signaling is activated with the loss of ADAM9, likely mediated by AKT activity

Previously, our lab reported that loss of ADAM9 in colon cancer cell lines inhibits AKT activation. Consistently, with siRNA-mediated KD of ADAM9, we observed downregulation of phosphor-Ser473 AKT, the active form of AKT (Fig. 18A). As I

mentioned earlier, FoxO is one of the signaling pathways downstream of AKT, AKT-mediated phosphorylation of FoxO facilitates its nuclear export and degradation, leading to inhibition of its function as transcriptional factors (Manning & Toker, 2017). In the cellular proteomic analysis, we found that the protein level of FoxO3, the only FoxO transcription factor detectable in HCT116 cells, is upregulated upon ADAM9 KD (unpublished data). We validated the protein level upregulation of FoxO3 with western blotting after siRNA-mediated KD of ADAM9 (Fig. 18B). A similar change was observed with a second siRNA (AD9-2), confirming that this regulation is specific for ADAM9 KD (Fig. 18C). However, the mRNA level of FoxO3 didn't show significant change in the RNA-seq analysis (unpublished data), which is confirmed by the RT-qPCR result (Fig. 18D). Combining the two observations above, we believe that FoxO3 is post-transcriptionally regulated by ADAM9.

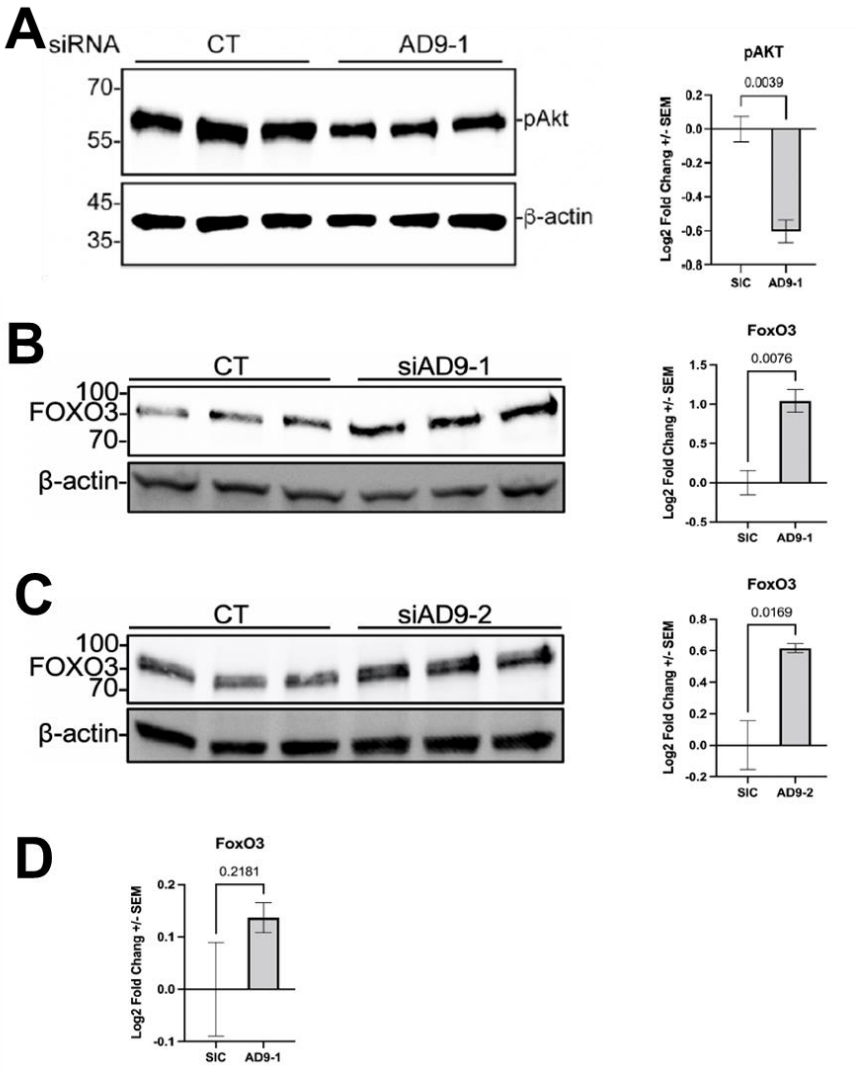

Figure 18. Loss of ADAM9 downregulates active AKT and upregulates FOXO3 protein

HCT116 cells were transfected with the indicated siRNAs (in biological triplicates), followed by western blotting, probing for phospho-S473 AKT (pAKT) (A) or FOXO3 (B, C); Density of the target protein was normalized against that of β-actin. (D) The same batch of cells from B were used for RT-qPCR to detect FoxO3 at the mRNA level. Log2 fold change is shown in mean +/- SEM; P values were calculated by unpaired student t-tests.

