FUNCTIONAL CHARACTERIZATION OF DENND5A/RAB6IP1 IN EARLY EMBRYONIC DEVELOPMENT

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ABSTRACT

Denn stands for differentially expressed in normal vs neoplastic cells, Dennd5a or Denn domain containing protein 5a is also named Rab6 interacting protein 1 (Rab6ip1), KIAA1091 (HUGE database). Rab6 is a small GTP-binding protein that involves in intra-Golgi transport. In a yeast two hybrid screen by Bruno Goud group in 1995, they used Rab6 as bait and found several interacting cDNA clones including Rab6 interacting protein 1 or Dennd5a. Several Dennd5a interaction partners have been discovered, Rab6, Rab11 and Sorting Nexin 1, which primarily locate Dennd5a to intracellular transport pathway. In Dr. Habas lab, Dennd5a was identified as a Daam1 interacting protein in a yeast two hybrid screen. Daam1 is a key player in non-canonical Wnt signaling and regulates vertebrate gastrulation and neural tube closure. I confirmed that Daam1 can physically bind to Dennd5a in immunoprecipitation assays using epitope tagged constructs. In the cellular level experiment, Dennd5a can colocalize with Daam1 on/near cell membrane and within punctate structures, however, Wnt5a and Wnt3a stimulations don't appear to affect their subcellular colocalization. In *Xenopus* in-vivo experiments, knockdown of Dennd5a by morpholino leads to unclosed blastopore and "open-back" defect during embryonic development. Interestingly, overexpression of Dennd5a by microinjection of RNA leads to very similar phenotype.

I dedicate this thesis to my parents and my sister.

I can’t thank enough for their love and unselfish support of my education.

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I came to US about six years ago with the pure heart of pursuing a higher degree even though worries filled since I might step into a totally different world. Pursuing a PhD was my whole goal of life; I come to realize little by little that this is only a part of my life just like everything else. If family and friends are the whole world for every person, friends are almost all when one is far away from home. I appreciate every bit of joy that my friends have brought me in the past years in USA. Taking this chance, I want to thank Eric who came the same year with me to the department, offered help and was also my study buddy, Nan Li, Ming Yi, Tao Guo who were my basketball buddies, Dr. Jiong Ma, Dr. Huan Li, Dr. Wangxi Luo who are not just doctors but also good chefs (they make me eat well), Yuko, Rich, Yanxun, Kaushik, Baihao, Noopur who I have been working with for so many years and are always ready to help, Xinda, TUCSC soccer club who make my Sundays always fun. I also want to thank Dr. Amini and Dr. Waring, who are nice and always willing to help and Dr. Spaeth who taught us a lot of skills to "handle" undergraduates. Life is full of ups and downs, losses and gains and I appreciate both knowledge and life experience that I have gained in the past years in graduate school. Finally, I thank Dr. Raymond Habas very much for giving me the chance to work in lab and get a deeper knowledge of science.

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CHAPTER 1

BACKGROUND

1.1 Review of Rab Proteins and Specifically Rab6 and Rab11.

Rab proteins comprise the largest family of monomeric small GTPases. Eleven Rab proteins are expressed in the yeast *Saccharomyces cerevisiae* but there might be more than 60 family members in humans as estimated from expressed-sequence tags (ESTs) and the sequenced human genome (1).

The first rab gene (YPT1) was identified in 1983 by D. Gallwitz et al. and it located between actin gene and tubulin gene in *Saccharomyces cerevisiae* (2). A. Tavitian and colleagues cloned the first homologs of SEC4/YPT in mammals, termed rab (**ra**s-like in rat **b**rain) genes (2).

Rab belongs to GTPase protein family and it cycles between GTP-bound or GDP-bound forms through specific regulatory proteins. These include guanine-exchange factors (GEFs), GTPase activating proteins (GAPs), GDP-dissociation inhibitors (GDIs) and GDP-displacement factors (GDFs).

Cells exchange chemicals and signals with each other and the extracellular environment through exocytic and endocytic pathways. These pathways are carried out through an elaborate system of vesiculo-tubular transport. Rab proteins together with their effectors coordinate consecutive steps of transport, such as vesicle formation, vesicle and organelle motility, and tethering of vesicles to their target compartment (1).

1.2 Different Gene or Protein Names of Dennd5a

- Denn(differentially expressed in normal vs neoplastic cells) domain containing protein 5a[**Dennd5a**]
- Rab6 interacting protein 1 (**Rab6ip1**), which has little to do with Rab6ip2 in terms of sequence
- **H**uman **U**nidentified **G**ene-**E**ncoded Large Proteins database (HUGE) name **KIAA1091**

In this thesis, the name Dennd5a will be used for the only purpose of clarity.

1.3 Discovery of Dennd5a Through Interaction With Rab6

Rab6 is a small GTP-binding protein that involves in intra-Golgi transport. In a yeast two hybrid screen by Bruno Goud group in 1995, they used Rab6 (**note**, I assume it is Rab6a because this group crystallized complex of Rab6a and its interacting partner Dennd5a (3).) as bait and found several interacting cDNA clones including Rab6

interacting protein 1 or Dennd5a (4).In 2007, Stephanie Miserey-Lenkei discovered through the yeast two-hybrid system that Dennd5a strongly interacted with the GTP-bound mutants of Rab6A, Rab6A' and Rab6B (Q72L) but not dominant-negative Rab6A and Rab6A' (T27N) mutants (5). In 2009, this interaction between Rab6a (with GTP) and Dennd5a was once again confirmed by crystallization of Rab6a and RUN1-PLAT domain (Details of Dennd5a domains will be discussed in subsection 1.4) of Dennd5a by Dr. Goud research group (3).

1.4 Dennd5a interacting partners and current study of the proteins

1.4.1 Rab6

There are **four** isoforms of Rab6 which are Rab6a, Rab6a', Rab6b and Rab6c. Rab6a is only 3 amino acids different from Rab6a' (Val^{62} to Ile, Thr^{87} to Ala and Val^{88} to Ala) (6,7).

In NCBI protein database, those two isoforms of human Rab6 gene were found as described above. Both Rab6a and Rab6a' were reported to localize to the cellular Golgi apparatus and position 87 amino acid of Rab6a is critical for binding to its effector, kinesin-like protein **Rabkinesin-6**, a Golgi-associated Rab6a effector (7). Elaine Del Nery (also from Dr. Goud lab) discovered that knockdown of Rab6a' by siRNA in HeLa

cells led to significantly lower cell confluency which suggested its potential role in cell cycle progression (6).

DENND5a can bind to GTP-bound form **Rab6a** using GST pull-down assays (8). DENND5a can also bind to empty/GDP/GTP-bound form Rab6 proved by GST pull-down; However, it should be pointed out that Dennd5a **only** bind to Rab6-GTPγS in GST pull-down experiments performed at higher stringency (>250 mM NaCl, data not shown)(5).

Rab6a is also reported to interact with human **myosin** $\mathbf{V_a}$ within the 35-amino acid region (residues 1169–1204) in which the position 1203 amino acid is crucial for this interaction (9). Class V myosins are large homodimers consisting of an amino-terminal actin-binding motor domain, a lever arm, and a carboxyl-terminal globular-tail domain (GTD) that mediates binding to cargo. Mammals have three myosin V isoforms, V_a, V_b and V_c.

Rab6b was first cloned from human melanoma cells and showed about **78%** similarity in nucleotide sequence to Rab6 (10) (This should be Rab6a since only Rab6a was cloned before 1997.). The deduced amino acid sequence of Rab6b showed 91% identity with the Rab6A protein and localized to Golgi complex and endoplasmic reticulum in overexpression study (11).

First “Rab6c” was characterized in human platelets by Fitzgerald and Reed in 1999 and the sequence of this human Rab6c is actually identical to that of human Rab6a’ described above by Echard in 2000(12). So, this should be the Rab6a’ expressed in human platelet. Later, Jidong Shan found ***wth3*** gene which was classified in to Rab6 family and named Rab6c. Rab6c has an elongated C-terminal region which contains 46 extra amino acids compared to its other family members (13). Further research by Joanne Young from Dr. Goud group claimed that Rab6c is a **primate-specific retrogene** derived from a Rab6a′ transcript and Rab6c protein is localized to **centrosomes** while Rab6a’ localizes to **Golgi** complex (14).

RAB6A, member RAS oncogene family (RAB6A) Human protein-coding gene RAB6A. Represented by 1072 ESTs from 312 cDNA libraries. Corresponds to 4 reference sequences (different isoforms). [UniGene 718381 - Hs.503222]

Table 1. Comparison of Rab6a in different species

Best Hits and Hits from model organisms		Species	Identity (%)	Length (aa)
NP_445818.1	ras-related protein Rab-6A	*R. norvegicus*	100.0	207
XP_001363135.1	PREDICTED: ras-related protein Rab-6A-like isoform 1	*M. domestica*	100.0	207
NP_001038105.1	RAB6A gene product	*G. gallus*	100.0	207
XP_001115437.1	PREDICTED: ras-related protein Rab-6A-like	*M. mulatta*	100.0	207
NP_001230202.1	RAB6A gene product	*S. scrofa*	100.0	207
NP_001180044.1	ras-related protein Rab-6A	*B. taurus*	100.0	207
XP_004016355.1	PREDICTED: ras-related protein Rab-6A	*O. aries*	100.0	207
XP_002708750.1	PREDICTED: RAB6A, member RAS oncogene family-like isoform 1	*O. cuniculus*	100.0	207
XP_001496149.3	PREDICTED: ras-related protein Rab-6A-like	*E. caballus*	100.0	184
NP_077249.1	Rab6a gene product	*M. musculus*	99.5	207
XP_003971157.1	PREDICTED: ras-related protein Rab-6A-like isoform 1	*T. rubripes*	99.5	207
XP_003456835.1	PREDICTED: ras-related protein Rab-6A-like	*O. niloticus*	99.5	207
NP_001080506.1	RAB6A, member RAS oncogene family	*X. laevis*	99.5	207
XP_003639844.1	PREDICTED: ras-related protein Rab-6A	*C. lupus familiaris*	99.5	207
NP_477172.1	Rab6	*D. melanogaster*	95.7	206
NP_510790.1	Protein RAB-6.2	*C. elegans*	94.6	204

RAB6B, member RAS oncogene family (RAB6B) Human protein-coding gene RAB6B. Represented by 425 ESTs from 149 cDNA libraries. Corresponds to reference sequence NM_016577.3. [UniGene 3526164 - Hs.715344]

Table 2. Comparison of Rab6b in different species

Best Hits and Hits from model organisms		Species	Identity (%)	Length (aa)
NP_776142.1	ras-related protein Rab-6B	*M. musculus*	100.0	207
XP_001364646.1	PREDICTED: ras-related protein Rab-6B-like	*M. domestica*	100.0	207
XP_001115381.1	PREDICTED: ras-related protein Rab-6B isoform 2	*M. mulatta*	100.0	207
XP_003895143.1	PREDICTED: ras-related protein Rab-6B isoform 1	*P. anubis*	100.0	207
NP_057661.3	RAB6B gene product	*H. sapiens*	100.0	207
XP_003132476.1	PREDICTED: ras-related protein Rab-6B-like	*S. scrofa*	100.0	207
NP_001094599.1	RAB6B gene product	*B. taurus*	100.0	207
XP_004003376.1	PREDICTED: ras-related protein Rab-6B isoform 1	*O. aries*	100.0	207
XP_002716636.1	PREDICTED: RAB6B, member RAS oncogene family	*O. cuniculus*	100.0	207
XP_001147918.2	PREDICTED: ras-related protein Rab-6B	*P. troglodytes*	100.0	194
XP_001496020.2	PREDICTED: ras-related protein Rab-6B-like	*E. caballus*	100.0	174
XP_003218408.1	PREDICTED: ras-related protein Rab-6B-like	*A. carolinensis*	99.5	207
NP_001086132.1	RAB6B, member RAS oncogene family	*X. laevis*	98.6	207
NP_001029351.1	rab6b gene product	*D. rerio*	96.3	214
NP_477172.1	Rab6	*D. melanogaster*	95.2	207
NP_510790.1	Protein RAB-6.2	*C. elegans*	94.6	203

1.4.2 Rab11

The mammalian genome encodes three Rab11 proteins, Rab11a, b, and c, which share high sequence identity (mouse proteins, Rab11a: Rab11b, 91% identity; Rab11a: Rab11c, 62% identity; Rab11b: Rab11c, 61% identity). Rab11c is better known as **Rab25** (15).

Rab11 has been reported to localize onto recycling endosomes and to play critical roles in recycling of all kinds of cargoes (such as transferrin, β_2-adrenergic receptors, E-cadherin et al) from early endosomes to cell membrane (16-18). Dr. Bruno Goud group also reported that Rab11 also influenced the endosome to Golgi trafficking (19). In 2004, Zhang et al discovered that **Sec15**, one component of **exocyst** complex, can interact with Rab11 preferentially **GTP restricted** state (20).

In 2007, Stephanie et al reported that Dennd5a can interact with Rab11a in the yeast two hybrid system and GST pull-down experiments; however, Dennd5a only interacts with **GTP-locked** mutant Rab11a but not with wild type Rab11a (5).

Notably, Rab11a and Rab11b both share **extremely high** level of sequence identity across species. For Rab11a, **99.5%** identity is shared by those of *Xenopus laevis* and *Homo sapiens*.

RAB11A, member RAS oncogene family (RAB11A) Human protein-coding gene RAB11A. Represented by 1372 ESTs from 404 cDNA libraries. Corresponds to 2 reference sequences (different isoforms). [UniGene 186722 - Hs.321541]

Table 3. Comparison of Rab11a in different species

Best Hits and Hits from model organisms		Species	Identity (%)	Length (aa)
NP_059078.2	Rab11a gene product	*M. musculus*	100.0	215
NP_112414.1	Rab11a gene product	*R. norvegicus*	100.0	215
XP_001367185.1	PREDICTED: ras-related protein Rab-11A-like isoform 1	*M. domestica*	100.0	215
NP_001005827.1	RAB11A gene product	*G. gallus*	100.0	215
XP_001103732.2	PREDICTED: ras-related protein Rab-11A-like	*M. mulatta*	100.0	215
XP_003901132.1	PREDICTED: ras-related protein Rab-11A isoform 1	*P. anubis*	100.0	215
XP_510490.2	PREDICTED: uncharacterized protein LOC453523 isoform 2	*P. troglodytes*	100.0	215
NP_004654.1	RAB11A gene product	*H. sapiens*	100.0	215
NP_001003276.1	RAB11A gene product	*C. lupus familiaris*	100.0	215
NP_001026958.1	RAB11A gene product	*S. scrofa*	100.0	215
NP_001033251.1	RAB11A gene product	*B. taurus*	100.0	215
XP_004010297.1	PREDICTED: ras-related protein Rab-11A	*O. aries*	100.0	215
NP_001087765.1	RAB11A, member RAS oncogene family	*X. laevis*	99.5	215
NP_001016481.1	rab11a gene product	*X. tropicalis*	99.5	215
XP_003209617.1	PREDICTED: ras-related protein Rab-11A-like	*M. gallopavo*	99.5	215
NP_001007360.1	rab11a gene product	*D. rerio*	99.1	214
NP_477170.1	Rab11, isoform B	*D. melanogaster*	96.3	213
NP_490675.1	Protein RAB-11.1	*C. elegans*	93.8	209

RAB11B, member RAS oncogene family (RAB11B) Human protein-coding gene RAB11B. Represented by 809 ESTs from 305 cDNA libraries. Corresponds to reference sequence NM_004218.3. [UniGene 2095415 - Hs.626404]

