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A Novel Role for wnt5b in Regulating Proliferation of Radial Glial Cells

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**A NOVEL ROLE FOR *WNT5B* IN REGULATING PROLIFERATION OF
RADIAL GLIAL CELLS**

A Thesis Presented

by

SEAN W. BURTON

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

May 2011

Neuroscience and Behavior

**A NOVEL ROLE FOR *WNT5B* IN REGULATING PROLIFERATION OF
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ABSTRACT

A NOVEL ROLE FOR *WNT5B* IN REGULATING PROLIFERATION OF RADIAL GLIAL CELLS

MAY 2011

SEAN BURTON, B.A., KENT STATE UNIVERSITY

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Directed by: Professor Rolf Karlstrom

The *wnt* family of secreted glycoproteins perform diverse roles from development through adulthood in both vertebrate and invertebrate organisms. These roles include the establishment of multiple body axes, cell polarity and migration during gastrulation, and prevention of tumorigenesis during adulthood. Much research has been performed to examine the function of the different *wnt* genes, however, some have received relatively little investigation.

One of the relatively unknown *wnt* genes is *wnt5b*. My work has been characterizing a zebrafish with a mutation in the *wnt5b* gene, looking for mutations that caused defects in axonal and glial patterning during embryonic development. Previously, *wnt5b* has been shown to influence planar cell polarity, and has been found to inhibit zebrafish fin regeneration and implicated as an inducer of type II diabetes. However, little research has been performed to investigate its role in the development of the nervous system.

Here I show that *wnt5b* plays a direct role in regulating proliferation of radial glial cells in the embryonic zebrafish spinal cord. Zebrafish with mutations in this gene

have a increased number of radial glial cells that are in m-phase, compared to wild type embryos. I also show that *wnt5b* gain of function is sufficient to reduce this number well below wild type levels. Here I will present findings that, when combined with what has been previously found about the roles of *wnt5b* and its link to leukemia and mammary tumors, suggests that *wnt5b* may be a candidate for further study as a route to cancer therapy.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Studies of human neuropathologies, such as nervous system tumors and neurodegenerative diseases, have branched out into the study central nervous system (CNS) development during embryogenesis. The reason for studying CNS development is that either the cause or the treatment or both of these pathologies is similar to the processes that established the nervous system in embryo. A relatively recent advance in the field of CNS research is the establishment of the zebrafish as a vertebrate model organism for such studies.

Neural and glial progenitor proliferation

The neuroepithelium and ventricular zone

Production of neurons and macroglia (as compared to microglia, which have hematopoietic origins) begin with neuroepithelial (NE) cells that line the ventricular zone of the inchoate neural tube, in a single, pseudostratified cell layer (Magdalena Götz & Wieland B Huttner, 2005). These NE cells have a number of characteristic features; apical-basal polarity within the neural tube, anchored regions at the apical surface, and endfeet that contact the basal surface (Magdalena Götz & Wieland B Huttner, 2005; W B Huttner & Brand, 1997; Wodarz & Wieland B Huttner, 2003). These cells' nuclei also undergo stereotyped movements along the apical basal axis of the neural tube as they proceed through the cell cycle; these movements are referred to as interkinetic nuclear migration (Sauer, 1935; T. Takahashi, Nowakowski, & Caviness, 1993).

The first wave of neurogenesis is spawned by these NE cells, while the later, majority of neurons and neural progenitors are produced by radial glia (Anthony, Klein, Fishell, & Nathaniel Heintz, 2004; Malatesta et al., 2003).

Progenitor pool transition from neuroepithelial cells to radial glia

The transition from NE cells to radial glia approximately coincides with the time that neurogenesis begins in the CNS [cite]. This transition is marked by the expression of several astroglial markers such as astrocyte-specific glutamate transporter (GLAST) and brain lipid binding protein (BLBP) (Feng, Hatten, & N Heintz, 1994; E Hartfuss, Galli, Heins, & M Götz, 2001; Shibata et al., 1997), as well as glial fibrillary acidic protein (GFAP) (Choi, 1981; Levitt & Rakic, 1980).