Even though FoxO3 is not among the DEGs in RNA-seq analysis, the FoxO signaling pathway was identified to be enriched by DEGs with ADAM9 KD, which could be a result of FoxO3 protein level change. So we compared our DEGs with the FoxO3-targeted genes identified by (Eijkelenboom et al., 2013) through genome-wide analysis via RNA polymerase II profiling, and found that the two gene lists share 21 genes in common, accounting for approximately 6% of the DEGs in our RNA-seq analysis with ADAM9 KD (Fig. 19A). We also calculated the statistical significance of the overlap by (Lund, 2010) and the p-value equals 0.006 which is considered significant. Based on this, we believe that KD of ADAM9 activates the FoxO signaling pathway. To further test this, we selected three FoxO3 target genes from the overlap, including MUC13, THBS1 and GDA, which encode Mucin 13, Thrombospondin 1 and Guanine Deaminase, respectively, and validated that their mRNA levels were indeed upregulated upon KD of ADAM9 with RT-qPCR (Fig. 19B). Our next question is whether the reduced AKT activity caused by loss of ADAM9 will promote FoxO3 nucleus localization. To address this question, we performed immunocytochemistry to look into the nuclear FoxO3, which is represented by the fluorescence intensity within the DAPI stained area, and we observed FoxO3 level upregulation in the nucleus with loss of ADAM9 than control (Fig. 19C).

