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Wnt5a Signaling Independently of the Planar Cell Polarity Pathway Resulting in Convergent Extension and Neural Tube Closure During Vertebrate Development

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WNT5A SIGNALING INDEPENDENTLY OF THE PLANAR CELL POLARITY PATHWAY RESULTING IN CONVERGENT EXTENSION AND NEURAL TUBE CLOSURE DURING VERTEBRATE DEVELOPMENT

by

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of a thesis submitted by

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ABSTRACT

WNT5A SIGNALING INDEPENDENTLY OF THE PLANAR CELL POLARITY PATHWAY RESULTING IN CONVERGENT EXTENSION AND NEURAL TUBE CLOSURE DURING VERTEBRATE DEVELOPMENT

Jared J. Barrott

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Vertebrate development is regulated by cellular communication by mechanisms of cell fate and cell behavior. These crucial mechanisms are regulated by cellular signaling and in the case of cell fate, cellular signaling results in transcription of developmentally important genes. Communication between cells can also result in regulation of cell behavior by acting on cytoskeletal elements rather than nuclear factors. One of the cellular signals that regulate both cell fate and cell behavior is the family of Wnt signaling molecules. Wnt5a is one of 19 Wnt molecules and has been previously demonstrated to play critical roles in many important processes in embryonic development as well tumor suppression. Despite many studies that lend credence to a

pathway that regulates cell behavior for Wnt5a rather than cell fate, the identity of the pathway(s) Wnt5a impinges upon remains unclear. Despite the possibility of Wnt5a signaling through multiple pathways, here, focus is given to the non-canonical Wnt signaling pathway, a pathway that regulates cell behavior, also known as the Wnt/Planar Cell Polarity (PCP) pathway.

The involvement of Wnt5a in the Wnt/PCP pathway was demonstrated with a genetic approach: crossing Wnt5a heterozygous mice with mice heterozygous for a component of the Wnt/PCP pathway to uncover genetic interactions in vivo. Hence, Wnt5a X Looptail (Lp) (Wnt/PCP) heterozygous crosses have been performed. Double heterozygotes for this intercross did not exhibit a decrease in viable progeny as compared to the decreased numbers of Lp heterozygotes. These observations demonstrated a lack of genetic interaction between Wnt5a and the PCP pathway. Wnt5a mutants possess phenotypes associated with deficits in the Wnt/PCP pathway, namely convergent extension (CE) defects and neural tube closure defects. However, upon further investigation of the increased penetrance of craniorachischisis in Wnt5a-/-;Lp+/-, Wnt5a mutants do not display the characteristic broadening of the neural floor plate commonly associated with Lp-/-. This supports that Wnt5a and PCP signaling are parallel pathways that have converged to regulate different aspects of CE and neural tube closure. Despite the complexity of Wnt5a and its potential involvement in multiple pathways, dissection of this will explain the broad range of phenotypes observed.

ACKNOWLEDGEMENTS

I would like to thank my committee members for their fortitude in sticking with me through the changes and revisions of my project. Dr. Barrow deserves my profoundest gratitude for the excellent mentoring he has provided in helping me advance my scientific reasoning abilities and technical skills from an elementary level to where I am at today. If it were not for Dr. Stark and Dr. Hansen, I would still be attempting to solve every mystery in the universe. Thank you for your critiques and counsel. Dr. Fields came through in an area where most scientists fall short, and that is in statistical analysis. I could not have done it without him. I would also like to thank the funding that Dr. Barrow has used for my research project and the funding made available by the Physiology and Developmental Biology department.

The list of fellow students in the lab that have assisted in the completion of this project could make this thesis look small, but I would like to thank Jed Kendall for his support and friendship in and out of the lab. Thanks to Joe Schramme and Khealynn Fernelius for their late night assistance. Thanks to Aubrey Budge, Jason Long, James Taylor, Jeannette May, Trevor Gessel, Patrick Saas, Tyler Moore, Josh Rainey, Tyler Swiss, Bryant Zollinger, Jaden Jones, Chris Thacker, Julie Landers, and especially Whitney Sowby and Kate Kmetsch who empathize with the graduate school experience.

Lastly and most importantly, I would like to sincerely thank my wife, Keri, for her support and understanding, putting up with several lonely nights while I worked in the lab. She continues to be my anchor in life to what is most important.

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INTRODUCTION

One of the complexities in understanding vertebrate development is how cells of equal potential differentiate into precise three dimensional organs and tissues with considerable variation within an individual, yet undeviating consistency from one individual to the next. How do cells communicate information about their relative position in relationship to the whole? Each cell, in combination with its unique ability to read the same DNA differently, requires cellular polarity that orients the cell to its position in space. Although the mechanisms of how cells communicate positional information throughout an organism are unclear, work in the field of cellular planar polarity is beginning to illuminate the shadowy unknowns of how three dimensional patterning is consistently established between individuals of the same species.

Planar Cell Polarity

The Planar Cell Polarity (PCP) pathway establishes uniform polarity of cells within a tissue and acts as a cellular compass to orient the cell to its position in space. When cells are polarized, they can undergo directed cell migration and cell division (Barrow, 2006; Zallen, 2007). This control of cellular behavior is critical for normal three dimensional patterning. One general mechanism of how polarity is established is by defining a field of cells that have equal potential to give rise to any cell of a given tissue. Once the field of cells is defined, a signaling center must be established that secretes morphogens, creating a gradient signal that cells can recognize and respond to individually. Based on the positional information that each cell receives, the cells take on a fate that correlates to their position within the field of cells (Barrow, 2006). PCP signaling propagates the

messages that cells receive from a signaling center to confirm positional information or even compensate for the loss of signal in adjacent cells (Lawrence et al., 2007).

Conserved PCP Signaling

The PCP signaling pathway was first discovered in Drosophila. In Drosophila, PCP signaling controls the uniform orientation of hair bristles on the body and omatidia of the eye (Barrow, 2006; Montcouquiol et al., 2006). By use of forward genetics, a set of core PCP proteins has been determined that act in a complex pathway that facilitates localized actin polymerization. The list of core PCP proteins includes transmembrane proteins, such as Frizzled, Strabismus, and Flamingo and intracellular proteins, including Disheveled, Prickle, and Diego (Tab. 1) (Lawrence et al., 2007; Montcouquiol et al., 2006; Ueno et al., 2003; Wallingford, 2006).

TABLE 1

Drosophila	Function/Location	Mouse
fz (frizzled)	Seven transmembrane-pass domains; Wnt receptor/cell membrane	Fz3, Fz6
Vang or stbm (Van Gogh or strabismus)	Two transmembrane-pass domains/cell membrane	Vangl1, Vangl2 (Looptail)
stan or fmi (starry night or flamingo)	Seven transmembrane-pass domains/cell membrane	Celsr1, Celsr3
dsh (disheveled)	Recruited to membrane by fz activates DIX and PDZ domains/cytoplasm	Dvl1, Dvl2
pk (prickle)	Recruited to membrane by Vang and activates LIM and PET domains; inhibits fz/cytoplasm	Prickle1, Prickle2
dgo (diego)	Interacts with Vang and fmi for stabilization/cytoplasm	Inversin
ds (dachsous)	Atypical cadherin/cell membrane	Dchs1
fj (four-jointed)	Type II membrane protein/Golgi and cell membrane	Fjx1
ft (fat)	Atypical cadherin/cell membrane	Fat-j

The prevailing hypothesis on how planar cell polarity is propagated throughout a field of cells is that Frizzled recruits Dishevelled to the cell membrane in autonomous fashion and non-autonomously recruits Flamingo, Van-Gogh, and Prickle to the membrane of the adjacent cell. Van-Gogh and Prickle autonomously prevent the Frizzled mediated recruitment of Dishevelled (Fig. 1) (Doudney et al., 2005; Lawrence et al., 2007).

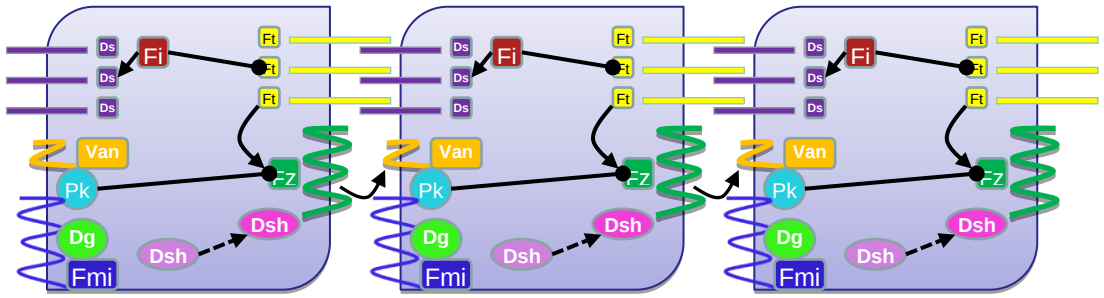


Figure 1: Asymmetrical protein distribution involved in PCP signaling. Ds is recruited by Fj. Ds non-autonomously recruits Ft on adjacent membrane. Ft promotes recruitment of Fz to the same membrane. Fz recruits Dsh to the membrane. Fz non-autonomously recruits Pk on adjacent, which recruits Dgo and Fmi to its side of the membrane. (⊣) – inhibitory (→) – fascilitory

The interactions of Frizzled and Flamingo and other PCP proteins lock in polarity on a cell-to-cell basis. However, a global signal is required to establish general polarity across a tissue. This global polarity involves the atypical cadherin molecules Dachsous and Fat as well as the Golgi protein Four-jointed (Tab. 1). The Dachsous/Fat polarity system is thought to establish universal polarity over a field of cells which in turn sets up cell-to-cell polarity through Frizzled/Flamingo interactions of adjacent cells. Despite the prevailing thought that the Frizzled/Flamingo system is dependent on Dachsous/Fat system, recent evidence suggests that these two molecular polarizing cues are independent (Lawrence et al., 2007). The Dachsous/Fat system is thought to regulate patterning in the developing fly embryo, while the Frizzled/Flamingo system has a more regulatory effect on polarity. These two similar, yet independent systems are thought to

be conserved in vertebrate species: a mechanism for controlling growth patterns and a mechanism for controlling polarity (Lawrence et al., 2007).

Convergent Extension and Neural Tube Closure

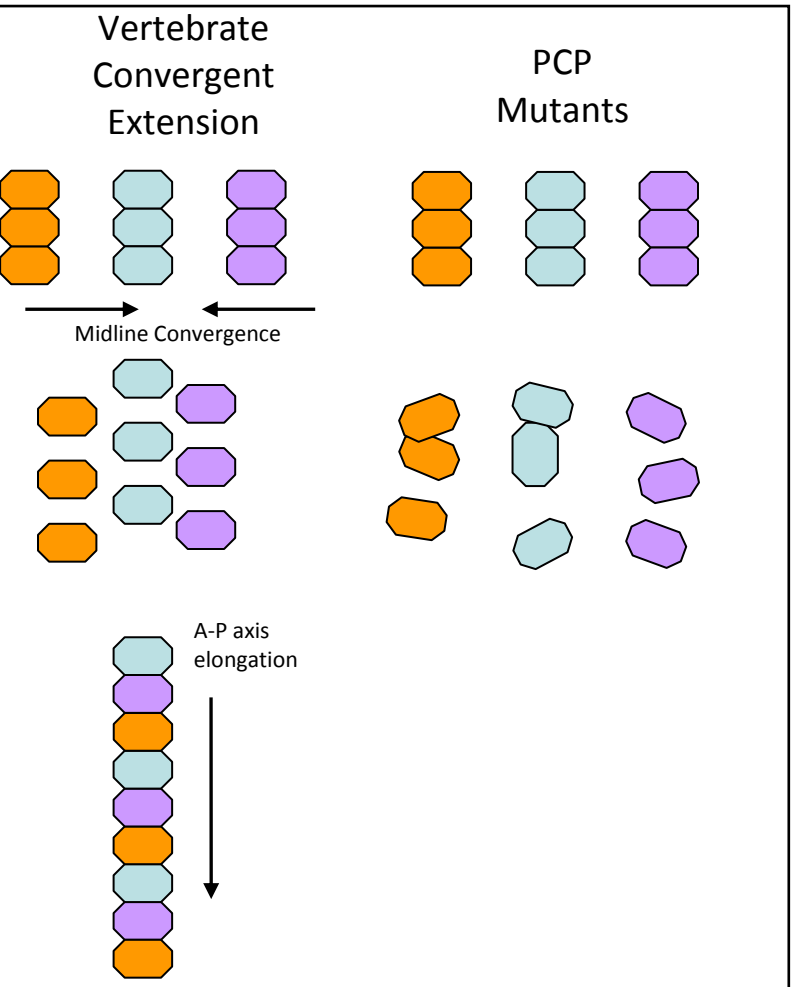


Figure 2: Convergent extension in wild type and in PCP mutants. Convergent extension results in midline convergence of cells and anteroposterior elongation.