Table 4. Comparison of Rab11b in different species

Best Hits and Hits from model organisms		Species	Identity (%)	Length (aa)
NP_033023.1	Rab11b gene product	*M. musculus*	100.0	217
NP_116006.1	Rab11b gene product	*R. norvegicus*	100.0	217
NP_001142168.1	uncharacterized protein LOC100274335	*Z. mays*	100.0	217
XP_001093925.1	PREDICTED: ras-related protein Rab-11B	*M. mulatta*	100.0	217
XP_003914857.1	PREDICTED: ras-related protein Rab-11B	*P. anubis*	100.0	217
XP_001147785.2	PREDICTED: ras-related protein Rab-11B	*P. troglodytes*	100.0	217
NP_004209.2	RAB11B gene product	*H. sapiens*	100.0	217
XP_854399.2	PREDICTED: ras-related protein Rab-11B	*C. lupus familiaris*	100.0	217
NP_001231804.1	RAB11B, member RAS oncogene family	*S. scrofa*	100.0	217
NP_001030468.1	RAB11B gene product	*B. taurus*	100.0	217
XP_004008616.1	PREDICTED: ras-related protein Rab-11B	*O. aries*	100.0	217
XP_002190133.1	PREDICTED: ras-related protein Rab-11B	*T. guttata*	99.5	217
XP_004068248.1	PREDICTED: ras-related protein Rab-11B-like	*O. latipes*	99.5	217
NP_001087959.1	uncharacterized protein LOC494642	*X. laevis*	99.5	217
NP_001011048.1	rab11b.1 gene product	*X. tropicalis*	99.5	217
NP_001012569.1	RAB11B gene product	*G. gallus*	99.5	217
XP_003198017.1	PREDICTED: ras-related protein Rab-11B-like isoform 1	*D. rerio*	99.1	217
NP_477170.1	Rab11, isoform B	*D. melanogaster*	95.3	213
NP_490675.1	Protein RAB-11.1	*C. elegans*	93.8	210

1.4.3 Daam1 (Disheveled Associated Activator of Morphogenesis 1)

Daam1 was found via a yeast two-hybrid screen by Habas et al to be the **intermediate** for Wnt/Fz/Dvl signaling to Rho (21). Daam1 belongs to the formin protein family, which is characterized by three major domains, termed the GTPase binding domain (GBD), Formin homology 1 (FH1) domain, and Formin homology 2 (FH2) domain (22). Also, Daam1 exists in an auto-inhibitory state, "locked" by the internal interaction between the diaphanous inhibitory domain (DID, part of the GTPase-binding domain [GBD domain]) and carboxyl-terminal diaphanous auto-regulatory domain (DAD); the binding of Dvl helps to unlock Daam1 (23). The Daam1 domain schematic is shown in Figure 8C (modified from Akira Sato et al. 2006). In 2006, Sato et al reported that Profilin1 interacted with the FH1 (formin homology) domain of Daam1 and was localized with Daam1 to actin stress fibers in response to Wnt signaling in mammalian cells (24). In 2007, the crystallized structure of FH2 by two research groups revealed a dimerized tethered or head to tail architecture in order to perform actin assembly activity (25,26).

In a yeast two hybrid screening with Daam1 as the bait, we identified a clone that can interact with Daam1. This clone was sequenced by our lab and identified as KIAA1091 or Dennd5a; I cloned the *Xenopus laevis* homologue of this gene. Confirmation of this interaction was through co-immunoprecipitation assays and the results are in Chapter 4.

1.4.4 Sorting nexin 1 (SNX1)

SNX1 has three domains, SNX domain, PX (phox homology) and BAR domain.

SNX1 can interact with Dennd5a in a genome wide yeast two hybrid screening using SNX1 as bait and this interaction was verified by GST pull-down experiment. Humberto Fernandes et al in 2012 reported that the second RUN Domain (RUN2) from DENND5a/Rab6IP1 binds to Sorting Nexin 1 (**SNX1**) by surface plasmon resonance analysis and is involved in the trafficking of vesicles at the level of Golgi. In contrast to the Rab6/RPRdel (the mutant RPRdel is show in Figure 1) complex, SNX-PX binding to Rab6/RPdel was 10-fold less (response units, RU) at twice the concentration (30 mM) relative to binding of Rab6/RPRdel (15 mM). Also, PX alone binds to Rab6/RPRdel as strong as SNX-PX (27).

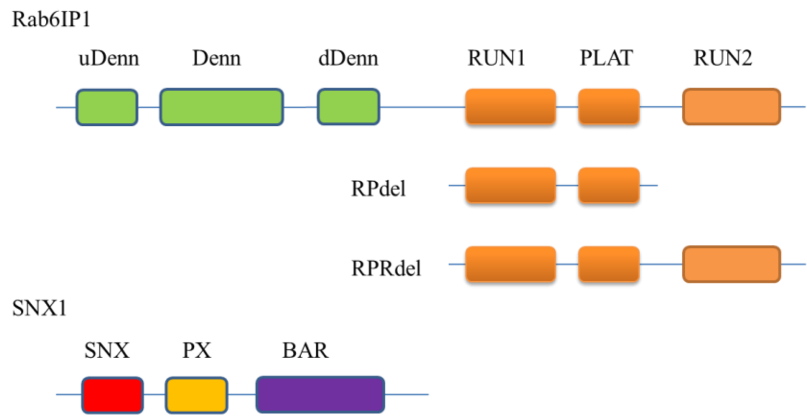


Figure 1. Schematic arrangement of domains for Rab6IP1 and Sorting Nexin 1 (SNX1).

PX domain is generally considered to bind various phosphatidylinositol phosphates, which potentially targets it to specific cellular membranes that are enriched

in these phospholipids (28-30). PX domain of SNX1 is also reported to be essential for its targeting to early endosome through binding to phosphatidylinositol phosphates (31-33).

Carlton et al propose that SNX1 associates with a microdomain of the early endosome from where it regulates tubular-based **endosome-to-TGN** retrieval of the cation-independent mannose-6-phosphate receptor (CI-MPR) (34). Bujny et al have established that suppression (siRNA) of SNX1 leads to a significant reduction in the efficiency of **endosome-to-TGN transport** of the Shiga toxin B-subunit (35).

It seems that the PX domain of SNX1 can bind both Dennd5a (Rab6ip1) and membrane of endosome. Besides this, Wassmer et al also reported that the spatial organization of the retromer pathway (early endosome-to-trans-Golgi network (TGN) transport) is mediated through the association of SNX1 with the proposed TGN-localized tether Rab6-interacting protein-1/**Dennd5a** (36). Even though Wassmer doesn't specify if PX domain of SNX1 binds to Dennd5a and endosome membrane at the same time, this appears to happen at the same time, which is still needed to be verified.

1.4.5 Rab39

Full-length cDNA of Rab39 was composed of 1251 bp and contained a complete open reading frame (ORF) of 636 bp potentially encoding a 211-residue protein with a calculated molecular weight of 23.7 kDa (37). There are two isoforms of Rab39, Rab39a (217 aa in length) and Rab39b (213 aa in length). They share ~**77%** identity of protein sequence from NCBI database.

In GDP-releasing assay, **Rab39** is sensitive to Dennd5a and Dennd5b (38). Dennd5a is very likely to be a GEF for Rab39 but not Rab6 (39).

Rab39a protein shares the highest homology with mouse Rah/**Rab34** (AB082987 in GenBank) and human Rab34 (NM_031934 or BC016841 in GenBank) at the N-terminal 192 aa (>**95% identity**), which has been shown to be localized to **membrane ruffles** and to be involved in **micropinosome** formation (37). Overexpression of Rab39a (not specified if it is Rab39A or Rab39B in the paper but from NCBI database its amino acid sequence is different from Rab39B.) enhanced **endocytosis** of FITC-BSA (tracer of endocytosis) as Rab5c was used as a positive control in HeLa cells instead of NIH3T3 cells(37). Rab39b is neuron specific in both fatal and adult brains. And Rab39b appears to colocalize with Golgi, Golgi associated vesicles and recycling endosome (40).

1.5 Snapshot of Dennd5a

1.5.1 Dennd5a Protein Sequence Similarity Among Different Species

Dennd5a protein in *Xenopus laevis* has 1287 amino acid (aa) while Dennd5a in *X. tropicalis* has 1284 aa. And Dennd5a of the two species shares ~94% similarity when aligned by NCBI BLAST. From Unigene of NCBI, Dennd5a in *X. tropicalis* shares identity of 91.1%, 90.5%, 85.3% and 49.2% with that in *Homo sapiens, Mus musculus, Dario rerio,* and *Drosophila melanogaster*, respectively.

1.5.2 Dennd5a Domains

Dennd5a has Denn (differentially expressed in normal vs. neoplastic cells) domain from sequence about 200~400 aa, uDenn domain upstream of Denn from sequence 1~150 aa, dDenn domain downstream of Denn from sequence 500~600, RUN domain followed by PLAT domain from sequence 875~1075 aa and a second RUN domain from sequence 1125~1287 aa. Overall, Dennd5a in *Xenopus laevis* shares about **88%** identity with that of *Homo sapiens*. However, Denn

uDenn Denn dDenn RUN1 PLAT RUN2

position
1~150 200~400 500~600 600~875 875~1075 1125~1287

Coverage
93% 100% 99% 99% 100% 100% 100%

Identity
86% **94%** 84% **93%** 88% **92%** 82%

Figure 2. Domain comparison of *Xenopus laevis* Dennd5a against human by DELTA-BLAST (Domain Enhanced Lookup Time Accelerated BLAST). Note, domain position or length in this analysis is a little wider than that from Conserved Domain Analysis in NCBI.

domain, dDenn domain and RUN-PLAT domain shares **94%, 93%** and **92%** identity with those of *Homo sapiens*, respectively.

The RUN acronym was coined after three members of the family: the RPIP8 protein, an effector of Rap2 (Bourne et al., 1991); UNC-14, a protein that plays a role in axonal elongation and guidance in *C. elegans* (Ogura et al., 1997); and NESCA (new molecule containing SH3 domain at the carboxyl terminus), an SH3 domain-containing protein cloned from human placental cells (3).

The PLAT domain, also referred to as LH2 (lipoxygenase homology domain 2) (Bate-man and Sandford, 1999), was named after three members in which this motif was discovered: polycystin-1, the product of polycystic kidney disease 1 (PKD1) associated with renal cysts and kidney disease (Sandford et al., 1997); lipoxygenases, multi-subunit enzymes involved in the biosynthetic pathway of hormones from lipid substrates (Gillmor et al., 1997); and alpha-toxin, a pore-forming molecule expressed by pathogenic bacteria (3).

1.5.3 Dennd5a Homologs

In Drosophila, its homolog is officially called Dmel\CG7852 and largely uncharacterized; the only phenotype reported by **flybase.org** is **viable** using a genome wide RNAi screen (41,42). In *Caenorhabditis elegans (C. elegans)*, its homolog is lst-6 (lateral signaling target) and it is reported by **wormbase.org** to expressed in nervous system, head and the following phenotypes have been reported as **NOT** observed in lst-6 : embryonic lethal, larval lethal, larval arrest, et al. No expression or phenotype data is reported by **zfin.org**. **Note, the above information was updated till 2015/03/25.**

CHAPTER 2

MOLECULAR CLONING OF DENND5A

The gene sequence of Dennd5a in *Xenopus laevis* is unknown while the gene sequence of Dennd5a in *Xenopus tropicalis* is known. Until recently 2015/04, the sequence was still unknown. In order to **design primers** to PCR the gene, I did BLAST using the known Dennd5a sequence. In Figure 1, BLAST result is shown using Dennd5a gene sequence of *Xenous tropicalis* as query and **Expressed Sequence Tag** of *Xenopus laevis* as the database. The four red hits (red bars) shows high to very high identity to the query sequence from 87% to 96%. The high identity percentages are good evidence that the hits fragments may come from the homologous gene of other species of *Xenopus*. Based on this, the reverse primer is easily picked.

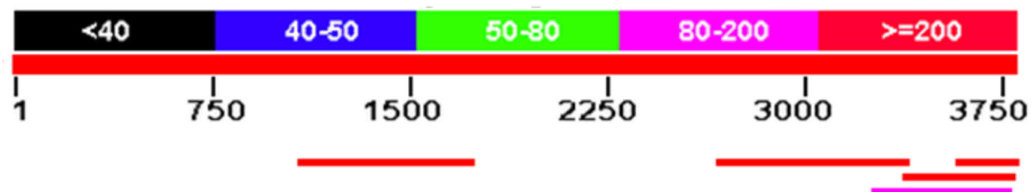


Figure 3. BLAST result using Dennd5a gene sequence of *Xenous tropicalis* as query and Expressed Sequencing Tag of *Xenopus laevis* as the database.

The forward primer is totally based on *Xenopus tropicalis* sequence which may **lower the success rate** of PCR because of the variations between the gene sequence

across the two species and nucleotide-complementary requirement for primer design especially in **the 3’ end** of the primer.

Another strategy is also used to take advantage of the middle fragment hit (spanning the position 1500): design primers against the middle fragment so that two PCR can be performed to get two fragments, with one spanning the start codon of the gene and the middle fragment part and the other spanning the stop codon of the gene and the middle fragment part; then the two fragments can be “stitched” together by a unique restriction site lying within the overlapping part (this part is within the middle fragment and luckily, there is a unique Xho I site.).

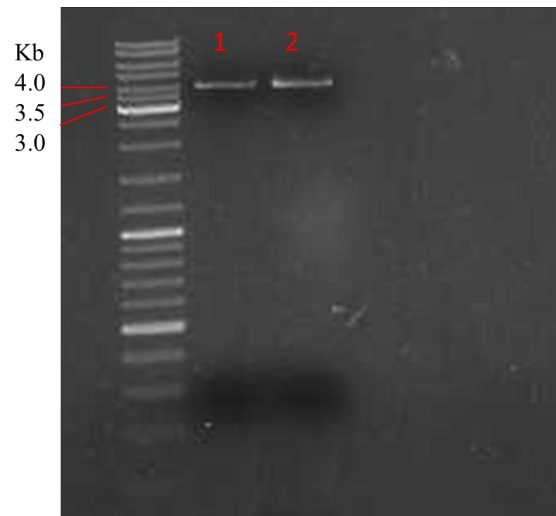


Figure 4. Dennd5a runs on 1% agarose gel. GeneRuler™ 10 kb DNA Ladder on the left. Lane 1 is Dennd5a DNA; Lane 2 is Dennd5a DNA digested by Stu I restriction enzyme.

Both strategies worked: one to PCR the whole gene open reading frame and the other to stitch two fragments covering start and stop codon and overlapping in the middle fragment through a unique restriction site. The Dennd5a gene was then cloned into pCS2-myc and pCS2-GFP vectors for later studies.

CHAPTER 3

TEMPORAL AND SPATIAL EXPRESSION PATTERNS OF DENND5A IN *XENOPUS* EMBRYONIC DEVELOPMENT

3.1 Introduction of *Xenopus* Embryonic Development

Xenopus, commonly known as the African clawed frog, is a genus of aquatic frogs native to Africa. There are two commonly used model organisms in this genus, *Xenopus laevis* and *Xenopus tropicalis*. *Xenopus laevis* is relatively bigger in size than *Xenopus tropicalis*. So is the size of their eggs, which are in the order of millimeters in diameter. The relatively large size is an advantage for microinjections. The genomic sequence of *Xenopus tropicalis* which is diploid was published in 2010 led by the Department of Energy's Joint Genome Institute (JGI) and the University of California, Berkeley. However, the genome sequence of *Xenopus laevis* which is tetraploid hasn't been fully dissected so far. For instance, Dennd5a sequence is known in *Xenopus tropicalis* but unknown in *Xenopus laevis*.