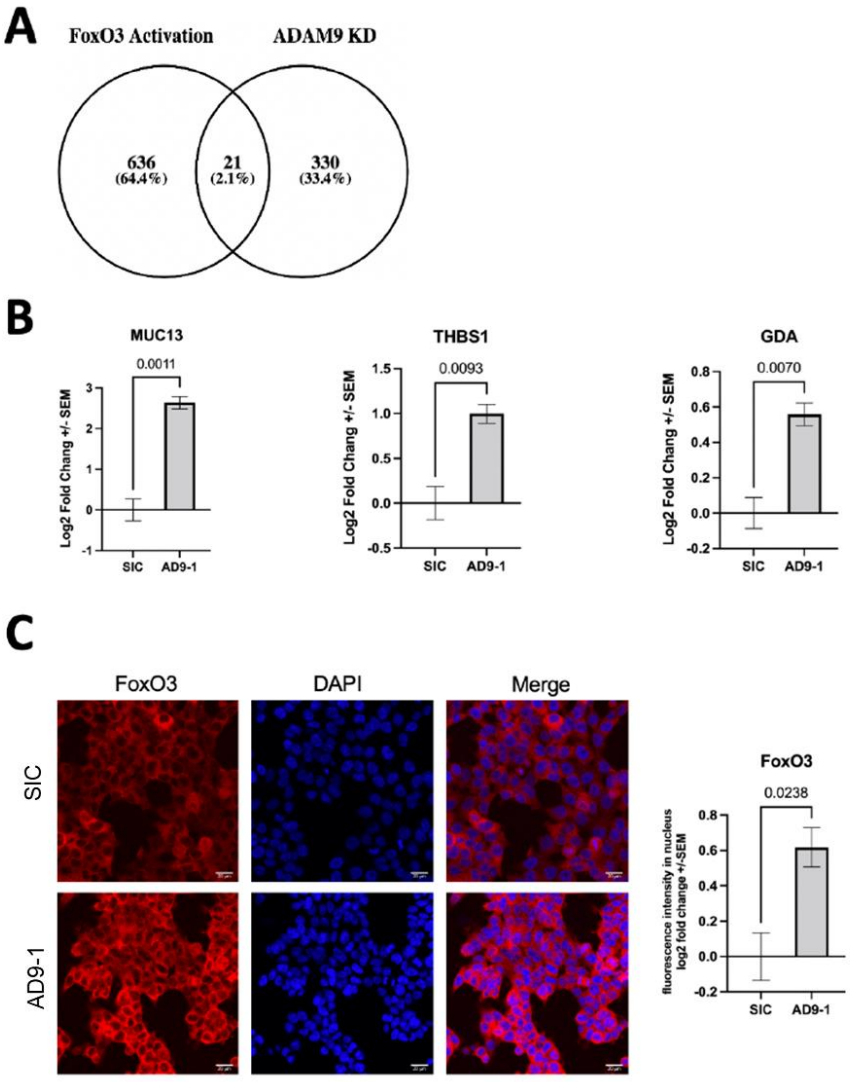

Figure 19. Loss of ADAM9 upregulates nuclear FoxO3 and promotes the expression of FoxO3-targeted genes

(A) a Venn diagram with the overlap between the RNA-seq DEGs and FoxO3 target genes identified with genome-wide analysis by (Eijkelenboom et al., 2013) with p-value = 0.006, calculated using (Lund, 2010). (B) The same batch of cells from Fig. 15 D was used for RT-qPCR to detect the mRNA levels of MUC13, THBS1 or GDA. (C) After transfection with indicated siRNAs, cells were processed for immunocytochemistry to detect nuclear FoxO3. Scale bar: 20um.

Log2 fold change is shown in mean +/- SEM; P values were calculated by unpaired student t-tests.

4.4 Discussion

ADAMs have been shown to play critical roles in modulating cell signaling pathways. The best characterized ADAMs are ADAM10 and ADAM17, as both have been implicated in NOTCH and EGFR signaling, mediated by proteolytic cleavage of NOTCH receptors and EGFR ligands, respectively (Sahin et al., 2004; Murthy et al., 2012; Toonen et al., 2016; Zbodakova et al., 2021). Despite the importance of ADAM9 in various diseases as described earlier, very little is known about the substrates and downstream targets of this protease. Here we combined the RNA-seq analysis with the cellular proteomics analysis to develop a non-biased approach to screen for the altered downstream pathways with loss of ADAM9, and we discovered that ADAM9 promotes both the mTOR signaling (unpublished data) and the FoxO signaling mediated by AKT activity in a cultured human colon cancer cell line.

Abnormal overexpression or aberrant activation of AKT has been reported to be associated with increased cell proliferation and survival in many cancers, mediated by its downstream pathways including Wnt/β-catenin signaling, mTOR signaling and FoxO signaling (Farhan et al., 2017; Song et al., 2019; Fleming-de-Moraes et al., 2022; Peng et al., 2022). As we found that loss of ADAM9 downregulates the protein level of active AKT, which has phosphorylation on