Since the discovery of PCP signaling in Drosophila, work in zebrafish and frogs revealed that a conserved PCP pathway controls important cellular processes in vertebrates (Montcouquiol et al., 2006; Wallingford et al., 2002). For example, convergent extension (CE) is a morphogenetic process regulated by conserved planar cell polarity proteins. In CE, mesodermal cells and prechordal plate cells converge medially toward the axial midline where the cells intercalate and extend the embryonic axis in anteroposterior (A-P) fashion (Fig. 2) (Copp

et al., 2003; Montcouquiol et al., 2006; Qian et al., 2007; Ueno et al., 2003; Wang et al., 2006; Ybot-Gonzalez et al., 2007). Neural tube closure is another developmental process

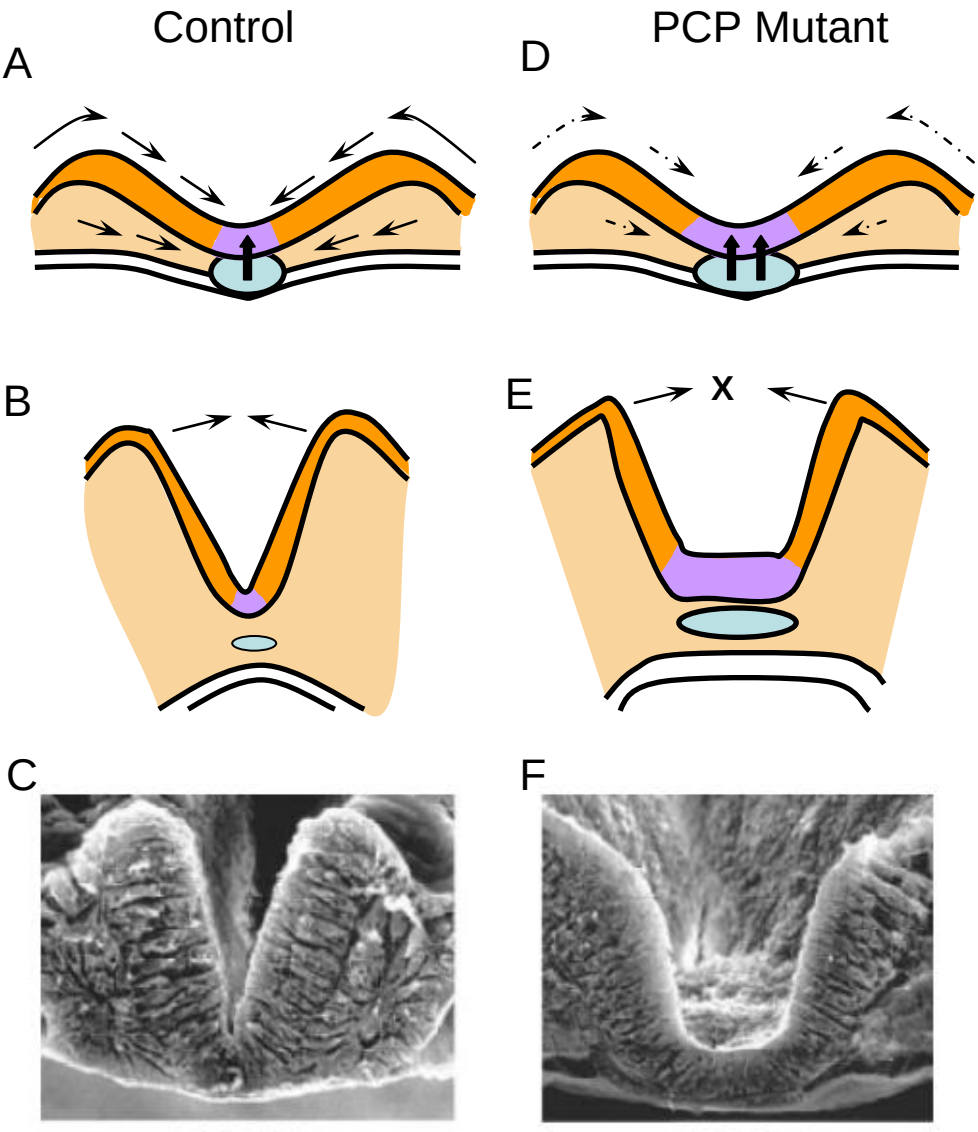


Figure 3: Neural tube defects exhibited in PCP mutants. Neural tube defects are a result of a lack of convergence (D). The notochord (light blue) is broader (D, E) resulting in a broader signal to induce a wide medial hinge point (purple) that exhibits a wide floor plate (E, F), displaying the characteristic U-shape rather than the V-shape found in wild type embryos (E8.5). EM photos (C, F) taken from Doudney et al., 2005.

regulated by components of the PCP pathway and is linked to CE of prechordal plate cells (Fig. 3) (Copp et al., 2003; Doudney et al., 2005; Montcouquiol et al., 2006; Qian et

al., 2007; Ueno et al., 2003; Wallingford, 2006; Wang et al., 2006; Ybot-Gonzalez et al., 2007).

Neural tube closure defects are among the most common congenital human birth defects. When closure defects occur at the level of the brain, it is termed anencephaly. Closure defects that occur in the lower portion of the spinal chord are termed spina bifida, and when closure defects occur throughout the brain and spinal chord, it is called craniorachischisis (Ueno, 2003).When machinery of the PCP pathway is eliminated from the embryo, a lack of CE and neural tube defects (NTDs) are among the hallmark phenotypes. Mice that are mutant in the conserved PCP proteins exhibit an abnormally wide and short body axis. This body axis defect is linked to the lack of CE, which is displayed in mutants by the ectopic presence of prechordal plate cells outside the midline restriction of the notochord (Wang et al., 2006). NTDs, specifically craniorachischisis, are associated with deficiencies in the PCP pathway. This NTD is most commonly associated with a broadened neural floor plate (Doudney et al., 2005). The other mutant phenotype that is used to distinguish members of the conserved PCP proteins is the misalignment of stereocilia of the inner ear (Qian et al., 2007; Wang et al., 2006).

Wnt5a-Mediated PCP Signaling

Although much of the machinery of the PCP pathway is being delineated in vertebrates, much less is known about the initiation of cellular polarity. Wnt signaling is proposed to mediate PCP signaling in vertebrates (Montcouquiol et al., 2006; Qian et al., 2007), but the identity of Wnt molecules involved in PCP signaling and how they might contribute to CE and neural tube closure remains unclear. This proposal is designed to

dissect the interactions of the PCP signaling pathway initiated by Wnt5a during CE and neural tube closure in the mouse.

Wnt5a mutants have many defects in common with the Wnt/PCP pathway. The obvious defect is the abnormally wide and short body axis (Yamaguchi et al., 1999). Recently, researchers reported that Wnt5a mutants displayed disruption in stereocilia alignment and an increased penetrance in craniorachischisis in Wnt5a compound mutants that were also deficient in one allele of a member of the conserved PCP genes (Qian et al., 2007). I will therefore be performing molecular and genetic experiments to further test whether Wnt5a signals through the PCP pathway. PCP signaling is critical for tissue development and organ function because it can establish cellular polarity and regulate morphogenesis in a field of cells (Barrow, 2006; Zallen, 2007). In order to determine the involvement of Wnt5a in PCP signaling, one needs to understand the overlapping components of the PCP signaling and Wnt signaling pathways. Known components of the PCP pathway such as Frizzled (Fz) and Dishevelled (Dsh; Dvl in mice) are associated with Wnt signaling pathways. Fz, Dvl, and other proteins that function in multiple pathways will help in understanding the overlay that occurs between signaling pathways (Gordon et al., 2006; Veeman et al., 2003).

Regarding the PCP pathway, we can investigate the phenotypes of mice where proteins such as Dvl1 and Dvl2, Looptail (Lp), and Celsr are disrupted (Barrow, 2006; Doudney et al., 2005; Qian et al., 2007; Wang et al., 2006). One important comparison between the Wnt/PCP pathway and the Wnt/β-catenin pathway is the control of cell behavior versus cell fate. The PCP pathway is thought to mediate cell behavior that is necessary for morphogenesis because Dvl signals to activate the JNK pathway and rho

kinase, which directly affect the cytoskeleton and cell polarity (Montcouquiol et al., 2006; Veeman et al., 2003). On the other hand, the canonical pathway mediates cell fate decision by activating β-catenin, which in turn acts as a transcription factor in the nucleus (Giles et al., 2003).

Wnt Signaling

Since the discovery of Wnt ligands over two decades ago, much research has been invested to determine the signaling pathways of these essential developmental molecules (Gordon et al., 2006). Due to the fact that over 19 genes encode the Wnt family of secreted proteins (Mikels et al., 2006), the study of Wnt signaling is

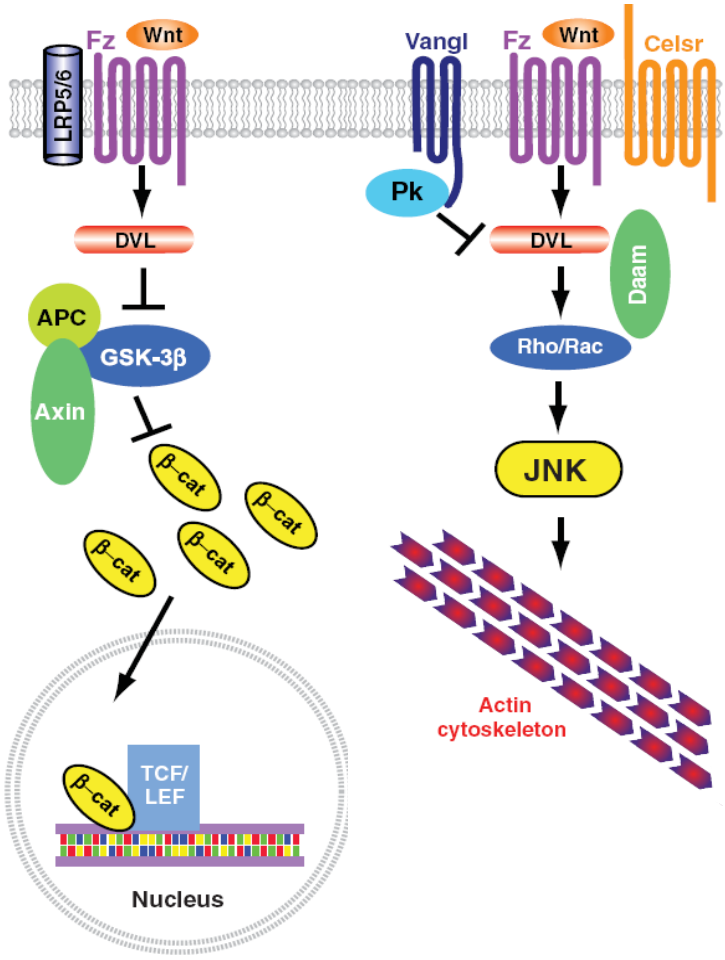


Figure 4: Diagram of canonical Wnt signaling and non-canonical Wnt/PCP signaling in mice (vertebrates). Binding of a Wnt ligand can result in gene transcription or polarization of the actin cytoskeleton depending on which Wnt initiates the pathway. Frizzled (Fz) and Dishevelled (Dvl) are involved in signaling in both pathways.