In Figure 5, *Xenopus* embryonic development stages are shown from fertilization, cleavage to gastrulation, organogenesis, larval stage. Cleavage stage is the mitosis process where cells quickly replicate themselves but there is no significant growth in size. This stage is marked by the formation of blastocoel the inner side of which is lined up by

blastomeres. Gastrulation is the stage when the one-layer blastula is transformed into three-layered gastrula through coordinated cell movement, specifically, convergence and extension. Movies that elaborate the process of gastrulation can be found at http://www.xenbase.org/anatomy/static/movies.jsp.

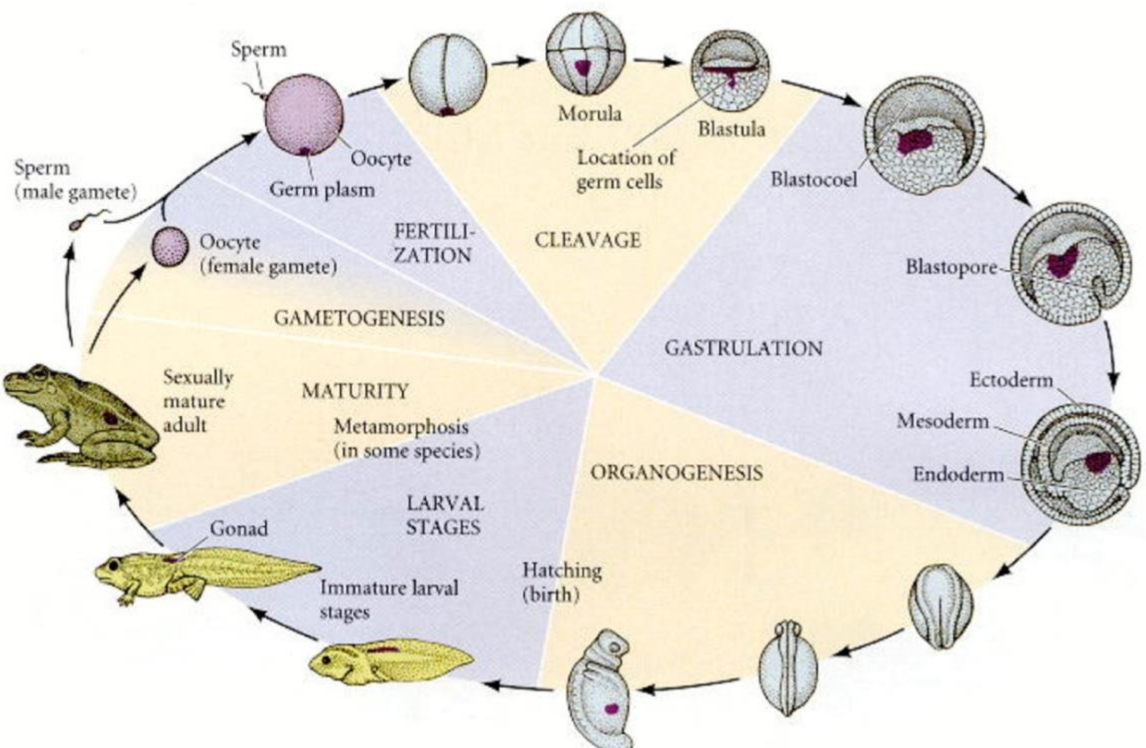


Figure 5. Xenopus embryonic development stages from fertilization, cleavage to gastrulation, organogenesis, larval stage. Image edited from http://sciwiki.ca/index.php/Biology_3338A:_Developmental_Biology

3.2 Temporal Expression of Dennd5a in *Xenopus laevis*

Temporal expression of a gene informs the mRNA level of the whole embryo (I use the whole embryo to extract RNA) and this doesn't distinguish the differences in individual tissues or organs.

No temporal expression data of Dennd5a have been reported either in human, mouse, frog or zebrafish so far. In order to investigate its expression pattern, RNA is extracted from frog embryos of specific stages and reverse transcription is performed using iScript™ One-Step RT-PCR Kit. Primers targeting the first ~394 bp of Dennd5a gene sequence are used, so that the size of the fragment is similar to that of ODC. ODC (Ornithine decarboxylase) is used as the internal reference gene which is reported to expressed relatively constantly even though EF1α (Elongation factor 1-alpha) is also recommended(43,44). Since my research is more focused on the early embryonic development, stages spanning gastrulation, neurulation, until stage 26 are selected for this study. In Figure 6, the expression of Dennd5a is on the upper panel and the expression of ODC is in the lower panel. As seen in the figure, the expression level of ODC does show a minimized variation; the expression of Dennd5a is shown in every stage selected including gastrulation stage 10~11 and neurulation stage 12 ~ 17 and also doesn't seem to vary too much except in stage 12 where no expression is observable. It may be due to the low expression of the gene itself or due to the quality of RNA extracted for RT-PCR.

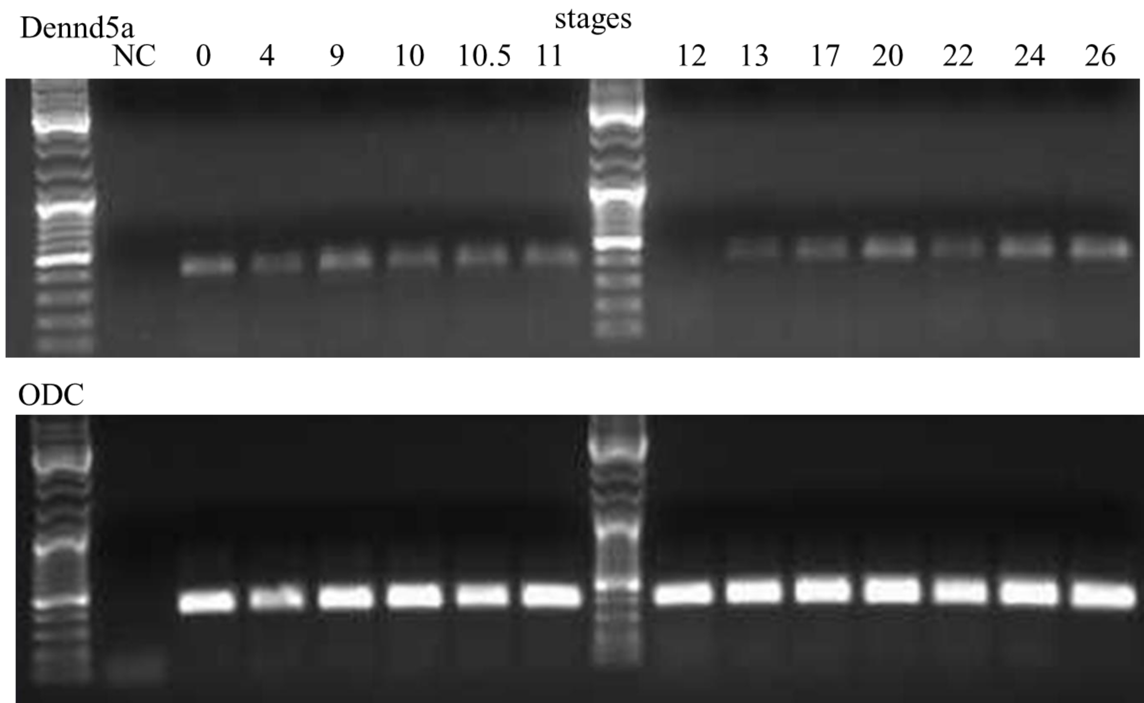


Figure 6. Denn5a expression pattern through RT-PCR. Upper panel, Dennd5a expression level; lower panel, ODC (Ornithine decarboxylase) internal reference gene expression. NC, negative control.

Another caution should be taken here: *Xenopus laevis* has an allotetraploid genome that have duplicated genes and these gene may still be very conserved sharing highly identical sequence and function (45,46); therefore, the fragment out of RT-PCR is possibly a mixture of two fragments from duplicated genes.

3.3 Spatial Expression of Dennd5a in *Xenopus laevis*

Since temporal expression doesn't distinguish the differences in different tissues or organs, the spatial expression through in-situ hybridization that detects mRNA expression level will fill up the missing information. pCS2-myc-Dennd5a is used to synthesize the full-length DIG (digoxigenin)-labelled mRNA through mMESSAGE mMACHINE® SP6 Transcription Kit and the length of the full-length Dennd5a RNA probe is about 4 KB. The size of the probe used is relatively large which may increase the difficulty of the probe penetrating the tissues of embryo and may also increase nonspecific binding through partial hybridization. As it's known there are two closely related genes in *Xenopus tropicalis*, Dennd5a and Dennd5b, the two of which share about 70% identity through BLAST. From Figure 7, the Dennd5a expression can be seen in the animal side of stage 8, in between the two neural folds, in spinal cord or neural tube, in forebrain and hindbrain, in **heart** and in eyes.

It is noteworthy that Daam1 mentioned in Chapter 1 is also expressed in the mouse heart and Daam1-deficient mice exhibit embryonic and neonatal lethality and

suffer multiple cardiac defects, including ventricular noncompaction, double outlet right ventricles and ventricular septal defects (47). Bao et al also reported that a single-copy deletion of Daam1 can lead to complex congenital heart defect (48). What is the significance of an interaction between Daam1 and Dennd5a that is confirmed through immunoprecipitation (data will be shown later.) in heart development is still unclear.

In wormbase, lst-6 (lateral signaling target), homolog of Dennd5a, is expressed in nervous system and head. No data is reported in zfin.org (zebrafish database).

In human protein atlas database(http://www.proteinatlas.org/ENSG00000184014-DENND5A/tissue), Dennd5a protein can be detected in most organ systems but not in blood and immune system and **skin**. Also, the Dennd5a protein expression discrepancy in cancer tissues and normal tissues is prominent especially in melanoma and **skin** cancer from the database.

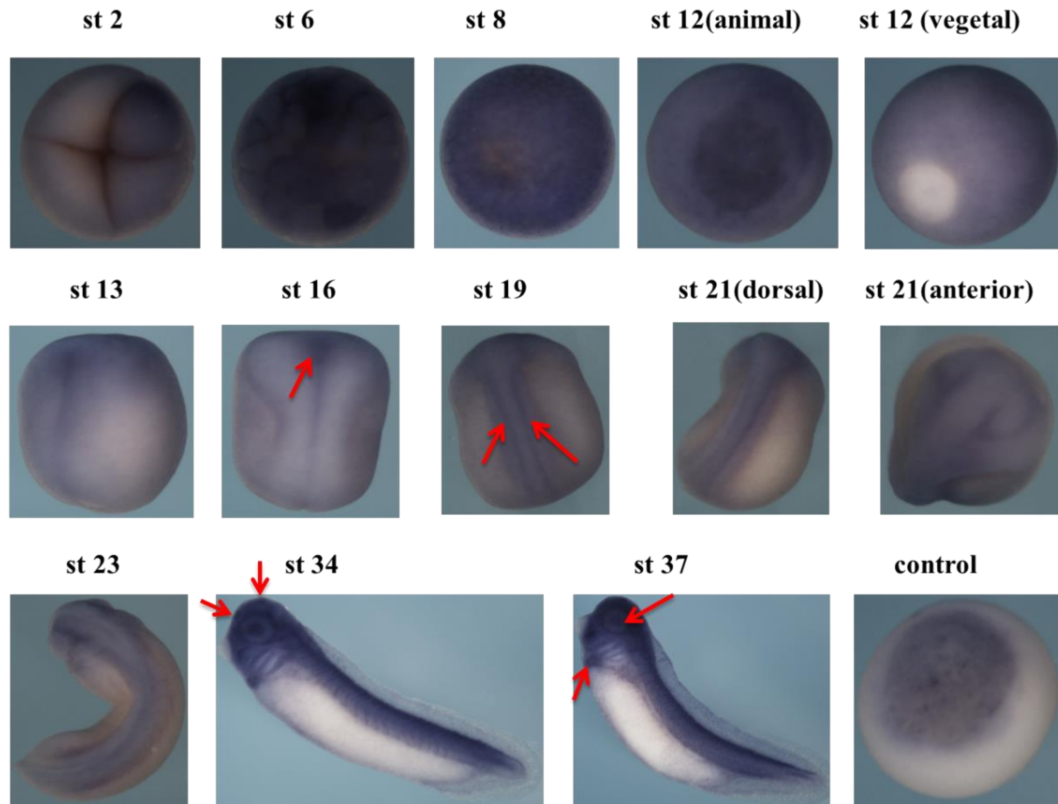


Figure 7. Dennd5a expression pattern through in-situ hybridization. Arrow in stage 16 points to the cleft between neural folds. Arrows in stage 19 point to the neural tube. Arrows in stage 34 point to brain area. Arrows in stage 37 point to eye and heart.

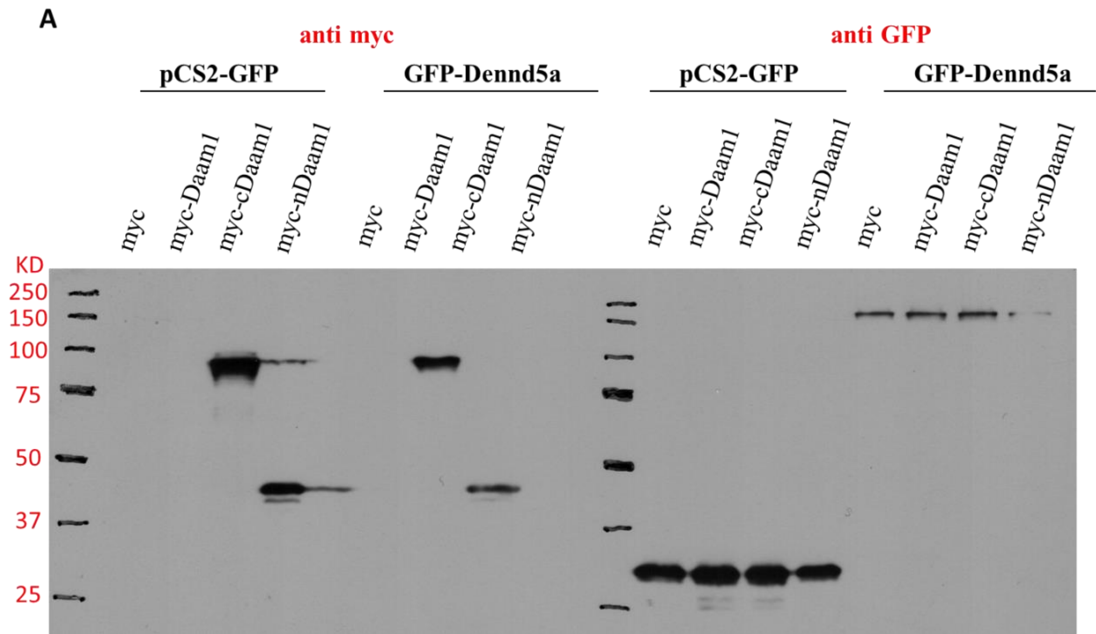
CHAPTER 4

DAAM1 IS ONE OF THE INTERACTION PARTNERS OF DENND5A

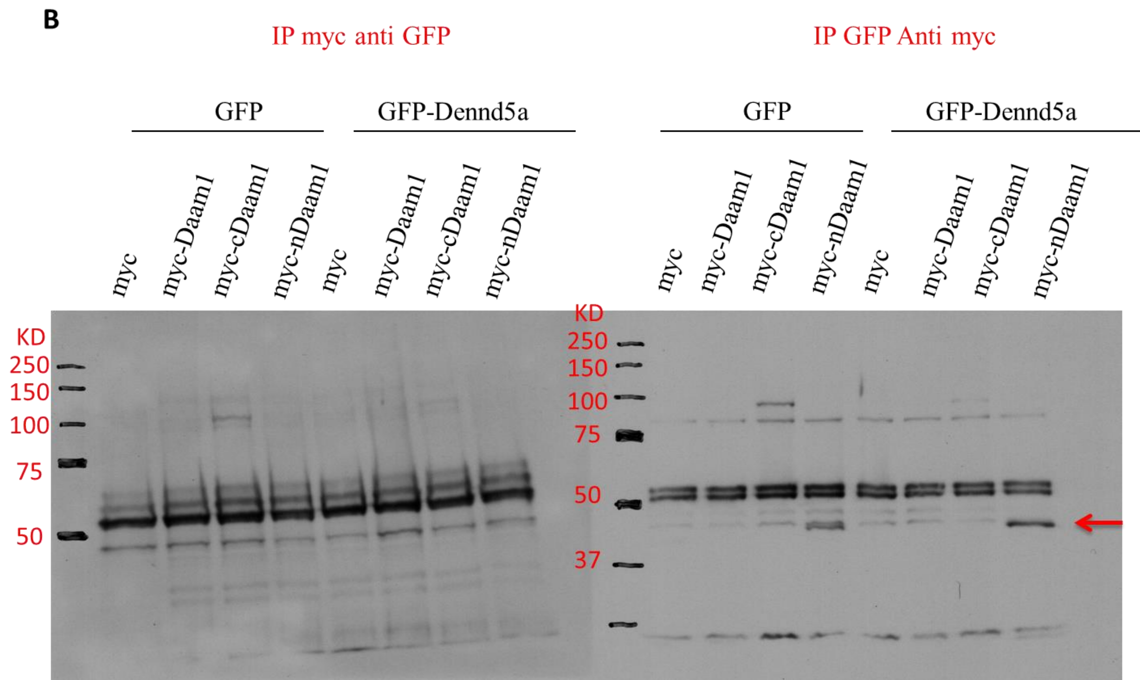
4.1 N-terminal Daam1 Interacts With Dennd5a in Immunoprecipitation (IP) Experiement