Ser473, the signaling pathways downstream of AKT are also potentially regulated by ADAM9 in HCT116 cells. As abnormal activation of Wnt/β-catenin signaling is related to cancer progression and poor prognosis in clinics (Yu et al., 2021), AKT can stabilize β-catenin through inhibition of GSK3 or direct phosphorylation of β-catenin to promote its cytosolic localization, to upregulate Wnt/β-catenin signaling (Fang et al., 2007; Manning & Toker, 2017; Shah & Kazi, 2022). Previously, our lab has reported that loss of ADAM9 reduces the protein level of active-β-catenin, indicative of nuclear active β-catenin and representative of Wnt activity, in HEK293T cells and another colorectal cancer cell line, SW620 (Chandrasekera et al., 2022). However, as HCT116 cells have aberrant activation of the Wnt/β-catenin signaling pathway due to a gain-of-function *CTNNB1* mutation that helps the encoded β-catenin protein with cytosolic accumulation, we didn't observe any significant changes in the protein level of active β-catenin or Wnt reporter assay using HCT116 cells, suggesting that Wnt/β-catenin signaling is not responding to the upstream alteration of AKT activity in this particular cell line.

Another major pathway downstream of AKT is FoxO signaling as AKT phosphorylates FoxO transcription factors to facilitate their nuclear export and degradation, thereby downregulating FoxO signaling (Manning & Toker, 2017). FoxO signaling was considered primarily as a tumor suppressor pathway due to its functions in promoting apoptosis and inhibiting proliferation, and abnormal inhibition of FoxO signaling is associated with acceleration of carcinogenesis via promoting metastasis and angiogenesis (Farhan et al., 2017; Liu et al., 2018).

Our RNA-seq data reveal an enrichment of the FoxO pathway upon ADAM9 KD, and our cellular proteomics results show a post-transcriptional increase of FoxO3 protein, a FoxO transcription factor, which were further validated with RT-qPCR of FoxO3 target genes and western blotting for FoxO3, respectively. We have also carried out immunocytochemistry using FoxO3 antibodies and found an upregulation of nuclear FoxO3 with ADAM9 KD, suggesting that ADAM9 regulates FoxO3 nuclear localization. Besides the two signaling pathways discussed above, the other major pathway downstream of AKT, the mTOR signaling was also found to be inhibited with loss of ADAM9 (Chandrasekera et al., 2022), which was further studied by other members of our lab.

Chapter 5

FUTURE DIRECTIONS

Explore more of the regulatory network downstream of DDX3 in NC

As we reported that DDX3 induces NC through promoting RAC1 expression and AKT activation, how RAC1 promotes downstream activation of AKT remains unclear. It has been reported that RAC1 can activate AKT mediated by its downstream effector p21-activated kinase 1 (PAK1) (Higuchi et al., 2008). As a member of the Ser/Thr protein PAK family kinases, PAK1 contains a C-terminal kinase domain and an N-terminal CDC42/RAC interaction/binding (CRIB) motif, also referred to as the autoinhibitory domain, which binds with each other upon the formation of trans-dimers to inhibit their kinase activities. Upon binding of the GTPase CDC42 or RAC1 with the CRIB motif, the PAK1 dimer will be disassociated resulting in the activation of both PAK1 monomers (Lei et al., 2000; Parrini et al., 2002). Independently of its kinase activity, the kinase domain of PAK1 can serve as a scaffolding protein to facilitate the recruitment of AKT to the cell membrane and activation by PDK1 (Higuchi et al., 2008). Additionally, an alternative kinase-dependent regulation was proposed by a separate study as PAK1 can phosphorylate AKT directly at Ser473 to promote its activation (Mao et al., 2008). To assess if RAC1 functions are dependent on PAK, we could generate an mRNA encoding the C-terminal kinase domain of human PAK1, which is 89% identical to the *X. tropicalis* ortholog, and test if it can rescue the

inhibition of NC induction and downstream signaling caused by RAC1 KD. If we could see the rescue here, one step further is to test the controversy of whether PAK activates AKT dependent on its kinase activity or serves as a scaffold protein to facilitate AKT activation. To differentiate between these two mechanisms, we could take advantage of a catalytically inactive mutant of the PAK1 kinase domain (p. Lys299Arg) (Sells et al., 1997), and compare its ability with that of the wild-type kinase domain to rescue the phenotypes caused by RAC1 KD.