Diagram adapted from Montcouquiol et al., 2006

complex and covers a broad range of developmental processes. Although distinct Wnts may act on identical receptors in the same target tissue, they have the ability to educe wide-spread and varied responses (Veeman et al., 2003).

The first pathway through which Wnt ligands were found to signal is the canonical Wnt or the Wnt/β-catenin pathway (Fig. 4). This pathway involves two membrane receptors Fz and low density lipoprotein receptor-related protein (LRP). Upon activation from a Wnt molecule, Dvl is released and activated so that it can inhibit phosphorylation and degradation of β-catenin. β-catenin can now enter the nucleus and act as a transcription factor and bind to lymphoid enhancer-binding factor 1 (Lef1) and T cell-specific transcription factor (TCF) (Gordon et al., 2006). However, in the murine system disruption of the canonical pathway yields results that differ from disruption of Wnt5a.

It is important to establish whether Wnt5a signals through the canonical Wnt signaling pathway or through one of the non-canonical Wnt signaling pathways (i.e. Wnt/PCP pathway). By conducting anatomical comparisons and finding similarities between mutant phenotypes of Wnt5a and the Wnt/β-catenin pathway, one can postulate whether further investigation is necessary to determine the involvement of Wnt5a in Wnt/β-catenin signaling.

For comparisons between the canonical Wnt signaling pathway and the Wnt5a mutant, Wnt3a mutants are used because Wnt3a has been shown to exclusively signal through the canonical pathway. Despite the fact that Wnt5a and Wnt3a mutants share some phenotypic similarities (i.e., shortened body axis), they differ significantly as well. For example, Wnt3a mutants demonstrate the presence of three neural tubes rather than one neural tube with flanking paraxial mesoderm (Takada, 1994; Yoshikawa, 1997). In

staining for paraxial mesoderm markers in Wnt5a mutant embryos, neural tube cross sections demonstrate the presence of these markers, albeit greatly disorganized (Barrow, unpublished data) (Appendix A). Along with these observations, other researchers have used BAT-GAL reporter genes as a molecular readout of the Wnt/β-catenin pathway and have found the pathway to be uninterrupted in Wnt5a mutants (Qian et al., 2007). This supports that there is not a direct one-to-one correlation between Wnt5a and the canonical Wnt signaling pathway and that Wnt5a signals independently of Wnt/β-catenin. In light of the evidence, Wnt5a should be readily classified as a non-canonical Wnt.

Wnt5a Phenotype

One preliminary method for discovering genetic interactions is to examine the defective phenotypes of mutant mice for a specific gene and search for mutant mice that share similar defects. Mutant mice that share defective phenotypes demonstrate that similar processes are affected and the two gene products could be working through the same pathway. In searching for the downstream components of Wnt5a signaling, there have yet to be reports of a mutant phenotype that matches perfectly with the Wnt5a mutant phenotype. The phenotype exhibited in the Wnt5a mutants is short, stubby limbs, face, ears, and genital tubercles (Yamaguchi et al., 1999). It also has a truncated anterior-posterior body axis, an open neural tube with about 40% penetrance, and severe hemorrhaging. As Wnt5a mutants have a shortened body axis and open neural tube similar to PCP mutants, we hypothesized that Wnt5a signals through the PCP pathway. As stated earlier, PCP pathway mutants demonstrate an open neural tube, as well as body axis truncations; however, the limbs and face of PCP pathway mutants appear to be

normal (Doudney et al., 2005; Wallingford, 2005; Wang et al., 2006), unlike the truncated face and limbs observed in Wnt5a mutants. This lack of phenotypic overlap between the Wnt5a mutant and the defective phenotype of PCP pathway mutants demonstrates that if Wnt5a is signaling through the PCP pathway, it does not do so exclusively. In Drosophila, exhaustive genetic studies have been performed and have provided strong evidence that PCP signaling occurs in a Wnt-independent fashion (Gordon et al., 2006; Wang et al., 2007). Despite the lack of 100% mutant phenotype overlap, Wnt5a still could be signaling through the PCP pathway, or in other pathways that regulate developmental processes.

Research Goal

In order to dissect the involvement of Wnt5a in PCP signaling, we performed mouse genetics experiments. We looked for exacerbations in the Wnt5a phenotype by crossing inbred mice heterozygous for Looptail (Lp) (Doudney et al., 2005) into a colony of mice that were heterozygous for Wnt5a. Lp mice represent a missense mutation in the Vangl2 gene, a core PCP gene. Therefore, by creating deficiencies in both the Wnt5a signaling pathway and the PCP signaling pathway (Lp mice), we examined the offspring of Wnt5a+/- X Lp+/- crosses in hope of finding a phenotype demonstrated in double heterozygous mice that is not found in either single heterozygote. A previous study used this same approach to discover the interaction of the tyrosine kinase receptor, PTK7, and Lp, representing the PCP pathway (Lu et al., 2004). Lu et al., found a 95% penetrance of spina bifida in the double heterozygote progeny; whereas the Lp single heterozygous mice displayed 10% penetrance, and PTK7 single heterozygous mice displayed no

penetrance in spina bifida defects. The observation that penetrance for spina bifida increased, along with other observations, supports a strong interaction between these two genes. We employed the same technique to find support for a genetic interaction between Wnt5a and the PCP pathway.

To further test the genetic interactions of Wnt5a and Lp, we generated Wnt5a mutants that were also heterozygous for Lp. We performed Wnt5a+/-;Lp+/- X Wnt5a+/- crosses and searched for exacerbations in the Wnt5a-/- phenotype where mutants were deficient in Lp(+/-) as well. We conducted additional experiments on Wnt5a mutants during earlier stages of development to ascertain an overlap in phenotype between Wnt5a-/- mice and mice that are mutant in members of the PCP pathway. We measured length-to-width ratios of E8.5 embryos to determine the involvement of Wnt5a in CE. We also stained prechordal plate cells in E8 -8.5 embryos for brachyury, also known as the Tail gene (T), to examine a broadened expression pattern of T-positive cells in Wnt5a mutants. Lastly, we observed neural tube cross sections of E8.5 embryos for the presence of a wide floor plate as seen in Lp mutant mice (Doudney et al., 2005). We performed these experiments to clarify the evidence that Wnt5a initiates PCP signaling.

This study has given better understanding to the involvement of Wnt5a in developmentally important processes such as CE and neural tube closure. As Wnt5a is required in development as well as in preventing tumors in the adult (Liang et al., 2003), it is critical that we understand its downstream signaling events to understand the molecular basis of the congenital defects and pathology associated with Wnt5a deficiencies.

RESULTS

Wnt5a+/-;Lp+/- Mice Exhibited Equal Viability to Lp+/-

Vangl2, an established component of the PCP pathway (Doudney et al., 2005; Ueno et al., 2003), is represented in inbred mice colonies as a missense mutation called Looptail (Lp). There is a myriad of published data on mutant phenotypes displayed in Lp mutant mice (Doudney et al., 2005; Montcouquiol et al., 2006; Qian et al., 2007; Ueno et al., 2003; Wang et al., 2006; Ybot-Gonzalez et al., 2007), hence, we used Lp mice to test the genetic interactions of Wnt5a and the PCP pathway. In searching for a genetic interaction, we crossed Wnt5a+/- with Lp+/- mice, and we observed 261 progeny. Lp+/- mice were distinguished easily by the presence of a loop in the tail. There is no phenotype to distinguish Wnt5a+/- from wild-type mice, therefore, PCR genotyping was used to determine the genotype of the offspring. In our observations, we expected a decreased number of progeny that where in any way Lp+/-. The decrease in Lp heterozygotes is explained by the presence of neural tube defects in the single heterozygous state that result in natural abortion of the fetus. If an interaction exists between Wnt5a and Lp, we would expect less progeny in Wnt5a+/-;Lp+/- mice or an exacerbation of the loop tail phenotype. No significant differences were observed between Wnt5a+/-;Lp+/- and Lp+/-. If anything is to be noted, there are more double heterozygotes that survived until birth and progress to maturity than Lp single heterozygotes (Tab. 2). Also there were no phenotypes observed in the double heterozygous progeny that were seen in either of the Wnt5a or Lp single heterozygotes. Occasionally, we saw Lp mice that displayed a tightly coiled tail in close proximity to the body, but this phenotype was observed in almost equal proportions between Lp+/- and

Wnt5a+/-;Lp+/- mice. Hence, we conclude that Wnt5a does not exacerbate the decrease viability demonstrated in Lp+/- progeny and Wnt5a+/-;Lp+/- mice lack a distinguishing phenotype that would indicate a genetic interaction.

Table 2

	Wild type	Wnt5a+/-	Lp+/-	Wnt5a+/-;Lp+/-
Observed	75	94	36	51
Expected	84.5	84.5	84.5	84.5

Wnt5a-/-;Lp+/- Displayed Increase Penetrance of NTDs

We attempted to view the evidence of a genetic interaction between Wnt5a and the PCP pathway during embryonic stages, we crossed Wnt5a+/-;Lp+/- to Wnt5a+/- in order to observe the presence of an exacerbated phenotype in Wnt5a-/-;Lp+/- embryos.

Table 3

	Wild type	Wnt5a+/-	Wnt5a-/-	Lp+/-	Wnt5a+/-;Lp+/-	Wnt5a-/-;Lp+/-
Observed	21	30	11 (5*)	18 (3)	31 (3)	16 (16)
Expected	15.9	31.8	15.9	15.9	31.8	15.9

The number in ( ) represents the subtotal that exhibited NTDs
***** *The 5 mutants seen with NTDs did not have enough tail to phenotype for Lp and the lack of primer sequences prevented clarification through PCR genotyping.*

Embryos were taken between embryonic days (E)11.5 – 14.5. There were no significant differences between the observed counts of embryos compared to the expected counts of embryos for each genotypic class when applying Mendalian genetics. Out of the 127 embryos observed, 16 of the embryos were Wnt5a-/-;Lp+/- and displayed 100%

penetrance of NTDs (Fig. 5, D). The increasing penetrance of NTDs observed between