Dennd5a was identified as a yeast two-hybrid screen where Daam1 was used as bait. IP was employed to confirm this interaction. Figure 8C shows domains of Daam1. Figure 8A shows the western blot of lysate; GFP-Dennd5a band is slightly above the 150 KD marker band and myc-Daam1 runs about 150 KD (not transfected for some reason in this experiment, will show in later experiment), myc-cDaam1 runs a little below 100KD and myc-nDaam1 runs between 50KD and 37 KD. Figure 8B is the IP result in both directions, IP GFP western blot anti myc and IP myc western blot anti GFP; the nDaam1 binds to Dennd5a. As it is known that nDaam1 has a GTPase binding domain, the significance of the binding is yet to be uncovered. It's reported that nDaam1 can collapse actin fibers while cDaam1 can induce stress fibers; nDaam1 can bind to Rho, preferentially GTP-bound Rho, the activation of which can induce stress fiber formation(21). Expression of a constitutively active C-terminal domain of Daam1 selectively inhibited endothelial cell proliferation, while and nDaam1 appeared to inhibit cell proliferation in a cell-unselective manner (49).

A



B



C

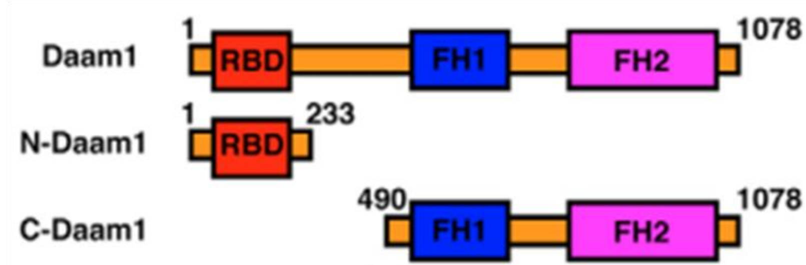


Figure 8. Dennd5a interacts with Daam1 in IP experiment. A) Western blot shows the lysate protein expression level. Protein markers show the size on the left; anti myc, anti c-myc primary antibody; anti GFP, anti GFP primary antibody; vectors that are transfected in each sample are labelled on top. B) IP shows N terminal Daam1 (nDaam1) interacts with Dennd5a. Protein markers show the size in KD (kilo dalton) on the left; IP GFP anti myc, IP with GFP antibody and anti c-myc primary antibody; IP myc and anti GFP, IP with c-myc antibody and anti GFP primary antibody; vectors that are transfected in each sample are labelled on top. Arrow points where nDaam1 band is. C) Domains of Daam1. RBD, Rho binding domain; FH, formin homology. Edited from Akira Sato et al 2006 Development.

4.2 The Interacting Between Dennd5a and Daam1 is Independent of RUN2 Domain

A deletion mutation occurred during cloning process probably because of the PCR error. The one nucleotide (adenine) deletion happens at position 3225 of *Xenopus* Dennd5a gene where there is five consecutive adenine; some research has reported that these **homopolymers** (that is, consecutive instance of the same base) may be one source of PCR error (50). QuikChange II XL Site-Directed Mutagenesis Kit was used to fix the mutation. The sequence of the fixed DNA was confirmed by sequencing results and western blot of the fixed Dennd5a was also done (Figure 9). The upper band in the blot is fixed Dennd5a and the lower one is Dennd5aΔRUN2. They are estimated ~ 20 KD different in size.

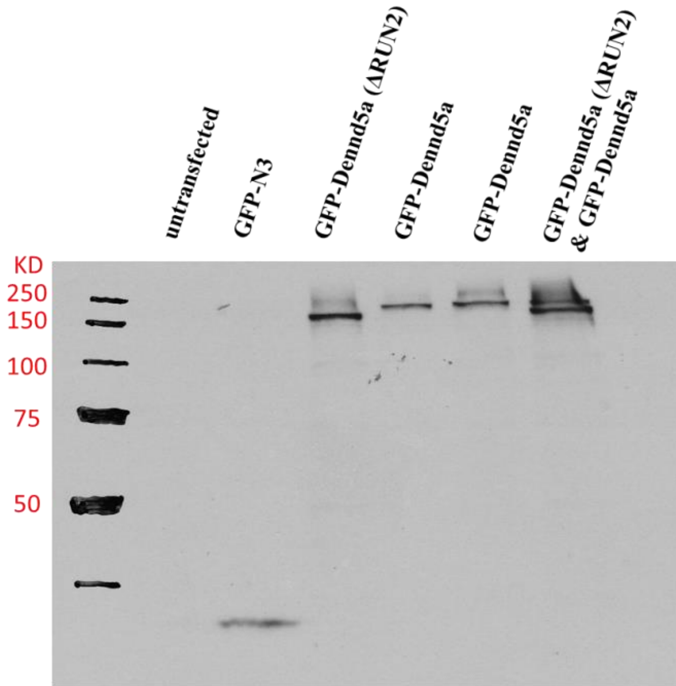


Figure 9. Western blot shows the expression of GFP-Dennd5a(upper band) and GFP-Dennd5a (ΔRUN2) [lower band].

The mutant Dennd5aΔRUN2 which lacks the second RUN domain was used in IP experiment to test its ability to interact with Daam1. Western blot shows the lysate protein expression level in Figure 10A. Figure 10B. IP shows N terminal Daam1 (nDaam1) interacts with Dennd5aΔRUN2; this interaction is shown in both directions, IP with GFP antibody blotting with anti myc and IP with myc antibody blotting with anti GFP. Since Dennd5aΔRUN2 doesn't contain the second RUN domain, it seems that RUN2 domain is not essential for the interaction between Dennd5a and Daam1. As mentioned in chapter 1 (1.3.4), RUN2 domain can interact with sorting nexin 1 (SNX1) (27).

Further investigation of which domain within Dennd5a is essential for the interaction can be done by cloning out individual domain and performing IP experiment or GST pull-down experiment.

A

anti GFP

GFP | GFP-Dennd5aΔRUN2

myc-Daam1 myc-cDaam1 myc-nDaam1 myc myc-Daam1 myc-cDaam1 myc-nDaam1

KD
250
150
100
75
50

anti myc

GFP | GFP-Dennd5aΔRUN2

myc-Daam1 myc-cDaam1 myc-nDaam1 myc myc-Daam1 myc-cDaam1 myc-nDaam1

B

IP GFP Anti myc

GFP | GFP-Dennd5aΔRUN2

myc-Daam1 myc-cDaam1 myc-nDaam1 myc myc-Daam1 myc-cDaam1 myc-nDaam1

KD
250
150
100
75
50

IP myc anti GFP

GFP | GFP-Dennd5aΔRUN2

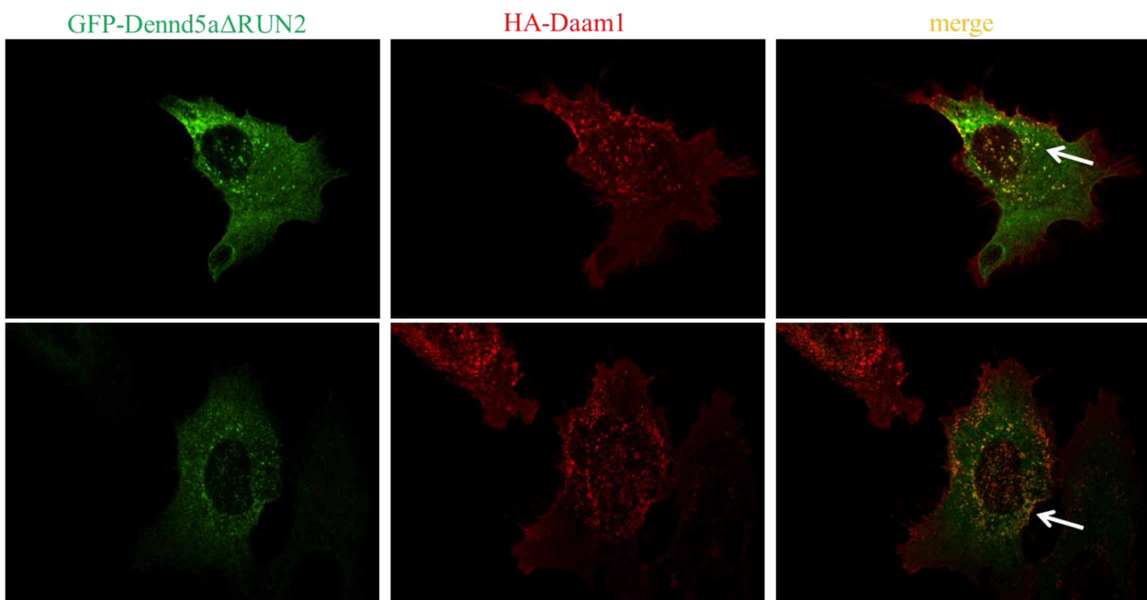
myc-Daam1 myc-cDaam1 myc-nDaam1 myc myc-Daam1 myc-cDaam1 myc-nDaam1

250
150
100
75

Figure 10. Dennd5a(ΔRUN2) interacts with Daam1 in IP experiment. A) Western blot shows the lysate protein expression level. Protein markers show the size on the left; anti myc, anti c-myc primary antibody; anti GFP, anti GFP primary antibody; vectors that are transfected in each sample are labelled on top. GFP-Dennd5aΔRUN2, Dennd5a without the second RUN domain. B) . IP shows N terminal Daam1 (nDaam1) interacts with Dennd5aΔRUN2. Protein markers show the size on the left; IP GFP anti myc, IP with GFP antibody and anti c-myc primary antibody; IP myc and anti GFP, IP with c-myc antibody and anti GFP primary antibody; vectors that are transfected in each sample are labelled on top. Arrow points where Daam1 (top left), nDaam1 (bottom left) and Dennd5aΔRUN2 (top right) bands are.

4.3 Dennd5a Colocalizes With Daam1/nDaam1 in HeLa Cells

The IP experiment above shows there is strong interaction between Dennd5a and Daam1/nDaam. So, to further prove this, vectors containing the two genes are transfected into HeLa cells and confocal microscope is used to check their subcellular colocalization. In figure 11A, the mutant GFP-Dennd5aΔRUN2 can colocalize with Daam1 on punctate or vesicle like structures. However, this kind of colocalization occurs in a very low frequency, lower than 10% estimated from my experiments. In figure 11B, wild type of Dennd5a is used; it can be seen Dennd5a colocalizes with nDaam1 in a low level in both short (7 hours) and long (21 hours) protein expression time and this colocalization is barely on those punctate or vesicle-like structures. The low level colocalization may be due to the *Xenopus* version of Dennd5a used in the immunostaining experiment where human cell line is used. Also, IP experiment where millions of cells overexpressing the tested proteins are collected is a cumulative result whereas immunostaining is individual phenomenon. It is also possible that the **factors** that lead to the colocalization of Dennd5a and nDaam1 are missed, so one interesting question here is when or what can cause the Dennd5a to colocalize on those vesicle-like structures.


A
GFP-Dennd5aΔRUN2
HA-Daam1
merge

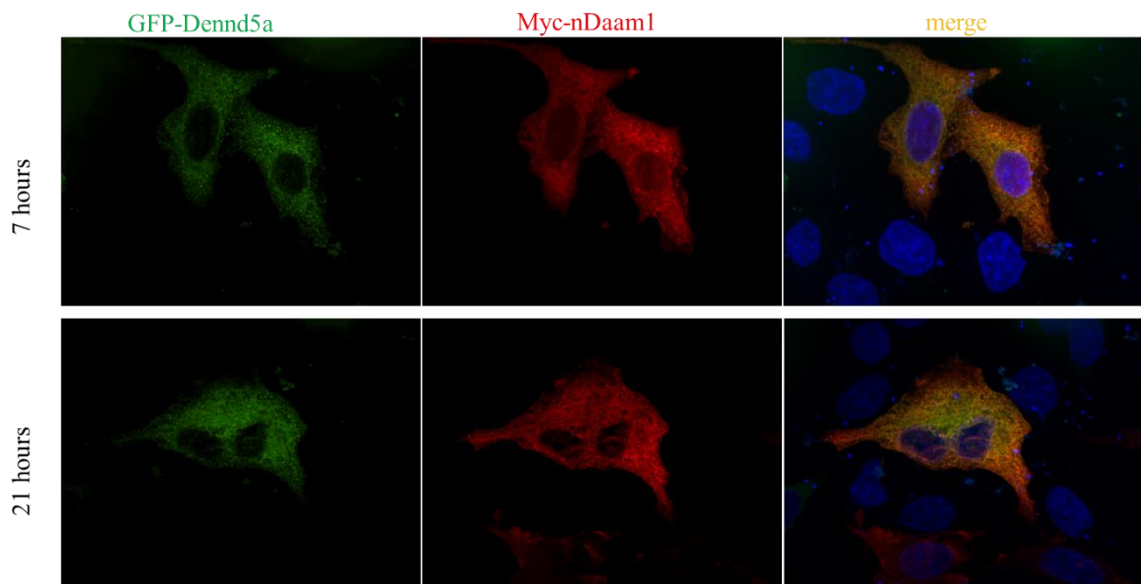

B
GFP-Dennd5a
Myc-nDaam1
merge
7 hours
21 hours

Figure 11. Dennd5a and Daam1 can colocalize with each other in cellular level. A) Confocal images show the localization of the mutant GFP-Dennd5aΔRUN2 and HA-Daam1 in HeLa cells without any treatment. The mutant can colocalize with Daam1 on the vesicle-like structures. Note, there images are one slice through the middle of the cell. B) Confocal images show the colocalization of GFP-Dennd5a and myc-nDaam1 in HeLa cells. The upper panel protein expression time is ~7 hours and the lower panel is ~21 hours. Note, each image is Z series maximum of stack images.

4.4 Final Note

All the IP experiments are performed by transfecting gene-containing vectors into 293T cells. Dennd5a gene is from *X. laevis*. However, Daam1 gene is from human. Both HeLa cell and 393T cell are derived from human tissues. Even though *Xenopus* Dennd5a protein shares ~89% identity with human Dennd5a, caution should be taken that this may affect the experiment sensitivity when we try to interpret experiment result, especially in subcellular level where we examine cells individually.