Similar to *DDX3X*, *RAC1* and *AKT*, two *de novo* gain-of-function mutations in *PAK1* were identified with patients exhibiting developmental delay, intellectual disability (ID) as well as NC-related defects including craniofacial dysmorphism, pigment defects and gastrointestinal anomalies (Harms et al., 2018). Interestingly, fibroblasts derived from one of the patients harboring the PAK1 variant with p.Tyr131Cys mutation have elevated phosphorylation of AKT that may contribute to the pathology (Harms et al., 2018), as patients with gain-of-function AKT mutations display similar macrocephaly phenotypes (Rivière et al., 2012; Akgumus et al., 2017). To test if PAK1 also functions in NC development, I have designed gRNAs for *pak1* and injected them together with CAS9 protein to achieve a mosaic knockout (KO) of *pak1* using the *snai2:eGFP* transgenic embryos. We did not observe a significant reduction of eGFP expression or craniofacial deficiencies in these tadpoles (data not shown), which might be due to functional redundancy from other PAKs (King et al., 2014). An alternative

candidate from the PAK family would be PAK3, as mutations in *PAK3* can lead to X-linked ID with facial dysmorphism (Gedeon et al., 2003; Peippo et al., 2007; Hertecant et al., 2017; Duarte et al., 2020). As the *X. tropicalis* PAK3 shares 93% identity with the human ortholog, we could perform LOF experiments for PAK3 using the *snai2:eGFP* transgenic embryos to evaluate if it functions in NC development. As both PAK1 and PAK3 are implicated in brain development (Huang et al., 2011), KD or KO of both PAK1 and PAK3 simultaneously will be an alternative option in case they share functional redundancy in regulating both the CNS and the NC.

Characterize the roles of RAC1 and AKT in NC using alternative systems

In this study, we showed that loss of RAC1 or AKT2 in *X. tropicalis* reduces the eGFP expression in *snai2:eGFP* transgenic embryos and disrupts craniofacial morphogenesis. As discussed earlier, we are not fully convinced that it's a bona fide NC effect due to the potential disruption of gastrulation. Due to the small size of *X. tropicalis* embryos and the limitation of our injectors, the latest stage that I can conduct injection is the 8-cell-stage and the one anterior dorsal blastomere injected also contributes to other cell populations besides the NC cells, such as the brain (Moody, 1987). To restrict the effect more specifically to the NC and minimize the side effects on the development of other cells and tissues, injection of one blastomere in 16-cell-stage embryos is more suitable. Besides the *in vivo* model, we can also conduct the LOF experiments *in vitro* using the NC cell line cultured in a dish. The O9-1 cells are multipotent CNC cells derived from E8.5

mouse embryos (Ishii et al., 2012), and have been used to identify regulators of CNC development by multiple studies (J. Wang et al., 2016; Tavares et al., 2017; Yan et al., 2022). Although culturing and maintenance can be challenging, O9-1 cells can be used to perform LOF studies with siRNA-mediated KD or CRISPR/CAS9-mediated genome editing, similar to regular adherent cell lines (Nguyen et al., 2018). As the *X. tropicalis* RAC1 shares 100% identity with both the mouse and human orthologs, we could perform RAC1 LOF experiments using the O9-1 cells to test if the eGFP reduction and the craniofacial defects in the *snai2:eGFP* line caused by loss of RAC1 *in vivo* are bona fide NC effects. While there are two AKTs in *X. tropicalis*, mammals have three AKTs, AKT1, AKT2 and AKT3, sharing 98%, 98% and 99% identity between the mouse and human orthologs, respectively. As *AKT1* and *AKT3* human mutations were identified to be associated with defects in the CNS and the NC (Rivière et al., 2012; Akgumus et al., 2017), we can begin with characterizing the roles of these two candidates in modulating NC development. As AKT1 share 83% identity with AKT3 in mouse (83% in human as well), suggesting potential functional redundancy between the two, KD or KO of both AKT1 and AKT3 simultaneously might be required to achieve a more drastic phenotype.