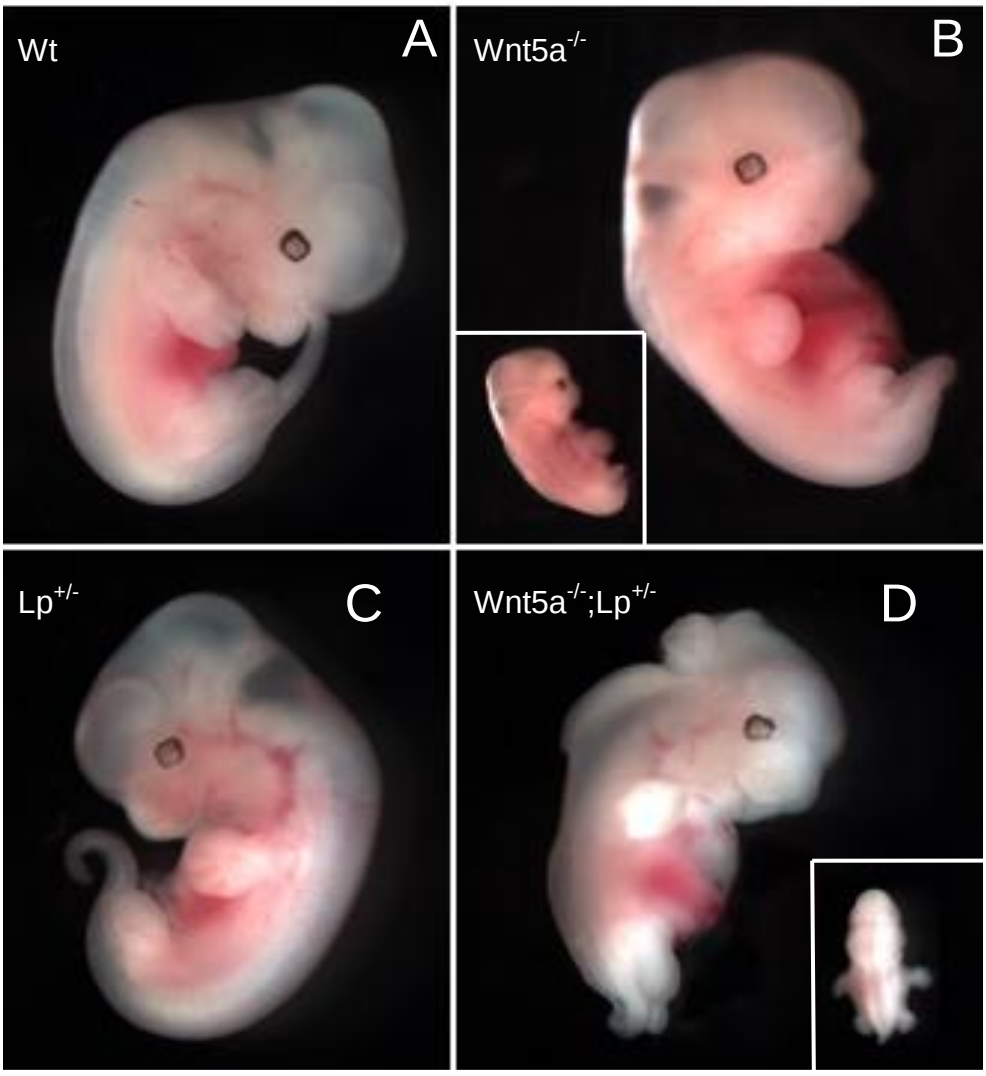


Figure 5: Neural tube defects demonstrated in Wnt5a-/-;Lp+/-. (A) Wt embryo at E12.5. (B) Wnt5a mutant displaying truncated phenotype in limbs, face, tail, A-P body axis. (C) Lp+/- with loop in the tail, (D) compound mutant with NTD – inserts of dorsal view.

Wnt5a mutants (5* of 11) and Wnt5a;Lp+/- compound mutants (16 of 16) supports a genetic interaction between the two pathways. Further evidence of this interaction is apparent in the increase in penetrance of NTDs between Lp+/- (3 of 18) and Wnt5a-/-;Lp+/- (16 of 16). However, Wnt5a and PCP signaling could be parallel pathways that

converge to regulate different aspects of CE that later gives rise to proper neural tube closure. The observation of increased neural tube defects is a general phenotype that can encompass several mechanisms of regulation and multiple pathways. In lieu of Wnt5a and PCP signaling in parallel pathways, we observed no disparity in the display of NTDs between Lp+/- and Wnt5a+/-;Lp+/- (Tab. 3). The more common approach of determining genetic interactions by searching for exacerbations in double heterozygotes compared to either of the single heterozygotes once again provides evidence that Wnt5a and the PCP pathway signal independently of one another.

To further elucidate the involvement of Wnt5a in the PCP pathway, we looked at CE, the developmental process that is primarily responsible for neural tube closure. We know that older Wnt5a mutant embryos (E11.5-14.5) display an abnormally short and wide body axis (Yamaguchi et al., 1999), but we wanted to determine the involvement of Wnt5a in CE at the earlier stages of gastrulation and axial elongation (E8.0-8.5). We also examined the effects of Wnt5b in our CE studies because Wnt5a/Wnt5b double mutants display an exacerbated phenotype to the already truncated phenotype found in Wnt5a mutants (Long, unpublished data) (Appendix B). Lp mutants and other components of the PCP pathway demonstrate defects in the early processes of CE. Studies have revealed that Lp mutants, as well as Dvl1/Dvl2 double mutants display significant disparity in length-to-width (LTW) ratios between mutants and control counterparts. These PCP mutants also demonstrate defects in the midline restriction of prechordal plate cells and broadened neural floor plates (Doudney et al., 2005; Wang et al., 2006; and Ybot-Gonzalez et al., 2007).

Smaller LTW Ratios in Wnt5a-/- Embryos

As previously demonstrated, PCP signaling is a required process for neural tube closure and axis elongation. PCP-mediated CE of neurepethelium and the underlying mesoderm is the process that precedes and ensures neural tube closure and axis elongation. As demonstrated in Xenopus, CE results in the convergence of medial cells towards the midline and intercalation of these cells to elongate the embryo in the anteroposterior axis (Wallingford et al., 2002). Throughout neurulation, the embryo continues to become thinner along the mediolateral axis and longer along the anteroposterior axis as revealed by an increasing LTW ratio (Wang et al., 2006; Ybot-Gonzalez et al., 2007). To verify whether Wnt5a is regulating some aspect of CE during neurulation, we measured the lengths and widths (see methods) to obtain a ratio for comparing Wnt5a mutants to control embryos at E8.5 (Fig. 6).

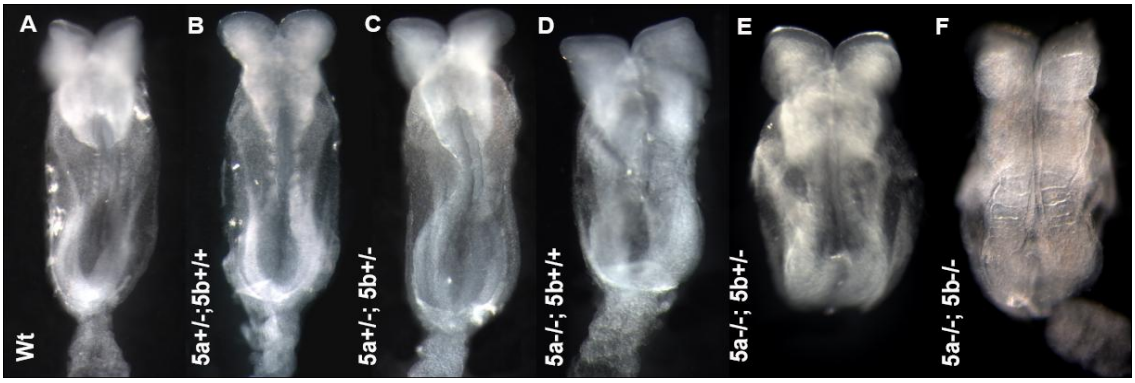


Figure 6: Various Wnt5a;Wnt5b mutants displaying convergent extension defects at E8.5 (6 somite stage). Wnt5b appears to not exacerbate the mutant phenotype at this stage of development, but later in development Wnt5b dramatically effects the truncated phenotype of Wnt5a mutants exhibits.

A single copy of the Wnt5a allele appeared sufficient to ensure proper morphogenetic movements associated with CE. As seen with other genes that are

important in the regulation of CE, Wnt5a-/- embryos displayed a significantly lower LTW ratio than control embryos that match up at the same somite stage of development (Fig. 7). Interestingly, Wnt5b, a paralog to Wnt5a, does not exhibit exacerbation in the LTW ratios measured in Wnt5a mutants (Appendix C). However, embryos that are deficient in both Wnt5a and Wnt5b exhibit exacerbated truncations in the body axis a few days later in development (Appendix B) (Long, F., McMahon, A., and Barrow, J., unpublished data). We can conclude that Wnt5a is essential in early processes of CE (E8.5). Despite that Wnt5b contributes a later role in axial elongation, it does not appear to be essential in CE as displayed in LTW ratios; neither does it exacerbate the mutant LTW ratio of Wnt5a (E8.5).

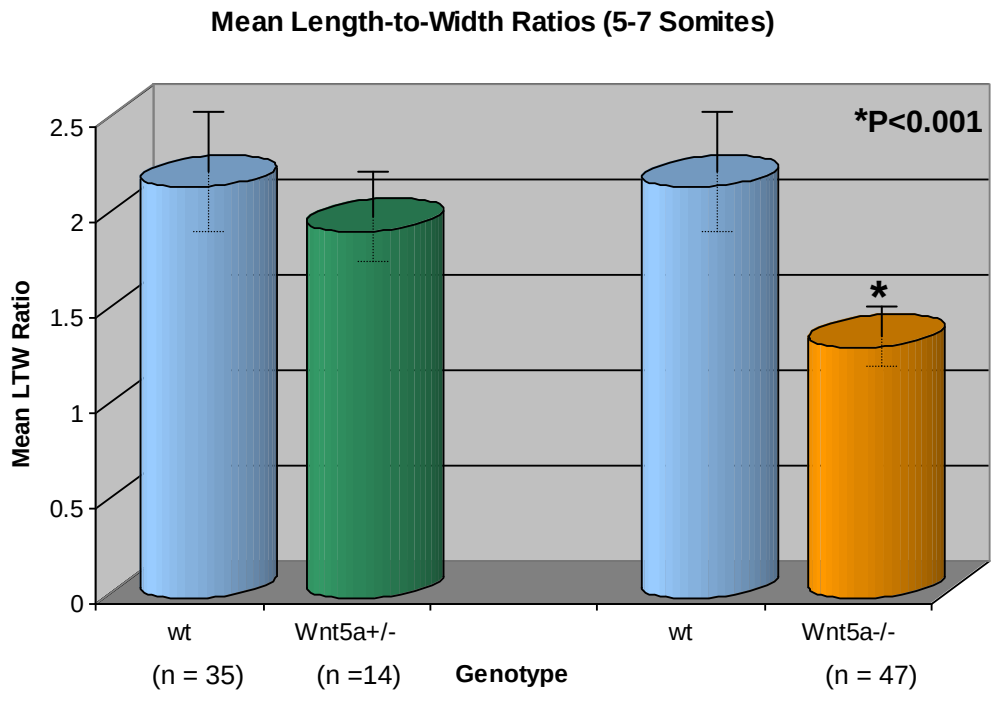


Figure 7: Graph of length-to-width (LTW) ratios of Wnt5a mutants. Wnt5a-/- display convergent extension defects as demonstrated by a significantly lower LTW ratio compared to wild-type embryos at the same stage of development.