CHAPTER 5

IS WNT SIGNALING A PLAYER IN THE INTERACTION OF DENND5A AND DAAM1?

5.1 Introduction of Non-canonical Wnt Signaling

The Wnt signaling pathway is a conserved network of proteins known for its crucial role in various aspects of cell fate determination, cell migration, cell polarity, neural patterning during embryonic development. Currently, three major Wnt signaling pathways have been discovered: canonical Wnt pathway or beta-catenin dependent pathway, noncanonical Wnt pathway or beta-catenin independent pathway, and Wnt/Ca^{2+} pathway(51). Non-canonical Wnt pathways are diverse and in many cases less characterized/defined. They are grouped into several categories such as planar cell polarity (PCP) that plays important role in convergent and extension movement, WNT/RAP1, WNT/ROR2, and WNT-GSK3-MT (52,53).

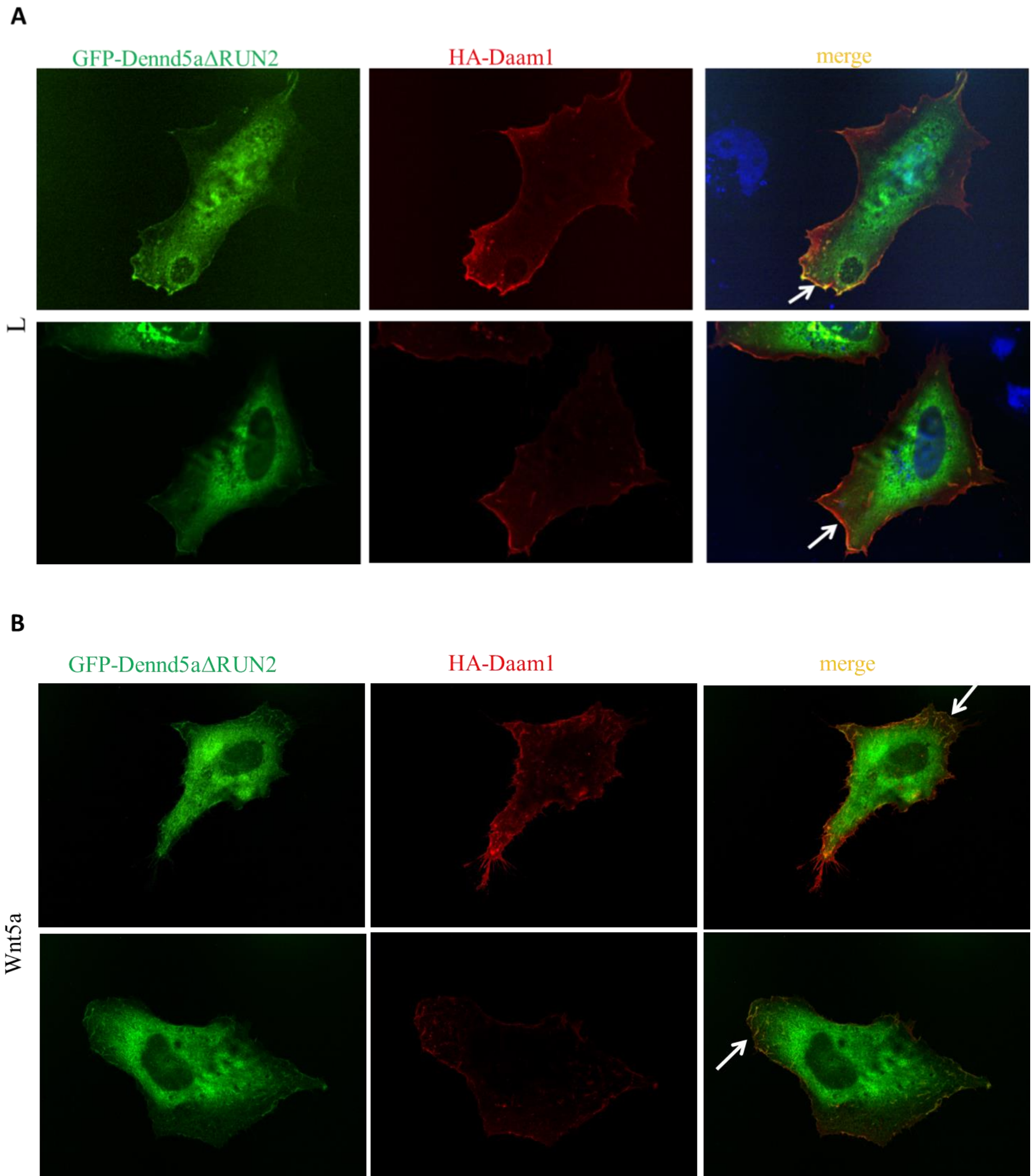
Nineteen Wnt genes and ten Frizzled (Fz) genes have been found in both the mouse and human genomes (54,55). It is accepted that the Frizzled (Fz) family of transmembrane proteins are cell surface receptors for Wnt proteins. Wnt molecules were initially classified into either the canonical subfamily (Wnt-1, Wnt-3a, and Wnt-8) or the noncanonical subfamily (Wnt-4, Wnt-5a, and Wnt-11) (54). However, this classification

may be oversimplified: in *Xenopus laevis* embryos, Wnt-5A induced axis duplication and an ectopic Spemann organizer in the presence of hFz5, a member of the ten Frizzled transmembrane receptors (56); in mammalian cells and *Xenopus* embryos, Wnt-1 in mammalian cells and Wnt-11 in *Xenopus* embryos activates both Rho through Dishevelled (Dvl)-Daam1complex and Rac in a Daam1-independent manner, and Rac activation mediates Wnt/Fz activation of Jun N-terminal kinase (JNK) (57); in a PC12 and N1E-115 cell model, Wnt-3a and Dvl regulate neurite formation through Rho-kinase (58).

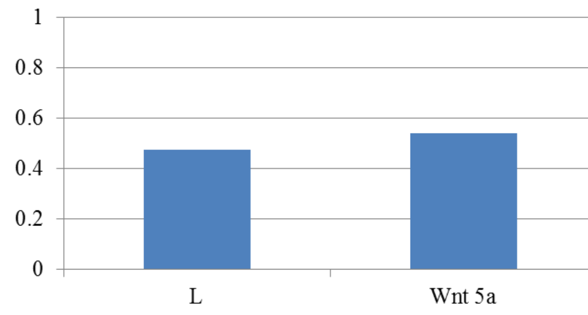
Several Rho-guanine nucleotide exchange factor (Rho-GEF) including p114-RhoGEF and Lfc (or GEF-H1) are critically involved in **Wnt3a** and Dvl-induced RhoA activation through interaction between their DH-PH domain of these Rho-GEF and **nDaam1** and interaction of between Rho-GEF and region around PDZ domain of **Dvl-1** (Dishevelled-1) (59). Weak-similarity GEF (WGEF) is reported to form a complex through interaction with nDaam1 and PDZ domain of Dvl-2 to activate Rho(60). Besides, Dennd5a is proposed as a GEF for Rab39 as it is sensitive to Dennd5a and Dennd5b(38). **So, it needs to be tested if Dennd5a works in a similar fashion like p114-RhoGEF and Lfc (or GEF-H1) or WGEF.**

5.2 Wnt5a

To investigate the effect of Wnt5a on the colocalization of Dennd5aΔRUN2 and Daam1, HeLa cells are starved in extremely low fetal bovine serum containing medium (FBS, no more than 0.5%) for ~20 hours before treatment with Wnt. In both L (control medium, Figure 12A) and Wnt5a condition medium (Figure 12B), Dennd5aΔRUN2 and Daam1 can colocalize beneath the cell membrane or close to cell membrane or cell membrane ruffle-like region as indicated by the arrows. The ratios of this colocalization under L and Wnt5a treatment are ~47% and 54%, which is not statistically different. It is needed to point out that Dennd5a used here has no second RUN domain which is proposed to bind to sorting nexin 1 (SNX1) through its PX domain (27). In Figure 12D, HeLa cells are incubated in 10% FBS medium, the mutant GFP-Dennd5aΔRUN2 mainly localizes within perinuclear area where Golgi complex and Endoplasmic Reticulum may reside, condenses on a structure pointed by arrow close to nucleus and it doesn't colocalize with actin fiber stained by phalloidin. Note, each image in figure 12D is one slice through the middle of the cell.


A
GFP-Dennd5aΔRUN2
HA-Daam1
merge
L
B
GFP-Dennd5aΔRUN2
HA-Daam1
merge
Wnt5a

C



D

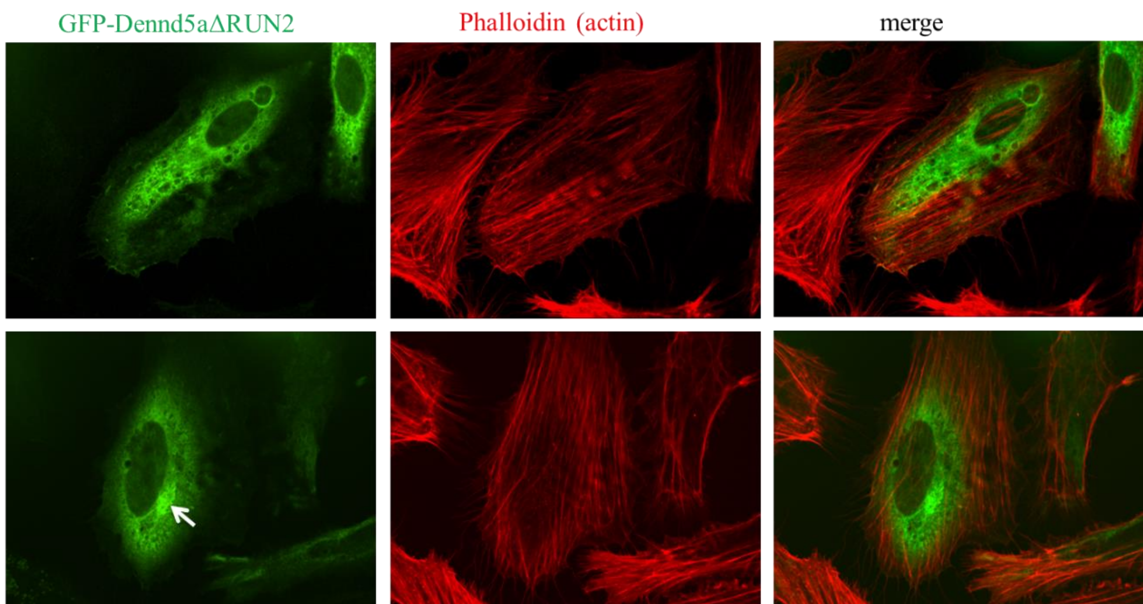
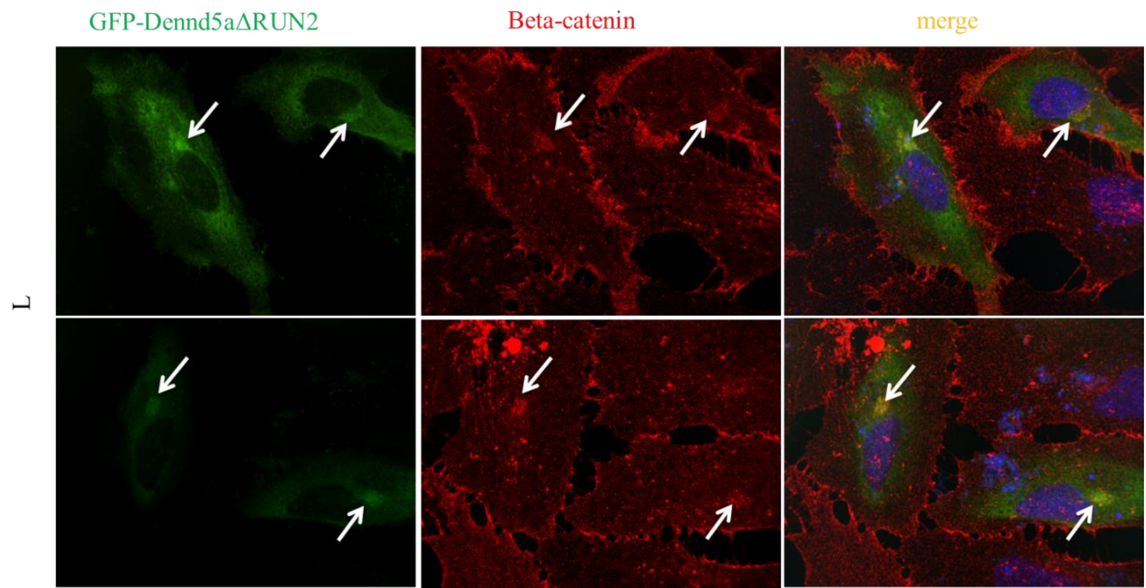


Figure 12. Wnt5a doesn't appear to affect the colocalization of GFP-Dennd5aΔRUN2 and Daam1. A) Confocal images show the localization of the mutant GFP-Dennd5aΔRUN2 and HA-Daam1 under L control medium in HeLa cells (cell goes through ~20 hrs starvation.). The mutant can colocalize with Daam1 along the cell membrane or close to cell membrane. Note, each image is one slice through the middle of the cell. B) Confocal images show the localization of the mutant GFP-Dennd5aΔRUN2 and HA-Daam1 under Wnt5a condition medium in HeLa cells (cell goes through ~20 hrs starvation.). The mutant can colocalize with Daam1 along the cell membrane ruffle-like structure. Note, each image is one slice through the middle of the cell. C) Wnt5a doesn't appear to affect cell membrane or close to cell membrane localization with wnt5a condition medium treatment. D) Confocal images show the localization of the mutant GFP-Dennd5aΔRUN2 in HeLa cells. The mutant mainly localizes within perinuclear area where Golgi complex and Endoplasmic Reticulum may reside, condenses on a structure close to nucleus and it doesn't colocalize with actin fiber stained by phalloidin. Note, each image is one slice through the middle of the cell.

5.3 Wnt3a

Figure 13A, Confocal images show the localization of the mutant GFP-Dennd5aΔRUN2 and beta-catenin under L control medium in HeLa cells (cell goes through ~20 hrs starvation.). The mutant shows an extremely similar localization pattern to that in 10% FBS medium; surprisingly, the mutant appears to colocalize with beta-catenin very nicely as pointed by arrows and this colocalization is highly consistent (almost all cells that are scanned by confocal microscope show this pattern.). However, Figure 13B. Confocal images show the localization of the mutant GFP-Dennd5aΔRUN2 and beta-catenin under Wnt3a condition medium in HeLa cells (cell goes through ~20 hrs starvation.). The mutant doesn't seem to be affected by Wnt3a and still shows an extremely similar localization pattern to that in 10% FBS medium; however, the mutant loses its colocalization with beta-catenin which goes to nucleus induced by Wnt3a as pointed by arrows, even the molecular mechanism is not fully understand (61-64).