Characterize the pathogenic mutations associated with DDX3X syndrome

Originally, I generated expression constructs harboring DDX3X mutants through site-directed mutagenesis and planned to transfect them to HEK293T cells, in which our lab reported previously to have canonical Wnt signaling downregulated

with DDX3X KD, to determine if they could alter canonical Wnt signaling. When overexpressing WT DDX3X in HEK293T cells, we did not see the upregulation of canonical Wnt signaling as expected even using the TOPFlash reporter assay which is very sensitive to the alteration of canonical Wnt signaling. As HEK293T cells have 3 copies of *DDX3X* in the genome, we speculated that it could be due to the abundance of endogenous DDX3X. This is also why we decided to create a DDX3X KO cell line and employ it in screening pathogenic DDX3X variants for alteration in downstream pathways. Since we have not successfully knocked out DDX3X, we asked collaborator Dr. Stephen Floor from UCSF for an inducible cell line that has DDX3X protein level >90% reduction upon inducer treatment, to minimize the expression of endogenous DDX3X. Using an inducible degron system, they tagged the endogenous DDX3X with a fragment of the Auxin Inducible Degron (mAID) in HCT116 cells expressing the auxin-responsive F-box protein. When the cells are treated with the synthetic auxin, the auxin-responsive F-box protein and the auxin will form a complex to induce ubiquitination and rapid degradation of the mAID-tagged DDX3X (Calviello et al., 2021; Venkataramanan et al., 2021). After receiving the cells very recently, I treated them with the auxin, Indole-3-acetic acid (IAA), and observed a drastic reduction of DDX3X in protein level. Next, we can overexpress the pathogenic DDX3X variants and determine if it will alter downstream pathways. As I mentioned earlier, HCT116 cells harbor an activating *CTNNB1* mutation leading to aberrant activation of canonical Wnt signaling, we probably cannot use the cells to characterize Wnt activity, but we

can still test if AKT activity or RAC1 expression will be altered upon overexpression of the variants.

Identification and characterization of ADAM9 substrates

Besides the RNA-seq and cellular proteomics analyses upon ADAM9 KD to identify the downstream signaling pathway regulated by ADAM9, our lab has also conducted a secretomics analysis comparing the secreted proteins between the control and ADAM9 siRNA transfected HCT116 cells. To identify potential ADAM9 substrates, we integrated the transcriptome, the cellular proteome and the secretome, to search for differentially expressed proteins (DEPs) that are upregulated at the cellular protein level but not at the mRNA level, and meanwhile downregulated in the secretome, upon ADAM9 KD. If it's a substrate of ADAM9, cleavage will be inhibited upon loss of ADAM9 so that the protein will stay on the cell membrane without having the ectodomain released to the extracellular matrix. To exclude the transcriptional targets of ADAM9, we filtered out the DEGs upregulated upon ADAM9 KD in the RNA-seq analysis. Interestingly, we found that another member of the ADAM family, ADAM17, is a post-transcriptionally target of ADAM9, with the cellular protein level reduction upon ADAM9 KD validated by western blotting (data not shown). As a metalloproteinase, ADAM17 cleaves the ectodomain of various proteins, ranging from signaling molecules, adhesion proteins and endocytic receptors (Calligaris et al., 2021). As both the cellular protein levels of ADAM9 and ADAM17 were reduced upon ADAM9 KD, the substrates we identified with the three data sets

could be direct targets of ADAM9 or ADAM17. To distinguish between the two, we purchased ADAM17 KO HCT116 cells (ab266873) and planned to KD ADAM9 in these cells to compare the cellular protein level and the secreted protein level change of potential substrates with that of WT HCT116 cells.

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