Wnt5a-/- Embryos Displayed Broadened Expression of T

Wnt5a-/- embryos were also tested for CE by observing T expression of prechordal plate cells, primordial notochord cells. During normal neurulation these cells align tightly on the midline of the embryo. Where cellular polarity is disrupted, these

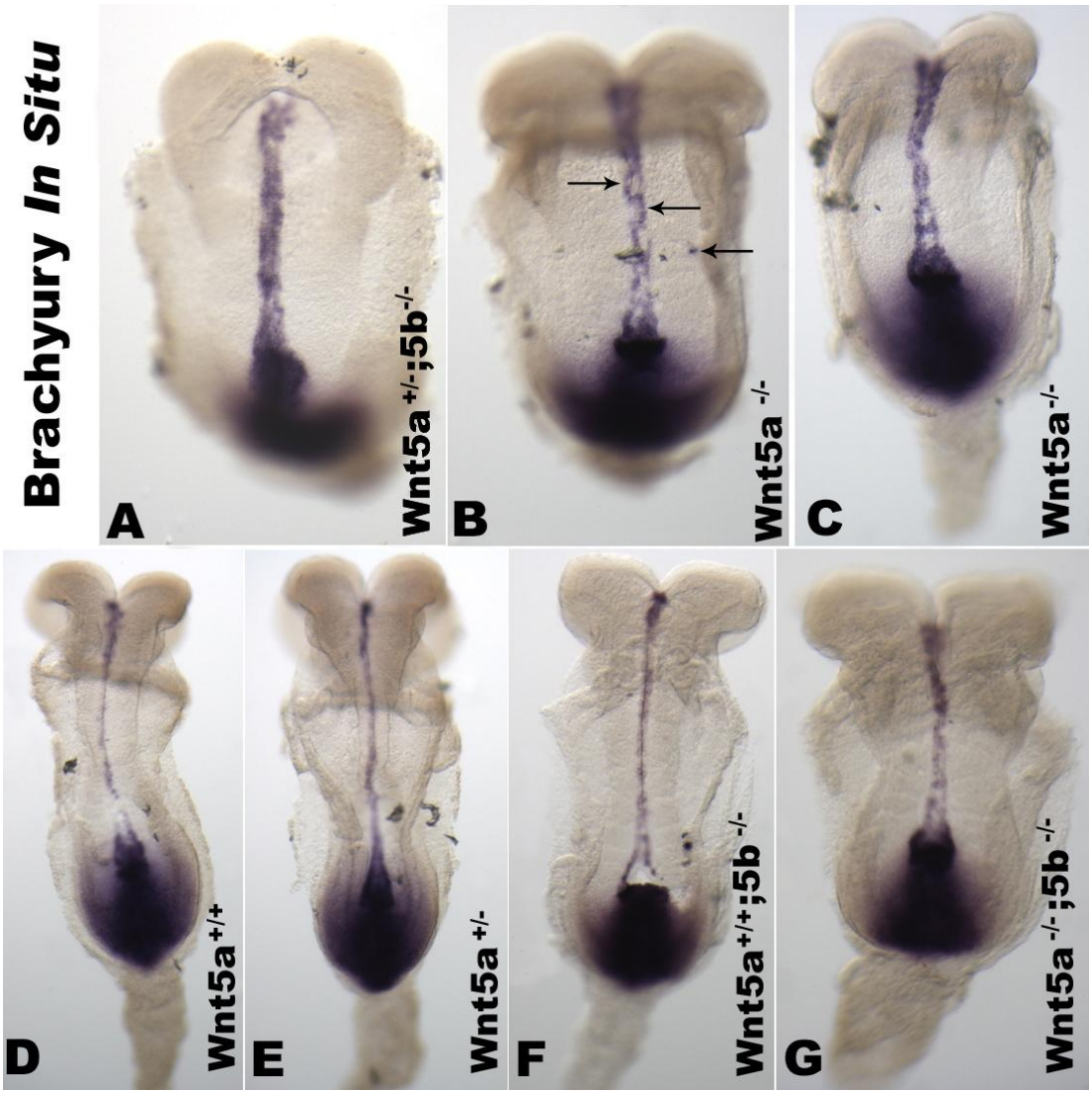


Figure 8: In situ hybridization analysis of Brachyury (T) in various genotypes of Wnt5a and Wnt5b at E8-8.5 stage embryos. T expression was maintained in all embryos, however, the expression domain became larger in Wnt5a mutants and Wnt5a;5b compound mutants. In (B) the prechordal plate cells (T-positive cells) are expressed in a zigzag like pattern with one extreme outlying cell.

cells display a broader T expression and in addition T-positive cells are found outside the notochord. In Wnt5a mutants, we observed a broader expression of T-positive cells (Fig. 8), however, the defects seen in Wnt5a mutants and Wnt5a/5b double mutants were not as severe as those seen in Dsh1/2 double mutants and Lp mutants (Wang et al., 2006). In the results presented by Wang et al., the embryos used were younger in development in the case of the Dvl1/2 double mutant and the Lp mutant. The prechordal plate cells appear in development in a dispersed manner, later to polarize and align along the midline. A few hours might be sufficient time for these cells to converge to their normal domain. In figure 8, the mutant embryos were younger than our control embryos (Fig. 8: B, C, D, and E are littermates; and A, F, and G are littermates), and although we compared mutants to control littermates, the age disparity, which was based on somite stages, might be the cause in the difference between expression patterns. However, the Wnt5a-/-;Wnt5b-/- and Wnt5a+/+;Wnt5b-/- (Fig. 8 C, F respectively) are comparable in somite stage, and the T expression domain is clearly broader in the Wnt5a/5b double mutant. Evidence is there that Wnt5a and Wnt5b play a role in the convergence of the prechordal plate cells, however, it might be parallel to that of PCP regulation. The less severe broadening of T-positive cells found in Wnt5a mutants again reiterates the lack of overlap between Wnt5a and the PCP machinery that Wnt5a is thought to initiate.

Wnt5a-/- Displayed No Floor Plate Abnormalities

One of the most distinguishing characteristics of NTDs found in Lp mutant mice is the wide neural floor plate (Doudney et al., 2005). We therefore examined neural tube sections of Wnt5a mutants for the presence of an abnormally wide neural floor plate. In

sectioning E8.5 embryos from Wnt5a+/- X Wnt5a+/- crosses, we observed 1 out of 9 Wnt5a mutant embryos that displayed a broadened floor plate at the axial level where neural tube closure initiates. The other eight Wnt5a mutant embryos displayed normal floor plates and normal patterns in the elevation process (Fig. 9). The observations that Wnt5a/5b double mutants do not exhibit a wide neural floor plate as do Lp mutants strongly suggest that Wnt5a mediates neural tube closure independently of PCP signaling.

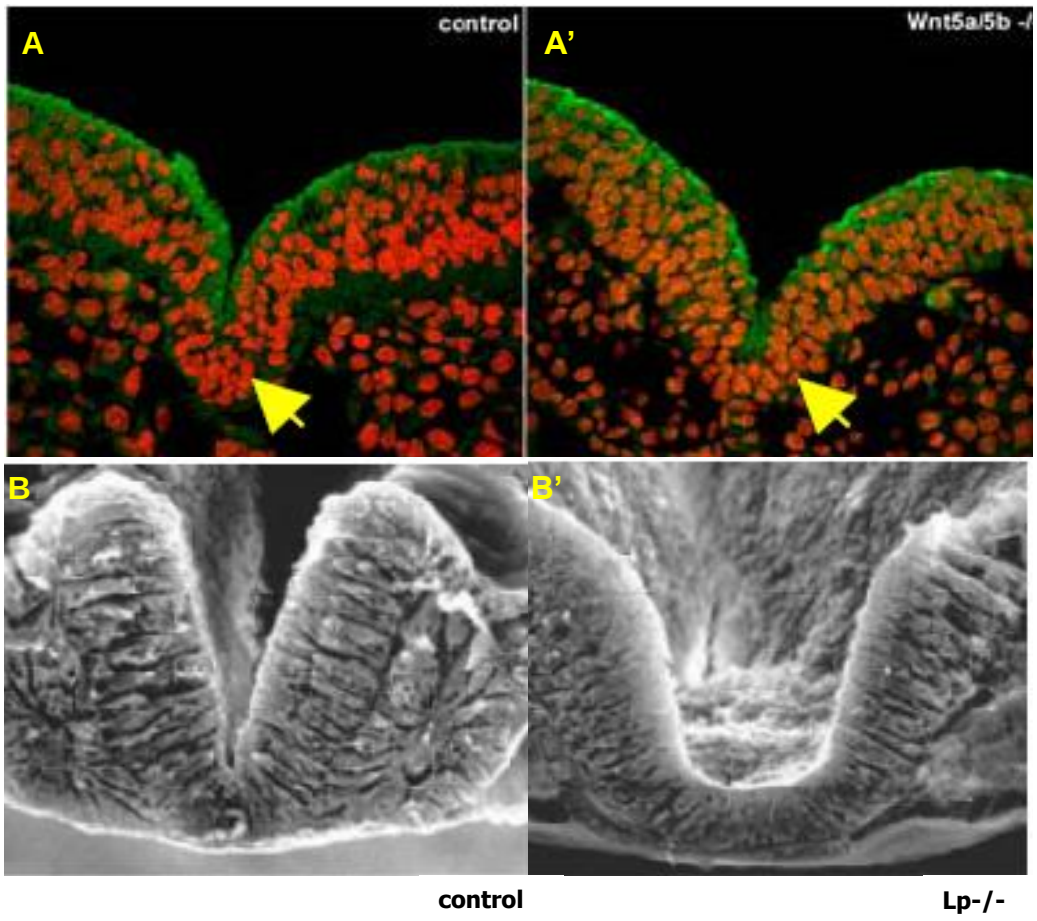


Figure 9: Neural tube cross sections of E8.5 embryos. A and A' show confocal images of neural tube stained with TO-PRO-3 and Phalloidin. A' is a cross section of a Wnt5/5b double mutant. As demonstrated the neural floor plate is similar to control (A) (yellow arrow). On the other hand Lp mutants (B') display wide neural floor plates compared to controls (B). EM images from Doudney et al., 2005.

Wnt5a-/- Displayed Disorganized Mesoderm

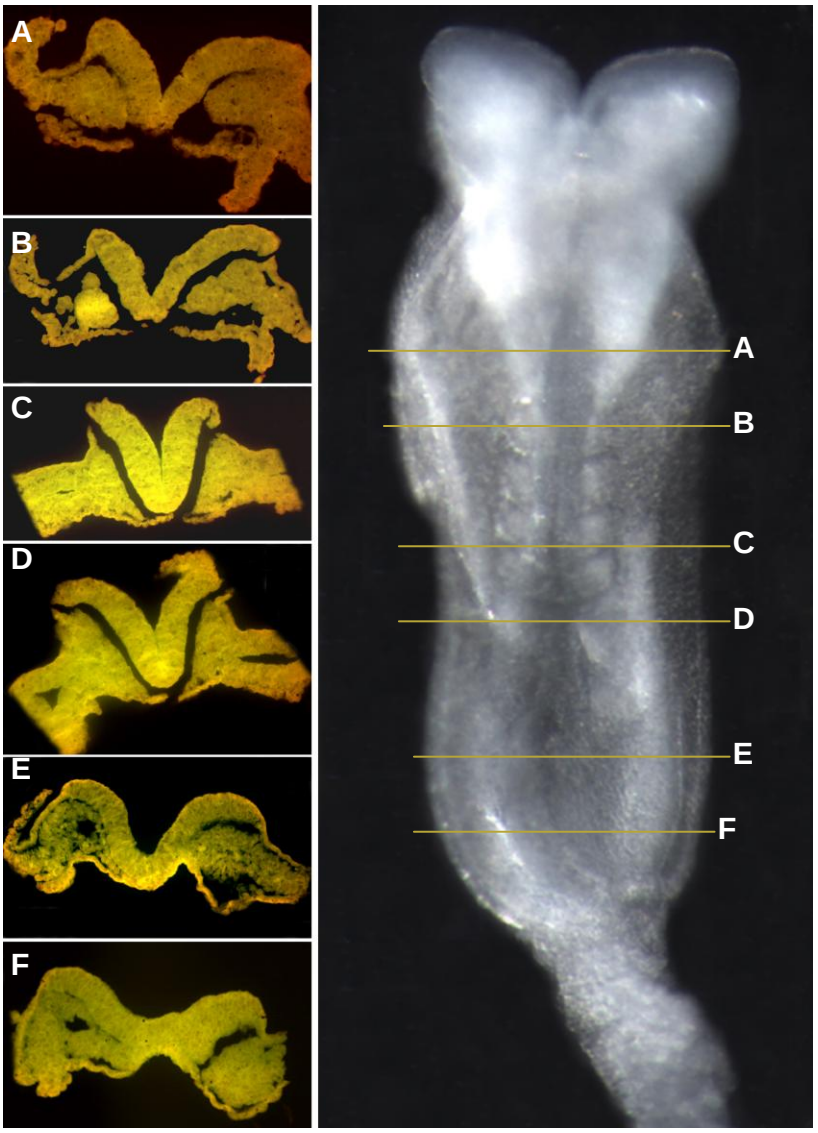


Figure 10: Neural tube sections in an E8.5 Wt embryo. In wild-type embryos the surrounding mesoderm is organized, assisting in proper neural tube closure.