A



B

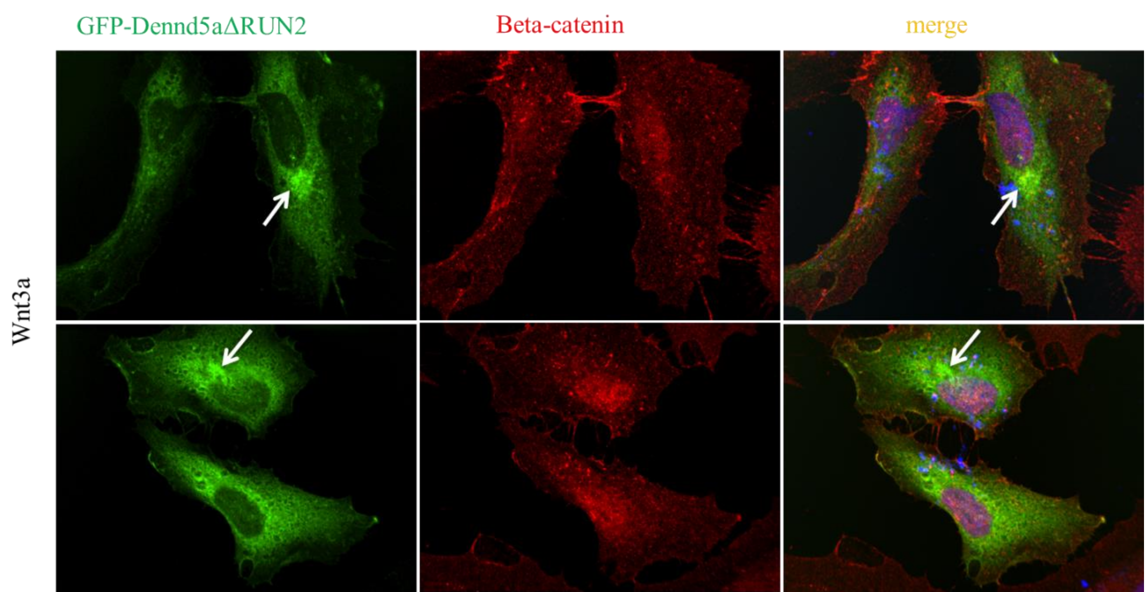


Figure 13. Wnt3a doesn't affect the localization of Dennd5aΔRUN2. A) Confocal images show the localization of the mutant GFP-Dennd5aΔRUN2 and beta-catenin under L control medium in HeLa cells (cell goes through ~ 20 hrs starvation.). The mutant shows an extremely similar localization pattern to that in 10% FBS medium; surprisingly, the mutant appears to colocalize with beta-catenin very nicely as pointed by arrows. B) Confocal images show the localization of the mutant GFP-Dennd5aΔRUN2 and beta-catenin under Wnt3a condition medium in HeLa cells (cell goes through ~20 hrs starvation.). The mutant doesn't seem to be affected by Wnt3a and still shows an extremely similar localization pattern to that in 10% FBS medium; however, the mutant loses its colocalization with beta-catenin which goes to nucleus induced by Wnt3a as pointed by arrows.

CHAPTER 6

DENND5A PLAYS A PIVITOL ROLE IN *XENOPUS* EMBRYONIC DEVELOPMENT

6.1 *Xenopus* Embryonic Development

Xenopus embryonic development starts from a fertilized egg (Figure 14. Stage 1, animal pole) marked by a dark spot on the animal side of the zygote. Immediately after fertilization, cortical cytoplasmic rotation and sperm and egg pronucleus occur. The first cell cycle takes a longer period around 90 minutes with gap phases. The following eleven cleavages occur at a shorter interval of 20- to 30-minute without gap phases; the embryo forms a ball of about 4000 cells, which is called blastula (Figure 14. stage 2 to stage 10) and encloses a fluid-filled blastocoel cavity. Normally the dorsal cells have a lighter pigmentation than the ventral cells and slightly smaller at stage 3. At stage 10, dorsal lip which runs from left to right above the yoke plug shows up and gastrulation starts. The gastrulation spans from stage 10 to stage 12. Several morphogenetic events happen including tissue specification (the formation of the three-layer gastrula including ectoderm, mesoderm and endoderm.), convergent extension and blastopore closure. Besides, the anterior-posterior, dorsal-ventral and left-right axes are also defined at gastrulation (65). Neurulation occurs from stage 13 to stage 18 during which period the neural plate folds and closes to for neural tube that will develop into nervous system later.

Organs need to be developed in order to be a fully functional individual. Organogenesis meaning the formation of organ happens from stage 19 (tailbud) to around stage 45 (free swimming tadpole).

For a real life staging of *Xenopus*, readers can also visit http://www.swarthmore.edu/NatSci/sgilber1/DB_lab/Frog/frog_staging.html.

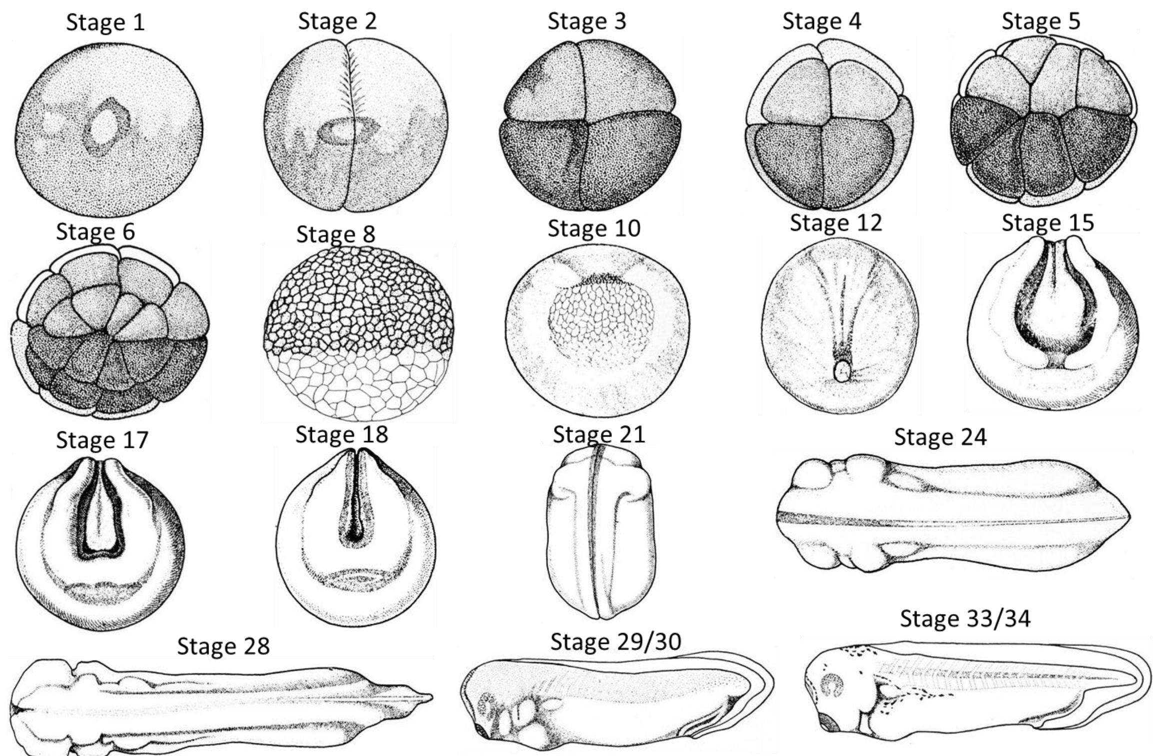


Figure 14. schematic shows developmental stages of *Xenopus*. Digitized images and developmental data from Nieuwkoop and Faber (1994) Normal Table of Xenopus laevis (Daudin). Garland Publishing Inc, New York ISBN 0-8153-1896-0.

6.2 Knockdown of Dennd5a Disrupts Normal Embryonic Development and Eventually Leads to Death as Embryos Develop into Later Stages.

In order to study the function of the gene of Dennd5a in early embryonic development, knockdown of Dennd5a is performed by injecting specifically designed morpholino oligonucleotides that target the gene's 5'UTR (upstream untranslated region). Morpholino oligonucleotides are short chains of about 25 morpholino subunits. Each subunit is comprised of a nucleic acid base, a morpholine ring and a non-ionic phosphorodiamidate intersubunit linkage. Morpholinos do not degrade their RNA targets, but instead act via an RNAse H-independent steric blocking mechanism (www.gene-tools.com).

The 5'UTR has to be cloned since the full genomic sequence map of *Xenopus laevis* is still under construction. 5'RACE (rapid amplification of cDNA end) was used to amplify and clone the fragment and stage 7 and 15 cDNA are used as the template. Interestingly, **two 5'UTRs** are amplified including the one for Dennd5a and the other one sharing ~89% identity and 81% coverage compared to 5'UTR of Dennd5a. The initial part (about 200 bp) of both genes are also obtained during 5'RACE; they share 92% identity and 97% coverage under BLAST of NCBI and the amino acid sequences coded by the initial part of both genes are almost identical. So, it's likely the other 5'UTR belongs to Dennd5b as it is in *Xenopus tropicalis*. It's also known that the genome of *Xenopus laevis* is allotetraploid resulted from whole genome duplication during evolution

millions of years ago (45,46). So, it is likely the two genes are a duplicated pair that may still possess similar function. We name this gene Dennd5b temporarily in this thesis. Therefore, the morpholino designed by gene-tools.com targets at 5'UTRs of both genes.

Dennd5a 5'UTR
5'GGGCCCCCGAAGGAGGATGACAGGCGACGGGA[GAGGAGGGCACGGGAGGTCTGA]CAGCGGCACT (**ATG**)AGCGGGGGAGGTGGAGGAGGTA3'

Dennd5a-related 5'UTR
5'CCCGGGGCCCCGGAGGAGGATGACAGGCGGGA[GAGGAGGGCACGGGAGGTCTGA]CAGCAGCACC (**ATG**)AGCGGGGGAGGAGGAGGAGGAG3'

Morpholino oligo sequence complementary to translation-blocking target:
5'TCAGACCTCCCGTGCCCTCCTC3'

Figure 15. Morpholino sequence is shown to target 5'UTR of both genes. The target region is in the bracket which have identical sequence. The start codons of both genes are in parenthesis. Note, only partial UTR sequence is shown here for both genes.

The efficiency of morpholino is also tested. A translatable fragment containing partial 5'UTR and the initial coding sequence of ~200 bp for both genes are cloned onto pCS2-myc vector. MO-5'UTR-Dennd5a&b, morpholino that target 5'UTR of both genes, and 5'UTR RNAs that have the morpholino binding sequence are co-injected dorsally into frog embryos at four cell stage (stage 3 in Figure 14). Embryos are lysed at stage 12/13 and western blot is performed to examine myc tag expression estimating the protein levels. Alpha tubulin (which is extremely conserved) is used as a loading control to make sure relatively the same amounts of proteins are loaded for each sample. Lane 1, 2, 5, 6 in Figure 16 show protein expression of myc-5'UTR-Dennd5a with and without morpholino. 5 ng/embryo morpholino (lane 5) decreases the protein expression to ~ half of its original level (comparing to lane 2 of Figure 16) and 10 ng/embryo morpholino

decreases the protein expression to ~ half of the level of 5 ng/embryo lane. Without surprise, MO-5'UTR-Dennd5a&b shows a similar effect on myc-5'UTR-Dennd5b. Lastly, MO-5'UTR-Dennd5a&b doesn't affect the expression of myc-Dennd5a RNA which doesn't have morpholino binding site (in lane 9). Even though the morpholino used here shows effective knockdown in vitro, antibody against endogenous Dennd5a protein is still necessary to quantitatively access the knockdown effect in vivo. There are only human or mouse antibodies against Dennd5a, but the human version protein sequence shared ~ 90 % identity with the frog version. Also, caution should be taken here that the knockdown phenotype may not solely be the result of loss of Dennd5a since both gene expression levels are affected in this case.

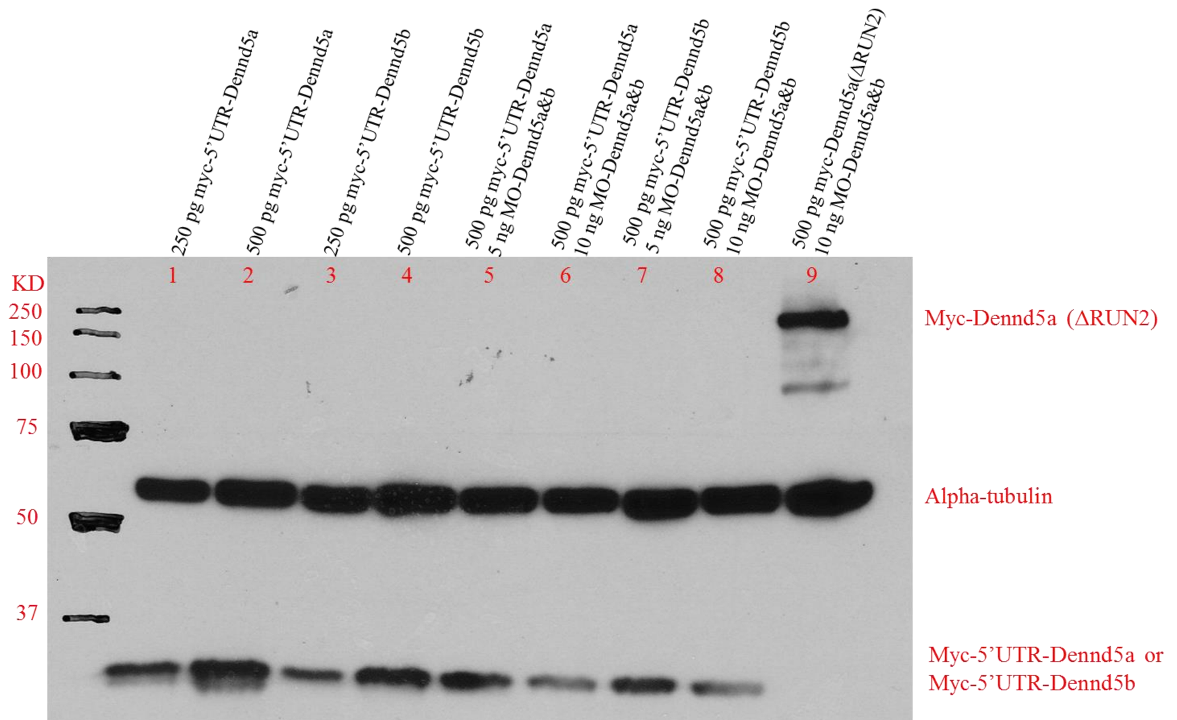


Figure 16. Morpholino and 5'UTR RNAs are co-microinjected into frog embryos to test its efficiency and specificity and results are shown in western blot above. Note, the size of myc-5'UTR-Dennd5a and myc-5'UTR-Dennd5b is almost identical. MO-Dennd5a&b means morpholino that targets 5'UTR of both Dennd5a and Dennd5b.

Loss of Dennd5a and Dennd5b leads to a severe phenotype. As shown in Figure 17, the blastopore is wide open with yoke exposed in stage 13; normally, in stage 13, yoke moves completely inside of embryo and blastopore closes completely during gastrulation; neural plate start to appear. In stage 22, the dorsal side of embryo is wide open even though there are cases of smaller opening (quantitative data will be shown later.). The open-back embryos can still develop into later stages; however, the death rate grows as well. The tadpoles also show reduced agility in swimming (data is not shown.) as the open back may very likely affect the development of neural tube and central nervous system, as it is no surprise since the expression of Dennd5a has been shown in neural tube/spinal cord through whole embryo in-situ hybridization.