Interkinetic nuclear migration

Nuclei of both NE cells and radial glia undergo the process of interkinetic nuclear migration (INM) as they progress through the cell cycle . INM is a cycle of stereotyped movements of the cells' nucleus along the apical/basal axis of the neural tube, with each phase of the cell cycle corresponding to one particular location of the nucleus along the axis. In the M-phase of the radial glial cell cycle, the nucleus is located at the most apical end of the cell. During G₁, the nucleus migrates basally, away from the ventricular surface. While the cell undergoes S-phase, its nucleus is located at the basal most point within the ventricular zone, near the ventricular-subventricular boundary. Finally, within the G₂-phase, the nucleus migrates apically, to the location at which mitosis will occur. An illustration of this movement is seen in figure 4.

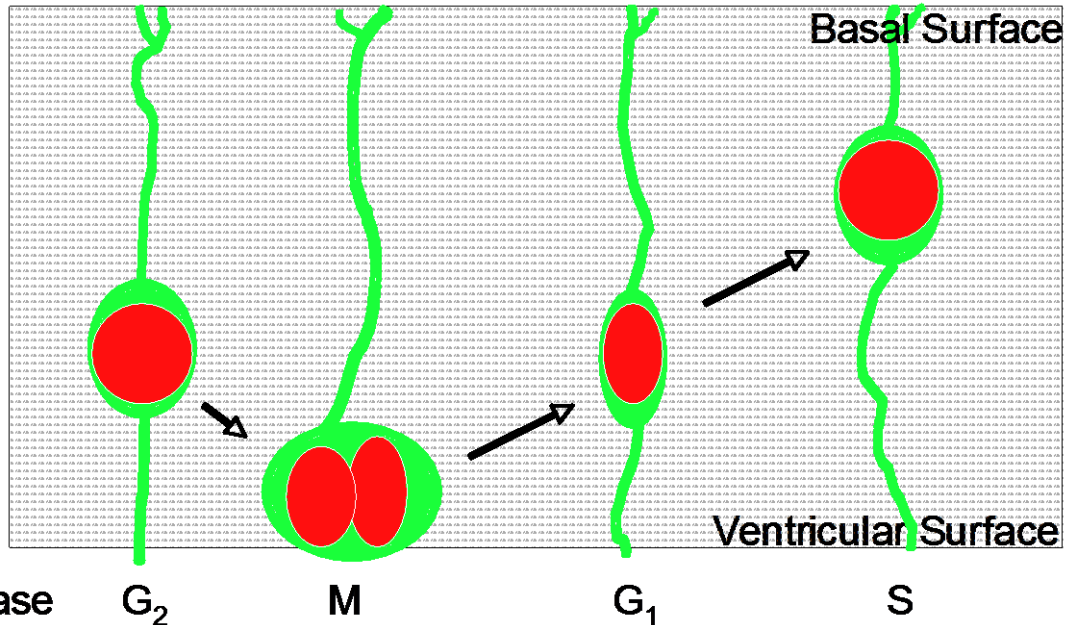


Figure 1: Interkinetic nuclear migration of radial glia in the neural tube/spinal cord. The nucleus of proliferating radial glia moves in relation to the ventricular zone throughout the cell cycle; being at the ventricular surface during m-phase and moving basally during s-phase.

Transit amplifying progenitors

Neural and glial progenitor migration along glial scaffolds

Neural proliferation during adult life

Cell cycle regulation

The cell cycle

Regulators of the cell cycle

[Intracellular] Promoters- Cyclins, CDKs, Cdc25s, Plk kinases...

Inhibitors- Wee1, Myt1, Cip/Kip members and Ink4 members...