In considering the different aspects of neural tube closure that Wnt5a might be regulating, we turned to forces outside the neural folds. The surrounding epithelium and mesoderm is a contributing force in neural tube closure (Montcouquiol et al., 2006). Immediately surrounding the neural folds in wild-type E8.5 embryos, we saw proper

condensation patterns of paraxial mesoderm that later give rise to the somites (Fig. 10).

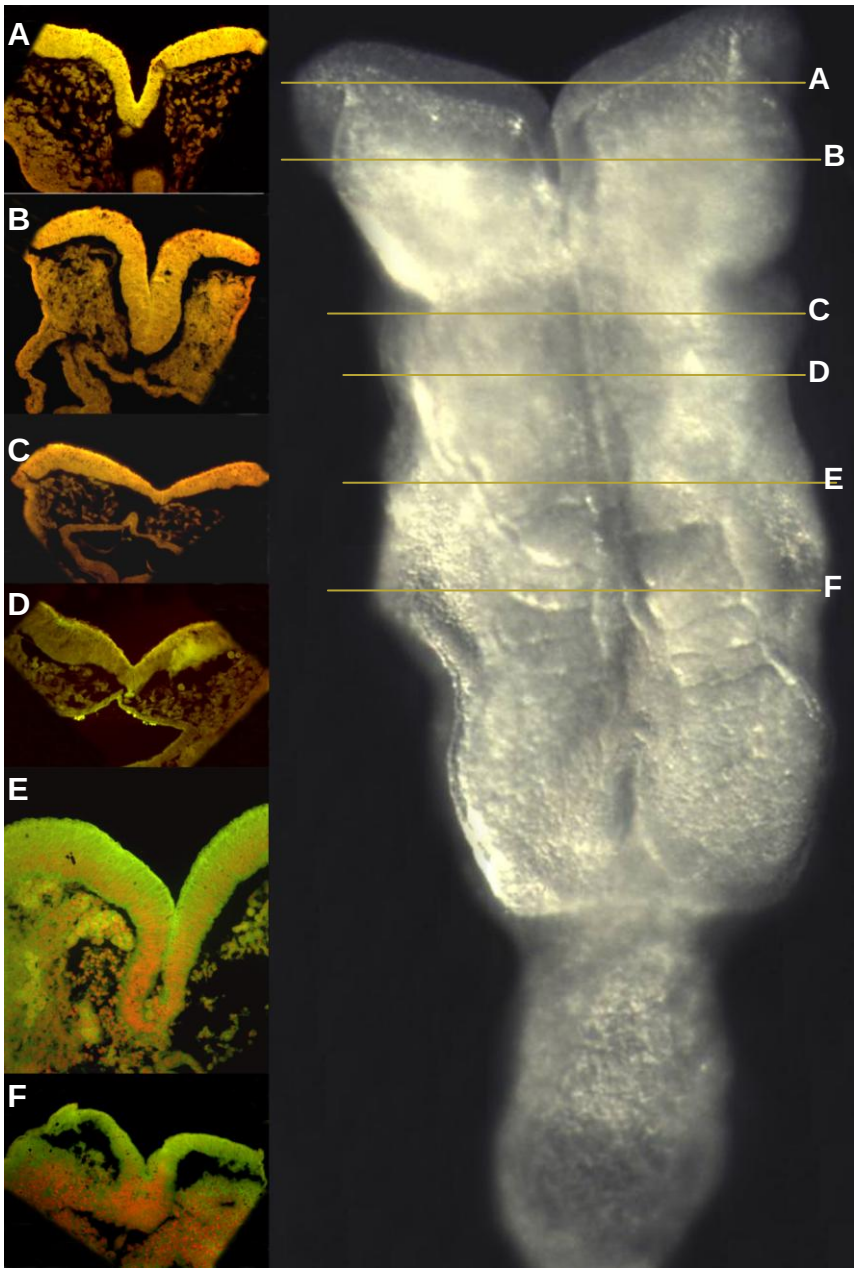


Figure 11: Neural tube sections in an E8.5 Wnt5a-/- embryo. In mutant embryos the surrounding mesoderm is disorganized, marking a loss of convergence/polarity, sometimes resulting in a failure to close the neural tube.

The cross sections of A and B in figure 10 represent the axial level where hindbrain neural tube defects occur. In these wild-type sections, paraxial mesoderm condensed in spherical patterns to either side of the neural groove. We observed a disruption in the organization of mesoderm surrounding the neural folds in Wnt5a-/- embryos at E8.5 (Fig. 11). Rather than observing well defined mesodermal

spheres on either side of the neural groove, as seen in the wild-type embryo, we saw dispersed mesodermal cells. The transverse sections represented in E and F of figure 11 are located at the axial level of the embryo where neural tube defects occur at the hindbrain. Albeit the mesodermal cells are present, the manner in which these cells are organized contributes to the convergent force to assist neural tube closure. This suggests that Wnt5a plays a role in neural tube closure by patterning the surrounding mesoderm in a manner so as to apply convergent force on the neural folds to bring them into proximity to each so that apposition can occur.

DISCUSSION

During development, tissue differentiation and organ development are orchestrated by an array of signaling molecules. The interactions between signaling pathways becomes increasingly more complex as genetic studies have shifted from Drosophila to understanding vertebrate species such as the chick, zebrafish, frog, and mouse. Despite the disparity in organ structures between the common developmental model systems named previously, there is under-appreciated conservation of the signaling pathways, the tools for orchestrating life, from the simplest of organisms to the most complex. For example, the same cassette of PCP genes that are responsible in Drosophila for proper orientation of omatidia in the compound eye are also essential for proper orientation of stereocilia in the inner ear of mice. Recent findings have shown that genes involved in PCP signaling are responsible for proper closure of the neural tube (Montcouquiol et al., 2006). However, the machinery involved is not as well delineated in vertebrate mammals as it is in Drosophila (Ueno et al., 2003). Recent evidence

suggests that Wnt5a mediates PCP signaling in the murine model system (Qian et al., 2007). In this study we examined mouse genetic interactions between Wnt5a and Looptail (Lp), Wnt5a's regulation of convergent extension (CE), and the control that Wnt5a exhibits on cells surrounding the neural folds. These are important studies and comparisons that should be considered in associating Wnt5a to the PCP pathway, in addition to the studies conducted to demonstrate Wnt5a's involvement in cochlear CE and polarization of stereocilia in the inner ear.

The Genetic Interactions between Wnt5a and Lp

To test for genetic interactions between Wnt5a and Lp, a known member of the PCP pathway, we crossed Wnt5a heterozygous mice to Lp heterozygous mice and examined viability in the progeny. To assess whether a genetic interaction is evident, a phenotype should be observed in the double heterozygous progeny that does not exist in either of the single heterozygotes. From the literature, Lp single heterozygous mice exhibited neural tube defects, which resulted in decreased viability of Lp+/- among the progeny. We observed a 45% decrease in the Lp single heterozygous progeny. We saw no decrease in the observed number of Wnt5a single heterozygous progeny compared to our expected values. In order for a genetic interaction to exist, there should be a greater decrease of Wnt5a/Lp double heterozygous progeny, however, we observed a 22% decrease among the double heterozygotes. This data gives no support to a genetic interaction between Wnt5a and the PCP pathway via Lp studies, but rather that Wnt5a and Lp are complementary in nature suggesting that Wnt5a and Lp lie in parallel pathways.

In the study by Qian et al., they reported increased penetrance of craniorachischisis in Wnt5a mutants that were likewise compromised by reducing the embryos to one copy of Lp. We wanted to validate this claim by crossing our viable Wnt5a+/-;Lp+/- mice to Wnt5a+/- mice. Observations of litters taken between E11.5-14.5, demonstrated increased penetrance of craniorachischisis in Wnt5a-/-;Lp+/- embryos. This result opened the door for a genetic interaction between Wnt5a and the PCP pathway, so we decided to study the mechanisms behind neural tube closure. We wanted more evidence to support the interactions between Wnt5a and the PCP pathway by observing for phenotypes of the PCP pathway that are evident during CE and early neural tube closure.

Neural Tube Closure

Neural tube closure is a complex process involving multiple steps in a discontinuous manner. The complexity of neural tube closure necessitates the regulation from multiple pathways. The following is a discussion on the steps leading to neural tube closure. Elevation and apposition of the neural folds occurs at three separate initiation sites that lead to apposition of both sides of the neural fold in both anterior and posterior directions along the embryo in a zipper-like manner (Doudney et al., 2005). The process begins with convergence of prechordal plate cells that align along the midline of the embryo, a process that is regulated by PCP signaling (Wang et al., 2006), and to some degree by Wnt5a signaling (Fig. 8). The notochord forms and secretes signals to the overlying ectoderm to become neurepithelium. The medial cells of the neurepithelium undergo apical constriction that results in the formation of the medial hinge point (Fig. 3).

Elevation of the neural folds follows the formation of the medial hinge point, which appears unaffected in PCP and Wnt5a mutants demonstrating another point of regulation in neural tube closure that is independent of PCP signaling. Evidence supports the role that Wnt5a organizes surrounding epithelial and mesodermal cells to force the neural folds to converge to the dorsal midline. Dorsal-lateral hinge points form to further assist the bending of the neural folds towards the dorsal midline. Interactions of epithelial cadherins ensure neural tube closure is completed as the neural folds appose at the dorsal midline (Doudney et al., 2005; Montcouquiol et al., 2006).

Wnt5a Mediates Convergent Extension

One of the crucial mechanisms of neural tube closure is the CE movements of neurepithelium and the underlying mesoderm. Studies in zebrafish, Xenopus, and the mouse demonstrate the conserved core PCP proteins in the fly regulate convergent extension. In a zebrafish study, Trilobite mutants (Lp homolog) displayed an inability of neural progenitor (prechordal plate) cells to re-intercalate after cell divisions, which in turned resulted in ectopic neural progenitor cells and open neural tubes (Ciruna et al., 2006). This suggests that PCP establishes polarity by regulating the plane of cell divisions in addition to regulating cell migration patterns. Our results indicate that Wnt5a is essential in convergent extension, which may support a link that PCP proteins are downstream machinery of Wnt5a. Our LWR measurements and T in situ of neurulating embryos demonstrate an overlap of phenotype of Wnt5a mutant embryos and embryos that are mutant for components of PCP pathway such as Lp and Dvl1/2 (Wang et al., 2006). Although, the overlap is not conclusive due to the less broad expression pattern

seen in Wnt5a mutants compared to PCP mutants presented by Wang et al. To further separate or compare, we need to conduct in-house studies of LTW measurements and T in situ analysis of Lp mutants. Even though Wnt5a mutants shares similar phenotypes to Lp mutants, there is the possibility that Wnt5a initiates a pathway parallel to the PCP pathway.

Neural Floor Plate Patterning

As evidenced in the Lp/Lp mutant, the floor plate is abnormally broadened, which appears to correlate with lack of convergence of the prechordal plate cells (Doudney et al., 2005). The cross sections of E8.5 Looptail mutants display normal elevation of the neurepithelial folds but fail to fuse as a result of the distance between the two folds. Wnt5a mutants appear to have no problems in the elevation process, which compares to components of the PCP pathway. However, Wnt5a mutant embryos display very low penetrance of a broad neural floor plate, the hallmark phenotype of neural tube defects in PCP mutants. Given the difference in neural tube defects between Wnt5a and PCP mutants strongly suggests that the two pathways although both involved in neural tube closure are involved in separate processes.