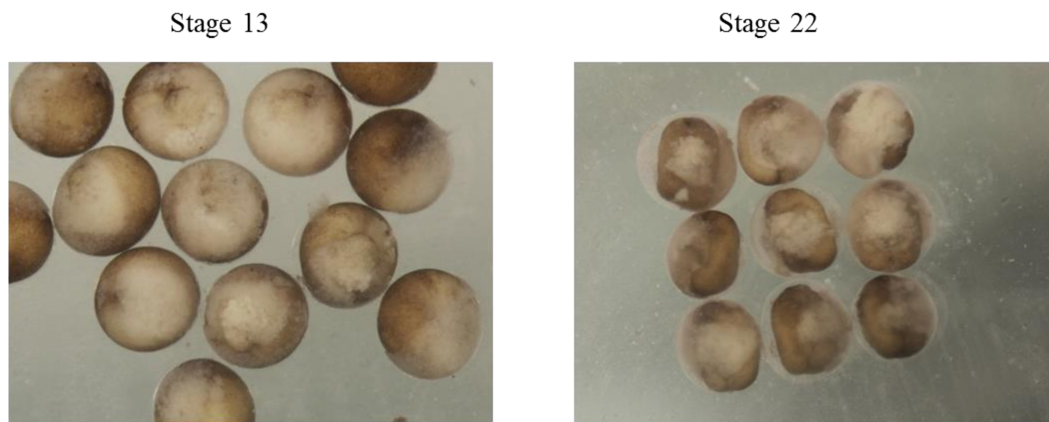


Figure 17. Phenotype is shown resulted from injection of morpholino targeting Dennd5a and Dennd5b. Unclosed blastopore is shown in stage 13 and opened back (dorsal side) is shown in stage 22.

The open-back phenotype correlates with MO_Dennd5a&b morpholino targeting both Dennd5a and Dennd5b genes in a dose-dependent manner (Figure 18). Injection here is all dorsal in four-cell stage (normally lighter smaller cells are dorsal, stage 3 in Figure 14). 2.5 ng/embryo MO_Dennd5a&b leads to ~15% embryos showing the phenotype, 5 ng/embryo MO_Dennd5a&b results ~45% embryos showing the phenotype and 10 ng/embryo MO_Dennd5a&b leads to ~80% embryos showing the phenotype. Even though ~16% of total embryos show the same phenotype in control morpholino group, that may come from injection itself or from the process of preparing fertilizing eggs for injection. In the uninjected embryos, this phenotype percentage at ~1% is much lower than any of the injection groups. We conclude that it is the loss of Dennd5a and Dennd5b that can lead to severe open back phenotype at stage 23/24. To make this conclusion more solid, antibody against Dennd5a and Dennd5b can be used to detect remaining protein levels of the defected embryos after in-vivo knockdown; besides, recue experiment can also be performed to make sure this phenotype is specific to Dennd5a and Dennd5b.

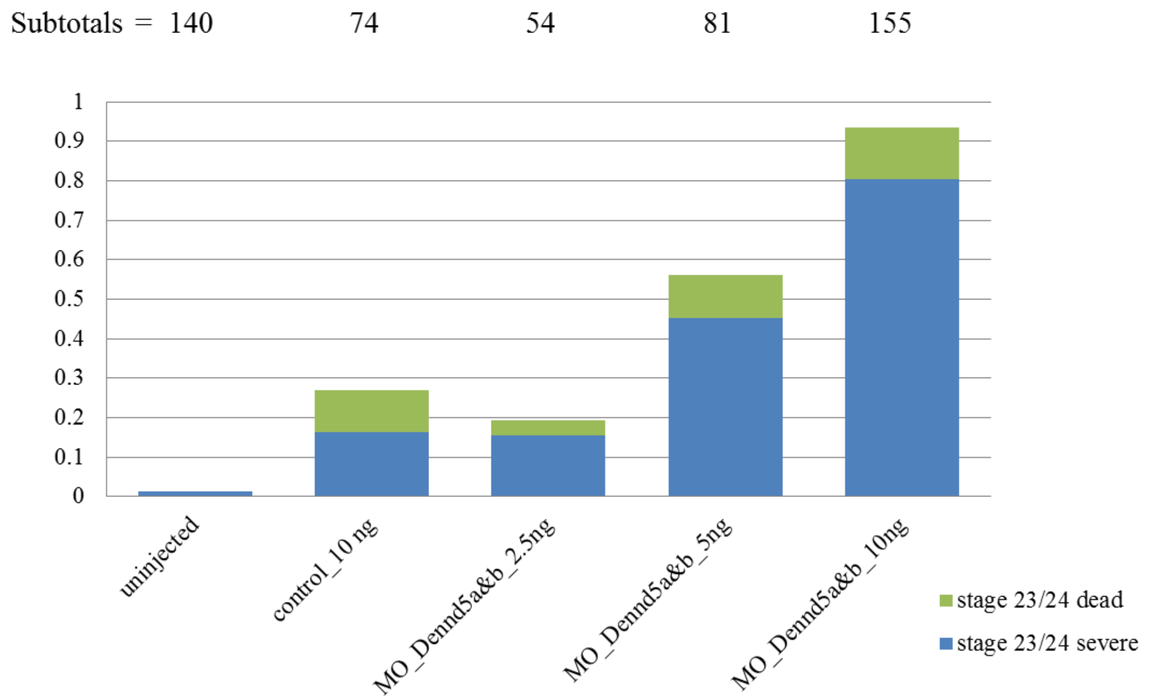
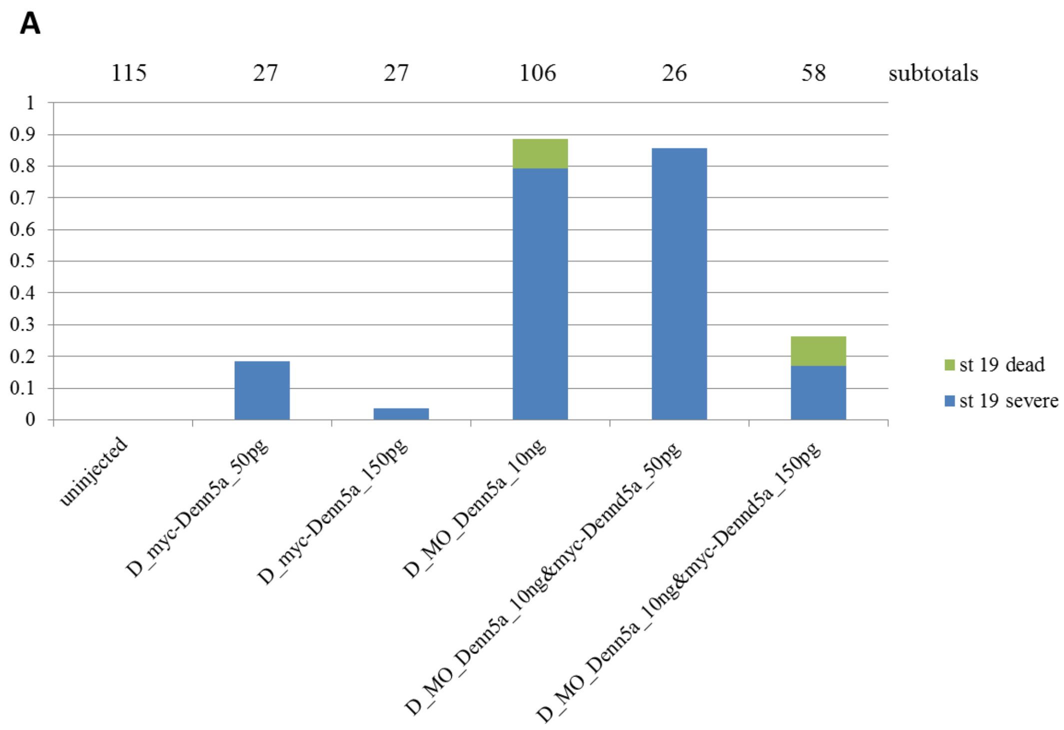


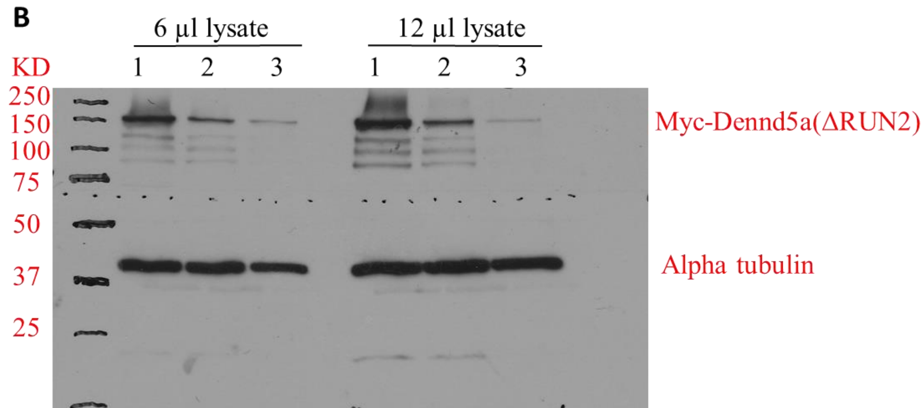
Figure 18. The ratios of “open back” phenotype are correlated to the dose of morpholino targeting Dennd5a and Dennd5b shown at stage 23/24. Note, morpholino is injected dorsally. All “open back” is considered severe. MO_Dennd5a&b_2.5ng means morpholino targeting both Dennd5a and Dennd5b is injected at 2.5 ng/embryo. Subtotals which are combined of repeated experiments for each group are listed on top of the figure.

In order to further prove that the open-back phenotype is caused by loss of Dennd5a and Dennd5b, we conduct a rescue experiment in which both knockdown morpholino and rescue RNA that doesn't have the morpholino binding site are microinjected into four-cell embryos dorsally. Note here we use the mutant RNA myc-Dennd5a(ΔRUN2) that lacks the second of RUN domain. The amount of RNA used is 50 pg/embryo and 150 pg/embryo both of which only give rise to low percentage (<18.5%) of the phenotype. In the group that both 10 ng/embryo MO_Dennd5a and 150 pg/embryo myc-Dennd5a(ΔRUN2) RNA is co-injected, the ratio drops from ~80% of the group without rescue RNA to less than 20%. In another group that both 10 ng/embryo MO_Dennd5a and 50 pg/embryo myc-Dennd5a(ΔRUN2) RNA is co-injected, the rescue doesn't seem to work only because most the embryos are dead and there isn't enough embryo for statistics.

I notice the embryos with open back aren't completely the same: some have some extension but not as extended as the wild type and others have no extension at all appearing "ball-like". Embryos are collected (note, for group 3 ball-like, 6 embryos are collected instead of 5 for the other two groups) as described in Figure 19B. As clearly shown in the western blot, the levels of myc-Dennd5a(ΔRUN2) in the group 2 and 3 are much lower than that of group 1 even though the same amount of RNA is injected. As tested before (Figure 16), MO_Dennd5a morpholino doesn't knock down the expression of myc-Dennd5a(ΔRUN2) that has no binding site for this morpholino. Besides, higher level of Dennd5a(ΔRUN2) leads to more anterior-posterior body extension but not as

extended as the wild type. To test if Dennd5a is directly involved in the convergent extension during gastrulation, Keller explant assay and animal cap explant assay can be performed.





1.myc-Dennd5a(ΔRUN2)_0.5ng Embryos with full extension

2. MO_Dennd5a_10ng & myc-Dennd5a(ΔRUN2)_0.5ng Embryos with some extension

3. MO_Dennd5a_10ng & myc-Dennd5a(ΔRUN2)_0.5ng Embryos with no extension (ball-like)

Figure 19. Dennd5a&b knockdown phenotype can be partially rescued by mutant myc-Dennd5a(ΔRUN2) RNA. A) RNA of myc-Dennd5a(ΔRUN2) can rescue the morpholino knockdown defect. Note, morpholino is injected dorsally ("D" is labeled). All "open back" is considered severe. MO_Dennd5a&b is the same as MO_Dennd5a. Myc-Dennd5a is the RNA synthesized in vitro. Subtotals which are combined of repeated experiments for each group are listed on top of the figure. B) The extension of embryo is correlated to the expression level of rescue RNA through western blot. The legend is at the bottom right. Group 1 embryos with open back but full extension are taken from injection of 0.5 ng/embryo myc-Dennd5a(ΔRUN2) RNA; group 2 embryos with open back but some extension are taken from injection of 10 ng/embryo MO_Dennd5a morpholino and 0.5 ng/embryo myc-Dennd5a(ΔRUN2) RNA; Alpha tubulin is used as loading control.

As mentioned before, MO_Dennd5a is the morpholino targeting both Dennd5a and Dennd5b, however, just Dennd5a RNA seems to rescue the phenotype, which may indicate the functional redundancy of the two genes as the genome of *Xenopus laevis* is allotetraploid from polyploidization during evolution. To further prove this hypothesis, morpholino that targets only Dennd5a or only Dennd5b can be designed and microinjected since both 5'UTRs are obtained.

As I try to identify the phenotype of the embryo in later developmental stage, I find that only a small fraction of the embryos with knocked down of Dennd5a and Dennd5b can live through till tadpole stage and also they are smaller in size which may be attributed to the exposed and "spilled" endoderm. Besides, they also show reduced swimming agility compared to the wild type.

Higher doses, > 20 ng/embryo MO_Dennd5a, are also injected dorsally. Nearly all embryos stop development and die around gastrulation stage appearing to be ball-like.

6.3 Overexpresion of Dennd5a Disrupts Normal Embryonic Development and Eventually Leads to Death as Embryos Develop into Later Stages.

In order to study the function of the gene of Dennd5a in early embryonic development, overexpression of Dennd5a is performed by injecting in-vitro synthesized RNA of Dennd5a.

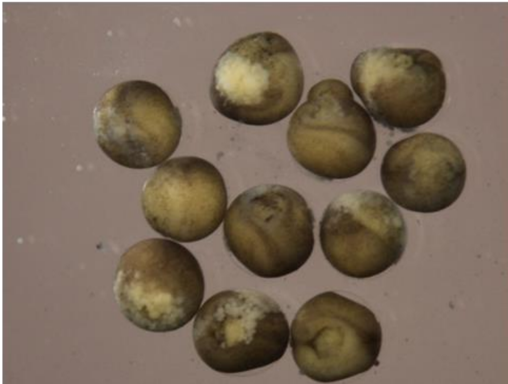
In Figure 20A, when myc-Dennd5a(ΔRUN2) RNA is injected dorsally, the neural tube of embryo can't close and the endoderm is widely exposed as shown in stage 18 and 22. Interestingly, this phenotype is extremely similar to that when MO_Dennd5a&b morpholino is injected. As the amount of RNA increases from 0.5 ng/embryo to 4 ng/embryo, the percentage of open-back embryo also increases as shown in Figure 20B.

GFP-Dennd5a(ΔRUN2) RNA is also injected both dorsally and ventrally and results are shown in Figure 21. As expected, dorsal injection of GFP-Dennd5a(ΔRUN2) show the same phenotype with that when myc-Dennd5a(ΔRUN2) is injected. In Figure 21B, the ratio of phenotype and the amount of GFP-Dennd5a(ΔRUN2) RNA are correlated in a dose-dependent manner which is also similar to that when myc-Dennd5a(ΔRUN2) is injected. However, when embryo is ventrally injected, a large amount of embryos show a sign of death, white and round embryo at stage 19. No other phenotype is distinguishable in ventrally injected embryos.

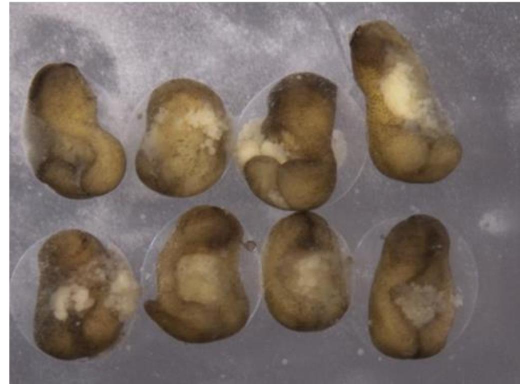
Notably, most embryos will die even before the tadpole stage. Even though a small number of embryos can survive, they have some obvious defect, for example, one eye or no eye (judged by black pigmentation). Rab11 is reported to play a role in fly eye development (66). Also, Rab11 is an interaction partner of Dennd5a or Rab6ip1 described in Chapter 1; besides, both Rab11 and Dennd5a are expressed in eyes (Dennd5a expression data in Chapter 3).

A

Stage 18



Stage 22



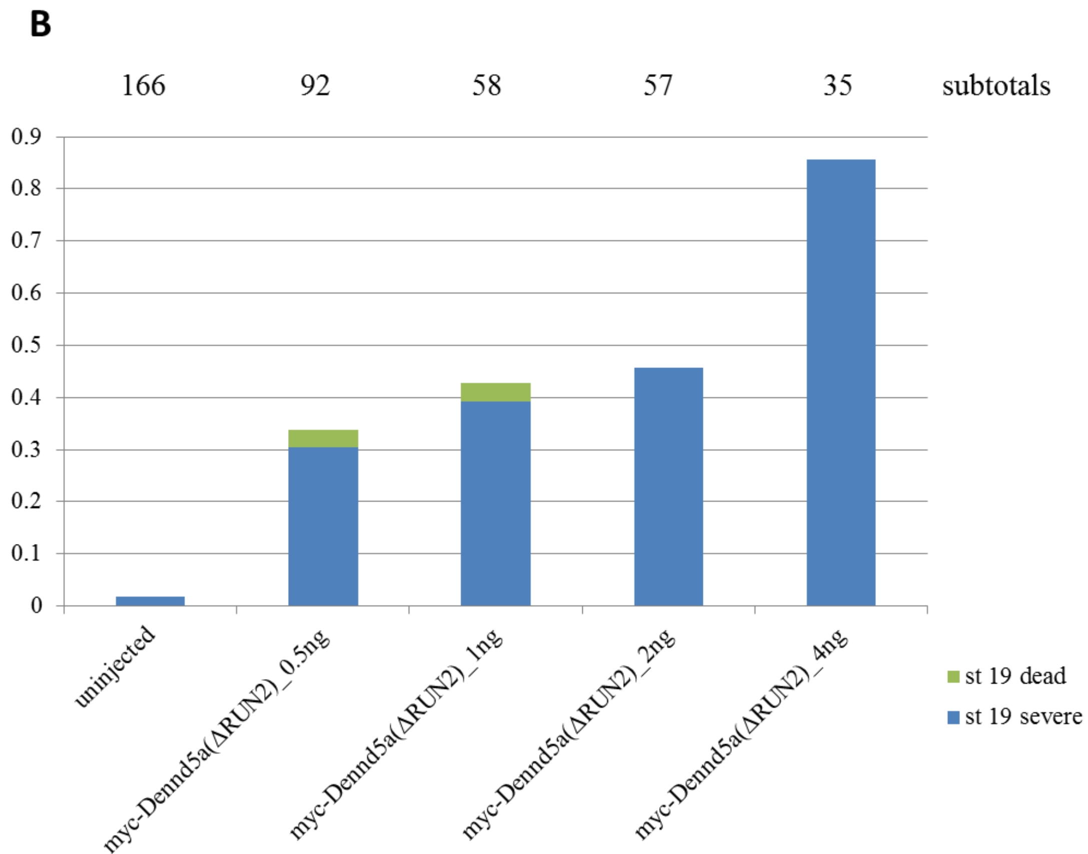
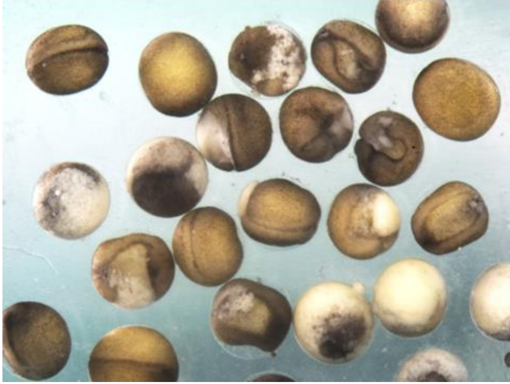


Figure 20. Overexpression of myc-Dennd5a(ΔRUN2) leads to open-back phenotype. A) Phenotype is resulted from dorsal injection of RNA myc-Dennd5a(ΔRUN2). Open back phenotype is shown in stage 18 and in stage 22. B) The open back phenotype is correlated to the amount of myc-Dennd5a(ΔRUN2) RNA injected dorsally. Myc tagged RNA is used in labs and no phenotype is reported.

A



B

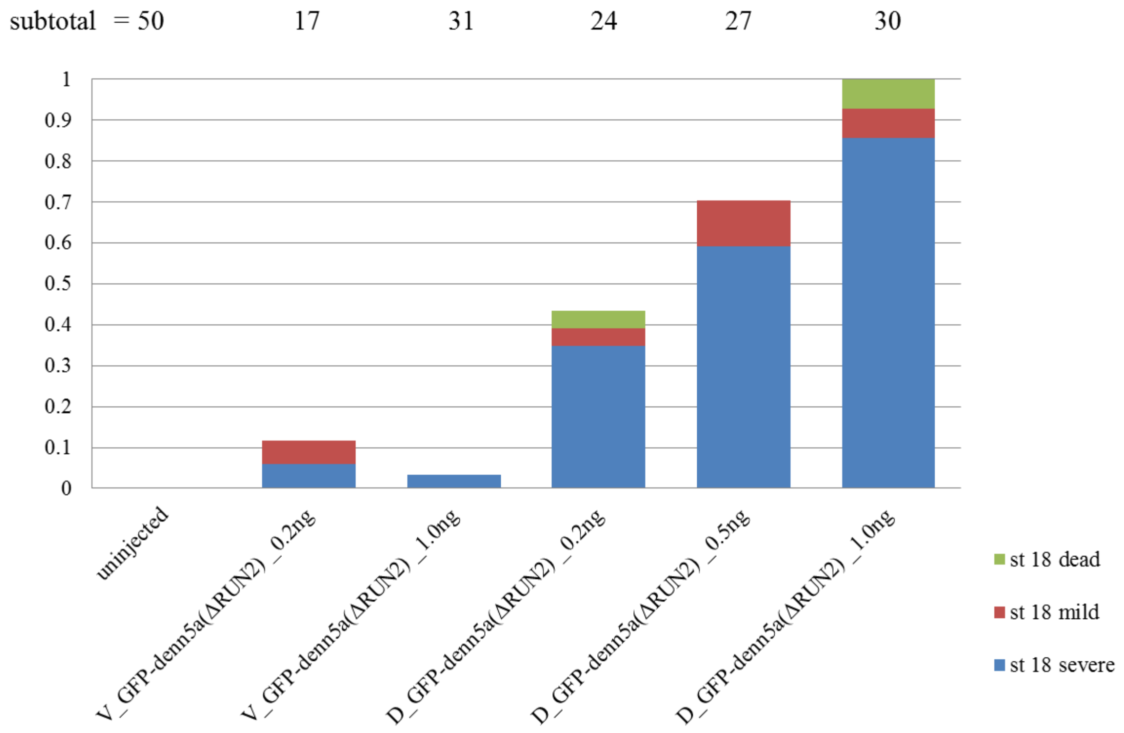


Figure 21. Overexpression of GFP-Dennd5a(ΔRUN2) also leads to open-back phenotype. A) Open back phenotype at stage 19 is resulted from dorsal injection of RNA GFP-Dennd5a(ΔRUN2). Ventral injection of RNA GFP-Dennd5a(ΔRUN2) leads to a large amount of death. B) The open back phenotype is correlated to the amount of GFP-Dennd5a(ΔRUN2) RNA injected dorsally but not ventrally. Note, ventral injection of GFP-Dennd5a(ΔRUN2) RNA leads to significant amount of death which is not reflected in this figure.

In this thesis, several Dennd5a interaction partners have been identified from literatures and they are Rab6, Rab11, sorting nexin 1 and Rab39. All the Dennd5a interacting partners except Rab39 are further confirmed by either GST pull-down assays or immunoprecipitation. Rab6 is extremely conserved across different species in terms of amino acid sequence; for example, Rab6a of *X. laevis* still shares ~ 99.5% identity with its human homologue. Similarly, Rab11 also shares very high identity with its homologues of different species. Rab6 is reported to be involved in inter-Golgi transport; Rab11 is reported to play a crucial role in endosome recycling to cell membrane; Sorting nexin 1 is reported to be critical in the endosome-to-trans-Golgi network (TGN) transport; the three interacting partners, Rab6, Rab11 and Sorting nexin 1, locate Dennd5a into the intracellular transport pathway.

In *X. laevis*, Dennd5a is expressed during all early embryonic developmental stages from cleavage, gastrulation, neurulation to tailbud stage in RT-PCR experiments. Dennd5a is also expressed in the neural fold cleavage, neural tube, heart, brain region and eyes in whole embryo in-situ hybridization experiments.

Dennd5a was identified as a Daam1 interacting partner in a yeast two hybrid screen. Daam1 is a key mediator in Wnt/Frizzled signaling to Rho and regulate gastrulation and neural tube closure in embryonic development. In immunoprecipitation experiments, Dennd5a can bind to amino terminal of Daam1 (nDaam1) and the RUN2 domain of Dennd5a is not essential in this binding. In subcellular experiments, Dennd5a

can colocalize with Daam1 beneath cell membrane and on vesicle-like structures in HeLa cells, which appears to be independent of Wnt5a treatment. Dennd5a can also colocalize with beta-catenin on a perinuclear structure and beta-catenin goes to nucleus under Wnt3a treatment while the localization of Dennd5a is not affected. A moderate colocalization of Dennd5a and nDaam1 is also observed.

Another closely related gene of Dennd5a was discovered in this study and I name it Dennd5b temporarily. From the initial ~ 200 bp of open reading frame, it is predicted that Dennd5a and Dennd5b share more than 90% identity in protein sequence. Note, *X. laevis* has an allotetraploid genome resulted from genomic duplication millions of years ago. Knockdown of both genes in *Xenopus* embryos using morpholino oligonucleotides leads to an "open back" phenotype in stage 18/19 and most of these defected embryos die before the tailbud stage. This defect can be partially rescued by a mutant form RNA of Dennd5a(ΔRUN2) which doesn't contain a functional RUN2 domain. Moreover, injection of Dennd5a(ΔRUN2) RNA into frog embryos results in very similar phenotype to that of knockdown of Dennd5a and Dennd5b genes. More work is still needed to elaborate how this happens in a molecular level.

CHAPTER 7

METHODS AND MATERIALS

7.1 Methods and Materials Used in Chapter 2

cDNA Synthesis

SuperScript III first strand synthesis system for RT-PCR (category #: 18080-051) is used to generate cDNA. Oligo(dT) is used as the primer.

PCR

Phusion high fidelity PCR kit (NEB #E0553S/L) is used for PCR reactions. A routine PCR reaction protocol is used which is supplied with the kit. Note, in all the PCR reactions, no DMSO is used. Primers are designed manually. Melting temperature is checked through OligoAnalyzer tool (https://www.idtdna.com/calc/analyzer). DNA product is run on 1% agarose gel with ethidium bromide.

BLAST (From NCBI Website)

Dennd5a sequence of *Xenopus tropicalis* is used as query to search against EST (Expressed Sequence Tags) database of *Xenopus laevis*.

7.2 Methods and Materials Used in Chapter 3

RT-PCR

Dennd5a primers are selected to target the initial ~400 base pairs from start codon. ODC primers also generate a fragment of ~400 bp. 30 cycles are used in PCR.

Whole embryo in-situ hybridization (Modified from Dr Harland and Dr Sargent's protocol)

Fix embryos in MEMFA for 1~2 hours at room temperature. Day 1, rehydrate embryos by submerging them sequentially from 100% methanol, 75% methanol, 50% methanol to 25% methanol. Neutralize embryos in PBST. Permeabilize embryos in proteinase K. Prehybridization, incubate embryos in hybridization buffer in 60℃. Hybridization, incubate embryos in hybridization buffer containing a specific probe. Day 2, wash embryos with 0.2X SSC. Incubate in MAB, 2% BMB blocking reagent and calf serum containing antidigoxygenin alkaline phosphatase (Roche) antibody overnight at 4℃. Day 3, wash with alkaline phosphatase buffer and add BM purple (Roche) to stain embryos. Bleach embryos with bleaching mix. Fix in MEMFA and prepare for photography.

7.3 Methods and Materials Used in Chapter 4 and Chapter 5

Immunoprecipitation (IP)

Dennd5a gene is subcloned into pCS2-myc vector and pCS2-GFP-N3 vector. pCS2 vector is primarily designed for expressing proteins in *Xenopus* embryos from either injected RNA or DNA; also it can be used for high levels of transient expression in a wide range of mammalian and avian cells. pCS2-myc has six consecutive myc epitopes, each of which has a polypeptide of MEQKLISEEDLNE. These vectors are transfected via PolyFect Transfection Reagent (Qiagen) into HEK293T cells. The standard protocol is used in the transfection kit. 0.5% NP40 is used to lyse cells and dissolve proteins; RIPA buffer has a relatively stronger lysing ability. Protein A/G PLUS Agarose (Santa Cruz Biotechnology, Inc.) is used for pre-clearing lysate and antibody binding. c-Myc (9E10) antibody (Santa Cruz Biotechnology, Inc.) and GFP antibody (Roche) are used to precipitate proteins and used in western blot.

Wnt conditioned medium

Wnt3a or Wnt5a conditioned medium producing cell lines are obtained from atcc.org and Wnt conditioned medium is prepared by lab colleagues. L for Wnt5a and Wnt5a conditioned medium contain 0.5% FBS serum while L for Wnt3a and Wnt3a conditioned medium contain 5% FBS serum.

Immunostainning

HeLa cells are plated onto BD BioCoat™ Fibronectin coated 60 mm cellware. For starvation, cells are incubated either in DMEM only with 1% P/S antibiotics or DMEM with 0.5% FBS and 1% P/S. For Wnt treatment, control cells are treated with L medium and experimental cells are treated with Wnt conditioned medium. Cells are fixed in 4% formaldehyde before staining. HA-probe (F-7) [Santa Cruz Biotechnology, Inc.] is used as the primary antibody. Nucleus is stained by DAPI. Actin fiber is stained by phalloidin red (Life Technologies). Goat anti-Mouse IgG (H+L) with Texas Red-X conjugate (Life Technologies) is used as secondary antibody. Glass cellware is embedded in Fluoro-Gel buffer with Tris (Electron Microscopy Sciences) for observation under Zeiss Axiovert 100 confocal microscope. MetaMorph and ImageJ are used for image analysis.

7.4 Methods and Materials Used in Chapter 6

5'RLM-RACE (RNA Ligase Mediated Rapid Amplification of cDNA End)

First choice RLM-RACE kit (Ambion) is used to amplify the 5' UTR (Untranslated Region) of Dennd5a. The kit is designed to amplify cDNA only from full-length capped mRNA which is an improvement to the classic RACE. Stage7/8 (between stage 7 and 8) cDNA and stage 15/16 (between stage 15 and 16) cDNA are used as template. Two different 5'UTR are obtained, presumably from Dennd5a and Dennd5b.

Embryo manipulation and microinjection

Priming frogs, human chorionic gonadotropin (Sigma Aldrich) is injected to frog the night before eggs are collected. Fertilization, eggs are fertilized by testes suspension. De-jellying, fertilized eggs are transferred to 3% cysteine (pH 7.6 ~ 7.8) to remove the outer transparent envelope. Needle preparation, borosilicate glass with filament (OD: 1.0 mm; ID: 0.5 mm) is pulled from the middle by Flaming/Brown micropipette puller Model P-97 (Sutter Instrument Co.). The needle is calibrated so that one single injection is 10 nL. Injection, eggs are injected dorsally or ventrally at four cell stage. Detailed description can also be found in the publication by Kato et al, 1999 (67).

Morpholino design

MO_Dennd5a&b morpholino that can decrease the protein expression level of both genes are designed and synthesized by gene-tools.com.

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