Wnt5a Patterns Surrounding Mesoderm

The neurepithelium receives polarizing cues to regulate various mechanisms of neural tube closure. The surrounding mesoderm also contributes to neural tube closure by physical force applied to the neural folds in order to bring them to close proximity for apposition to occur (Doudney et al., 2005; Montcouquiol et al., 2006; Wallingford, 2005).

From our results, Wnt5a appears to affect morphogenesis, the size and shape, of paraxial mesoderm that flanks the neural folds. When morphogenesis is disrupted, the force to bring neural folds together is lost. This demonstrates an alternative mechanism of Wnt5a to regulate neural tube closure, instead of regulating the convergence and extension of the neural floor plate as seen in core PCP proteins.

Conclusions

Although our understanding of Wnt5a's involvement in PCP signaling is incomplete, we now have a better view of detailed phenotypes that Wnt5a displays during early gastrulation and neurulation. Regardless of the common phenotype of neural tube defects between Wnt5a and the core proteins of the PCP pathway and the increased penetrance of craniorachischisis in Wnt5-/-;Lp+/-, there is more convincing evidence that these two pathways are independent of one another. The classical definition of a genetic interaction states that a double heterozygote should exhibit and exacerbated phenotype to that of both single heterozygotes. As observed in the Wnt5a/Lp double heterozygote progeny, no unique characteristics were discovered. Also the lack of phenotypic overlap, especially the difference in neural tube morphology, supports that Wnt5a regulates CE to ensure proper neural tube closure through a parallel pathway to that of the PCP pathway. This supports an evolutionary conservation of the PCP pathway in that like Drosophila, Wnt ligands do not mediate PCP signaling. In regard to the theory that most signaling pathways are conserved, studies involving Ror1/2 and Ryk receptors in the mouse are bringing to light the receptors that bind to the Wnt5a ligand (Oishi et al., 2003; Schambony et al., 2007). Additional investigation is necessary to understand if these

receptors mediate patterning in developmental processes such as CE and neural tube closure. Further knowledge will enable us to imply the mechanisms behind disease states where Wnt5a is compromised.

Wnt5a Implications in Human Disease

Most important are the correlations between misexpression of Wnt signaling and human disease or birth malformations and how correction of Wnt signaling in cells can cure such diseases as lymphoma (Liang et al., 2003) and defects resulting from open neural tube (Montcouquiol et al., 2006, Qian et al., 2007; Ueno, 2003). It has been shown that Wnt5a has oncogenic potential in mammary cells (Veeman et al., 2003); however, a recent study has given evidence that Wnt5a down regulates B-cell proliferation and that a loss of function or gene mutation in Wnt5a can lead to B-cell lymphomas and myeloid leukemia (Liang et al., 2003). Also Wnt5a has been shown to be expressed at higher levels as melanoma progression increases (Giles et al., 2002). The function as a tumor suppressor and many other novel functions of Wnt5a have yet to be elucidated due to the unknowns of its pathway. My aim in this research was to discover complementary molecules in the Wnt5a signaling pathway, specifically those associated with PCP signaling, so that clinically applicable functions of Wnt5a can be unlocked.

MATERIALS AND METHODS

Mouse Strains and Genotyping

Mice carrying Wnt5a mutation were obtained from Andy McMahon's lab at Harvard University and genotyped using the following set of primers: 5a neo (5'-GGG AGC CGG TTG GCG CTA CCG GTG G), wnt5a-for (5'-GAC TTC CTG GTG AGG GTG CGT G), wnt5a-rev (5'-GGAGAA TGG GCA CAC AGA ATC AAC). The following reaction for PCR was used: 94^{o}C for 30 seconds, 55^{o}C for 20 seconds, 72^{o}C for 30 seconds; 35 cycles. Looptail mice were a gift from Emory University and were genotyped according to the presence of a loop in the tail.

Length-to-Width Ratio (LWR) Measurement in Neurulating Embryos

Embryos derived from appropriate crosses were dissected at E8.5, presence of a seminal plug the following morning marked E0.5. Yolk sacs were removed for PCR genotyping. Embryos were fixed and stored in 4% paraformaldehyde at 4^{o}C. Before fixing in paraformaldehyde, embryos were transferred to an empty dish and allowed to extend naturally. After counting somite numbers, embryos were photographed with an Olympus U-CMAD3 camera mounted on an Olympus SZX12 dissection microscope. Images of the dorsal view of each embryo were acquired and imported into Adobe Photoshop 7.0 and length-to-width measurements of the trunk of the embryo were made as follows. On each embryo, a two lines was drawn from the base of the left and right side of the headfold to the base of the allantois. The average of these two lines was used for the length measurement. The width measurement was obtained by drawing at least three lines posterior to the headfold from one lateral side to the other lateral side and

averaging the measurements of the total number of widths taken. A total of 170 embryos were taken and measured and statistical analysis was performed by using the Kruskal-Wallis test, non-parametric ANOVA test.

In Situ Hybridization

Embryos were fixed in 4% paraformaldehyde and processed for in situ hybridization. Preparation of digoxigenin-labeled probes was carried out according to the manufacturer's protocol (Roche). Embryos were collected in PBS, fixed in 4% paraformaldehyde overnight, dehydrated, and stored in 100% methanol. After rehydrating embryos through a 75, 50, and 25% methanol series, embryos were bleached with a 4:1 mixture of PBT and 30% hydrogen peroxide. Embryos were incubated with 10 μg/ml of proteinase K for 2 min, washed twice with fresh 2 mg/ml glycine, washed twice with PBT, refixed in 4% paraformaldehyde and 0.2% glutaraldehyde for 20 min, and incubated in pre-hybridization solution (50% formamide, 5X SSC pH 4.5, 1% SDS, autoclave H_20, 50 μg/ml tRNA, 50 μg/ml heparin) at 68^oC for 1 hour. Embryos were incubated in hybridization solution (pre-hybridization + 5 μl/ml brachyury probe) overnight at 68^oC. The next day, embryos were washed twice with solution I (50% formamide, autoclave H_2O, 5X SSC pH 4.5, and 1% SDS) at 68^oC for 30 minutes, then equilibrated for 10 minutes in a 1:1 mix of solutions I and II at 68^oC. Embryos were washed three times in solution II (10% 5*M* NaCl, 1% *1M* Tris-HCl pH 7.5, 0.1% tween-20, and autoclave H_2O) at RT. Embryos were then treated with 100 μg/ml RNaseA in solution II for 1 hour at 37^oC, and subsequently washed in solution II for 5 minutes at RT. Embryos were washed twice in solution III (50% formamide, 2X SSC pH 4.5, and

autoclave H_2O) for 30 minutes at 68^oC. Then the embryos were washed three times in MBST and then place in a preblock solution (10% heat-inactivated sheep serum) for 2.5 hours at RT, embryos were treated with a 1:5000 dilution of anti-digoxigenin Fab fragments (Roche) overnight at 4^oC. After thorough washing with NTMT (2% 5*M* NaCl, 10% 1*M* Tris-HCl pH 9.5, 1*M* $MgCl_2$, 0.1% tween-20, and autoclave H_2O), the hybridization products were visualized using BM purple (Roche) as a substrate. The following probes were used in the in situ expression studies: Brachyury.

Embryo Collection, Embedding, and Sectioning

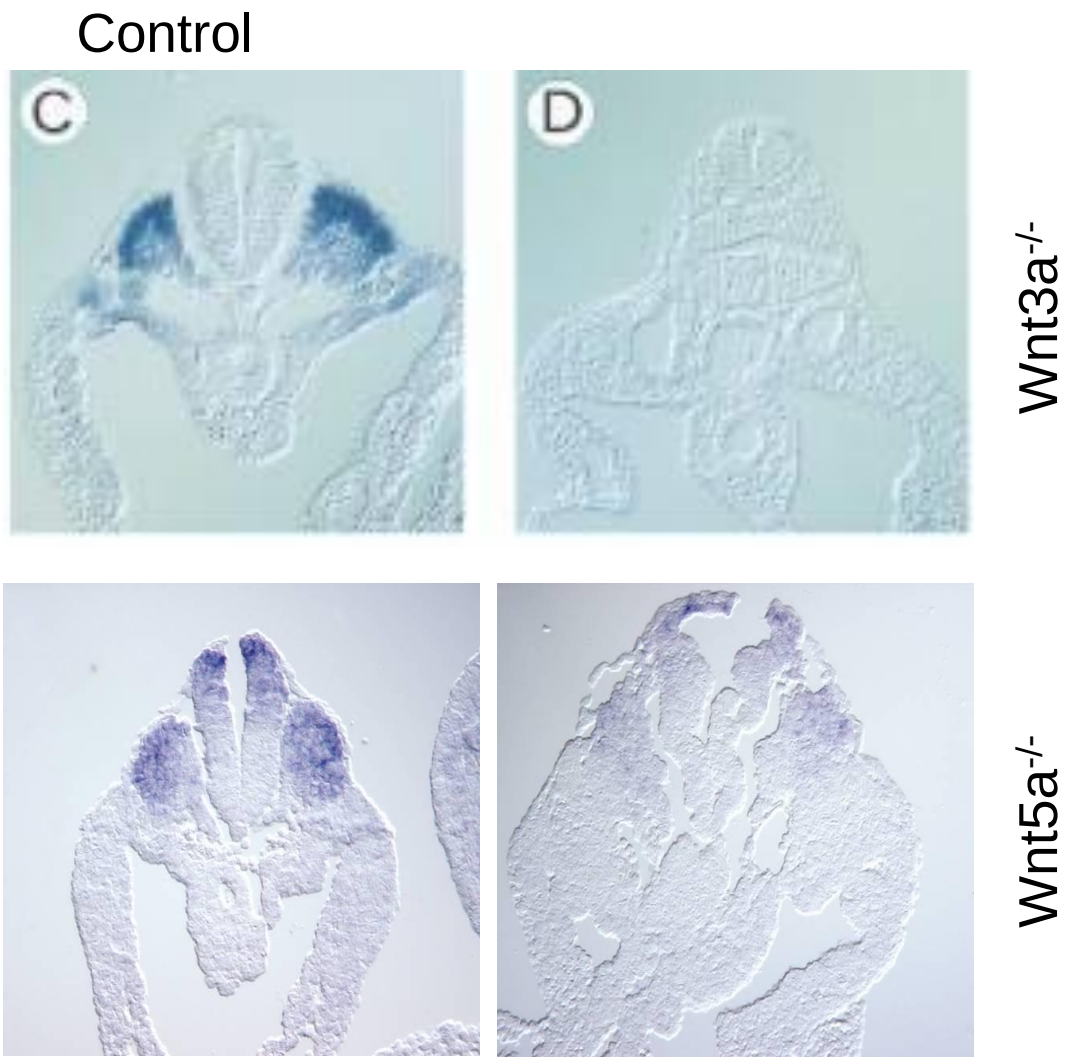
Embryos were dissected into PBS and fixed in 4% paraformaldehyde at least for 24 hours. For analysis of tissue sections, embryos were processed, embedded in paraffin wax, and sectioned at 6μm. First, embryos were washed in PBS and then dehydrated in a 25, 50, 75% methanol series. Embryos were stained with eosin and then finished the dehydration in a 100% methanol wash. Embryos were washed twice in xylene for 3 minutes each and then washed in a 1:1 solution of xylene and paraffin for 6 minutes. Solution was replaced with paraffin wax four more times at 12 minute intervals. Finally, embryos were set in an embedding cartridge overnight and then placed on the cryostat for sectioning.

Immunohistochemistry (for Paraffin-embedded sections)

Sectioned embryos were dewaxed in xylene and rehydrated through a 100 (2X), 75, 50, 25% ethanol series and then stored in PBS. Then slides were treated for antigen un-masking by boiling the slides for 15 minutes in antigen retrieval solution (5mM

EDTA and 1mM Tris-HCl pH 8). Antigen retrieval solution with slides was then cooled on ice until RT and then slides were transferred to a humidified chamber where blocking solution (5% FBS in PBTriton) was applied to each slide for 30 minutes. Slides were treated with a 1:250 dilution of Alexa Fluor 488 phalloidin (Invitrogen) in 2% FBS in PBTriton for overnight at 4^{o}C. The following day, slides were washed three times in PBT and replaced in the humidified chamber and a 1:800 dilution of TO-PRO-3 iodide (642/661) (Invitrogen) in PBT was added to each slide for 10 minutes at RT. Slides were washed three more times in PBT and then excess fluid was removed and Vectashield was added and the slides were coverslipped and analyzed using confocal microscopy.

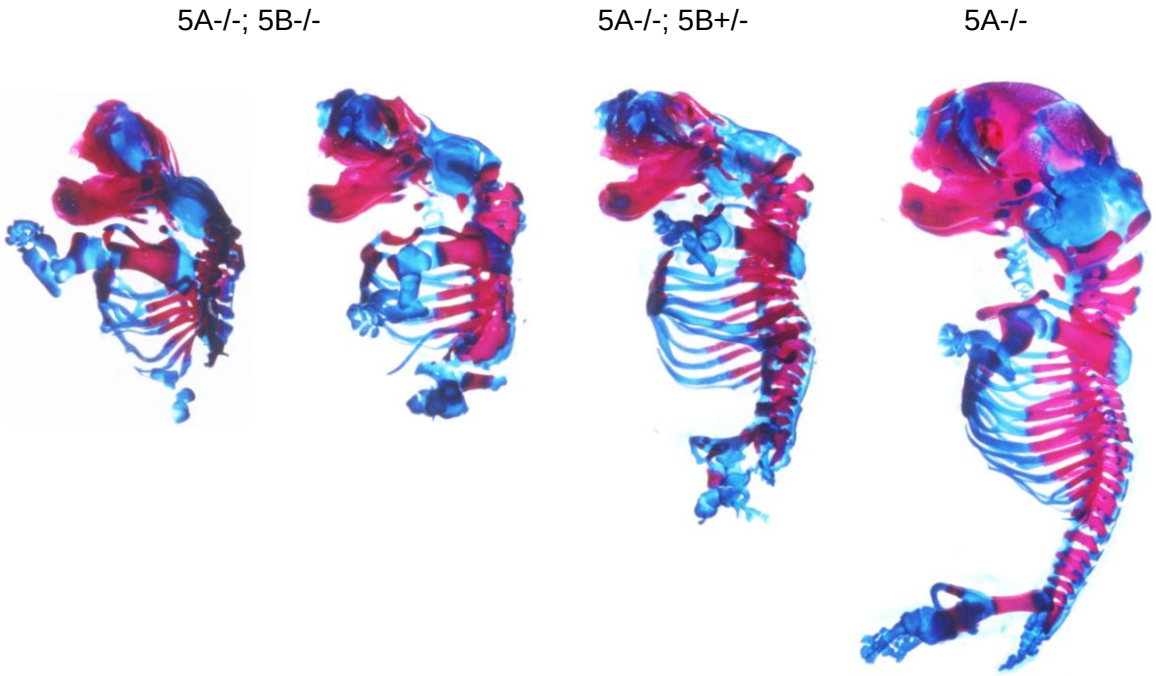
APPENDIX A

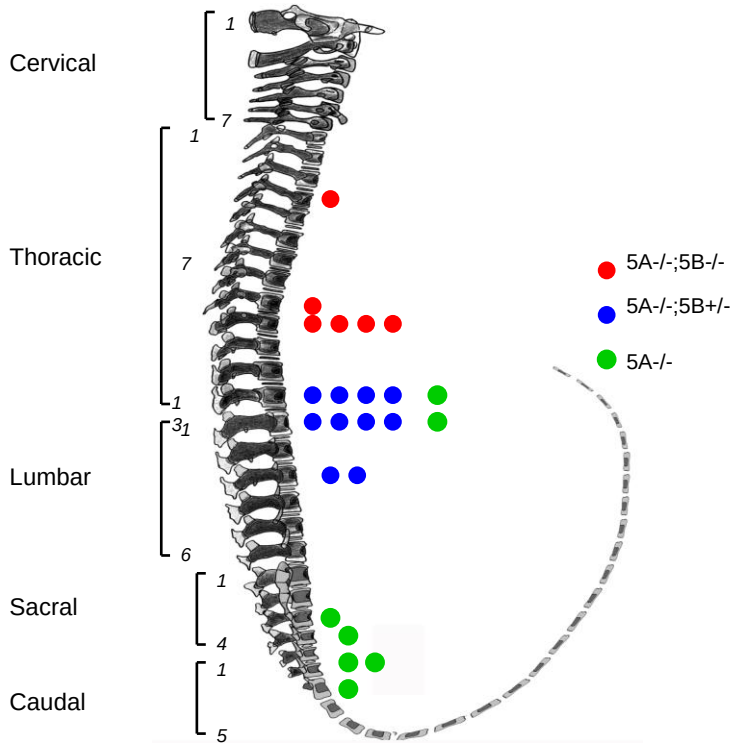


Neural tube cross sections of E8.5 embryos. Pax3 in situ staining for paraxial mesoderm marker is completely lost in the Wnt3a-/-, whereas the Wnt5a-/- expresses the marker, however, in an organized manner.

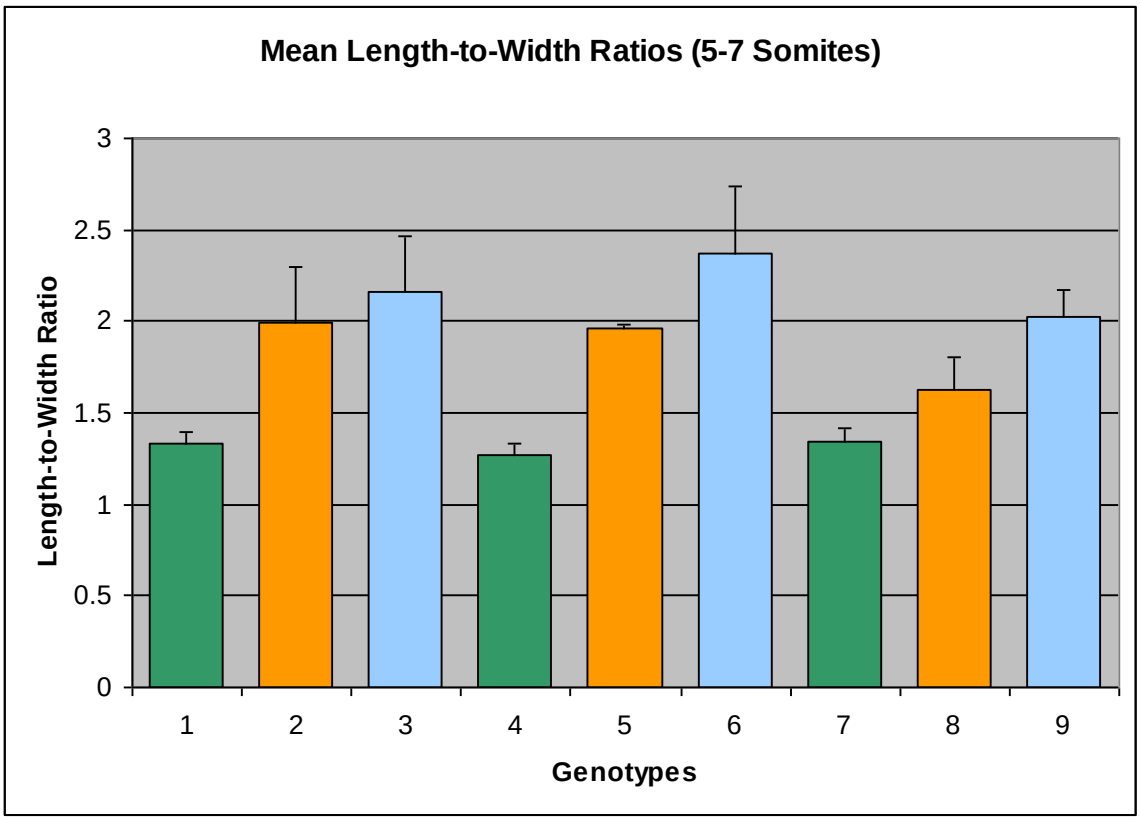
APPENDIX B

Exacerbated Phenotype in Wnt5A-/-; 5B-/-





APPENDIX C



Genotypes

1 – Wnt5a-/-
2 – Wnt5a+/-
3 – Wnt5a+/+

4 – Wnt5a-/-;Wnt5b+/-
5 – Wnt5a+/-;Wnt5b+/-
6 – Wnt5a+/+;Wnt5b+/-

7 – Wnt5a-/-;Wnt5b-/-
8 – Wnt5a+/-;Wnt5b-/-
9 – Wnt5a+/+;Wnt5b-/-

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Education

M.S. in Physiology and Developmental Biology, GPA 3.87 *August 2008*
Brigham Young University, Provo, UT
Thesis: "Wnt5a signaling in the planar cell polarity pathway resulting in convergent extension and neural tube closure during vertebrate development"

B.S. in Biology, GPA 3.52 *April 2006*
Brigham Young University-Idaho

General Education credits earned during high school, GPA 3.67 *1998-2000*
Chattanooga State Technical Community College

Research

Brigham Young University, Provo, UT

Masters student in the laboratory of Jeffrey Barrow *2006-present*
Planar Cell Polarity Signaling and Embryonic Limb Development

Melaleuca, Inc., Idaho Falls, ID

Intern in the laboratory of Jim Kurtz *Summer 2005*
Chemical Surfactants

Teaching Experience at Brigham Young University

Teaching Assistant to Dr. Bruce Woolley in "Physiology of Drug Mechanisms" *2007-8*

Teaching Assistant to Dr. Sterling Sudweeks in "Adv. Physiology Laboratory" *2006-7*

Technical Skills

PCR genotyping
Electrophoresis
Immunohistochemistry
In situ hybridization
Animal handling – snipping, sedating, euthanizing
Mouse husbandry
Embryo extraction
Tissue dissection
Histology
Confocal microscopy
Light microscopy
Cell culture
Microinjecting
Fluorescent dye tracking
Bead implantation
Ectoderm extirpation
Tissue grafting
Plasmid amplification
Plasmid digests
mRNA probe transcription
Autoclaving and dish washing
Preparing stock solutions and diluting stock solutions for protocol use
Proficient in reading protocols and troubleshooting equipment in experimental procedures
Proficient in Microsoft Office programs (Word, PowerPoint, and Excel-Data analysis and data analysis plus statistical software)
Proficient in Adobe Photoshop
Proficient in literature search engines connected to the internet

Awards/Scholarships

Graduate Student Researching Assistantship, Brigham Young University	2007-8
Graduate Student Teaching Assistantship, Brigham Young University	2006-8
BYU Alumni – Chattanooga Chapter Scholarship	2003-8
Jack Wheatley Scholarship	2003-6

Presentations, Publications, and Papers

Poster: Annual Meeting of the American Society of Cell Biology, "Identifying the Wnt5a Signaling Pathway," Jared J. Barrott1, Jed J. Kendall1, Fanxin Long2,3, Andrew P. McMahon2, Jeffery R. Barrow1,2 (1Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT 84602; 2 Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138; 3 Department of Molecular Biology and Pharmacology, Washington University, St. Louis, MO 63110). Washington, D.C., December 2007.

Presentation: BYU Department Seminar, "The Effects of Wnt5a on Embryonic Axis Elongation and Limb Patterning and Outgrowth." March 2007.