[Extracellular]

Wnts, Notch, Shh, EGF, TGF α , FGF

The role of wnts in cell cycle regulation

CHAPTER 2

MUTAGENESIS SCREEN FOR AXONAL AND GLIAL DEFECTS

The Hopkins lab at MIT conducted a large scale insertional mutagenesis screen in zebrafish with the goal of identifying mutations in genes that are necessary for embryonic development (Gaiano et al., 1996). The result was the discovery of 315 genes whose functions are necessary for proper embryonic development in the zebrafish (Golling et al., 2002; Adam Amsterdam et al., 2004). We took a subset of these mutants and performed a screen with the intent of identifying mutants with specific defects in CNS development, in particular, axonal and glial patterning (Barresi et al., 2010). We began our screen with 274 of the Hopkins screen mutants and identified 115 mutants with axonal and/or glial defects (Barresi et al., 2010). 40 of these mutants were omitted from the screen because they were necrotic and may have had defects due to this necrosis rather than mutation specific defects (Barresi et al., 2010). 24 of the remaining mutants were eliminated for having very broad morphological defects; of the remaining 51 mutants, 25 of them had reliably repeated phenotypes and these were subjected to further analysis (Barresi et al., 2010). These data, along with a break down of the different classes of mutant phenotypes, are represented in figure 1 below.

QuickTime™ and a
decompressor
are needed to see this picture.

Figure 2: Overview of Mutagenesis Screen Numbers. This table shows the number of mutations found that fall within each category. The 315 distinct loci were originally identified in the Hopkins screen. The 25 loci with repeated phenotypes may fall into more than of the defect categories. Adapted from (Barresi et al., 2010).

Three primary areas were observed for defects in CNS patterning: the forebrain, hindbrain and spinal cord/trunk (figure 2). For a more detailed review of our screen see (Barresi et al., 2010). Within this range of mutant phenotypes, a novel class of mutants was discovered. These mutants have significant changes in the number of large Glial Fibrillary Acidic Protein (Gfap) positive cell bodies (figure 3); either an increase (e.g., *arnt2*, *kif11*, *esco2*) or a decrease or complete loss (e.g., *ppp1r12a*, *pou5f1*, *mak16*) of

these cell bodies (Barresi et al., 2010). There are a number of possible reasons for the observed changes including gain or loss of apoptosis, changes in regulators of the cell cycle, or a change of cell fate for these cells' precursors. This change in cell numbers could result in several outcomes such as the loss of another cell type or disorganization of the CNS.

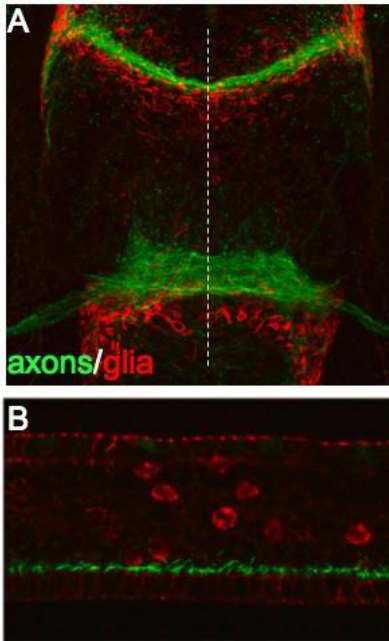


Figure 3: Screening the forebrain and spinal cord for axonal and glial defects. Fluorescent Immunohistochemistry labeling axons in green (acetylated tubulin) and glia (Gfap) in red. (A) A ventral view (anterior up) of the forebrain in a wild type embryo at 42hpf. This view of the forebrain shows two axonal commissures bridging the midline (white dotted line); the anterior commissure (AC) at the top of the image, and the post-optic commissure (POC) at the bottom. The optic chiasm is also shown here but is difficult to discern since it is in front of the POC in this view. (B) A lateral view (anterior left) of the wild type spinal cord at 42hpf. This image is taken at the depth of the ventricular zone. Gfap+ cell bodies seen here center around the ventricle. These cell bodies are only detectable when the cells are in m-phase.

When our screening of these mutants was completed, I undertook further characterization a single screen mutant as my thesis work. I performed preliminary comparisons of Gfap+ cell body numbers between wild type embryos and mutant embryos from the radial glial class of mutants described above. Based on preliminary tests which implied significance in the increase of the number of large Gfap+ cell bodies observed in the spinal cord, combined with my interest in secreted cell signaling molecule such as the Wnts, I decided to further characterize the defects of the *hi1780b/wnt5b* mutant.

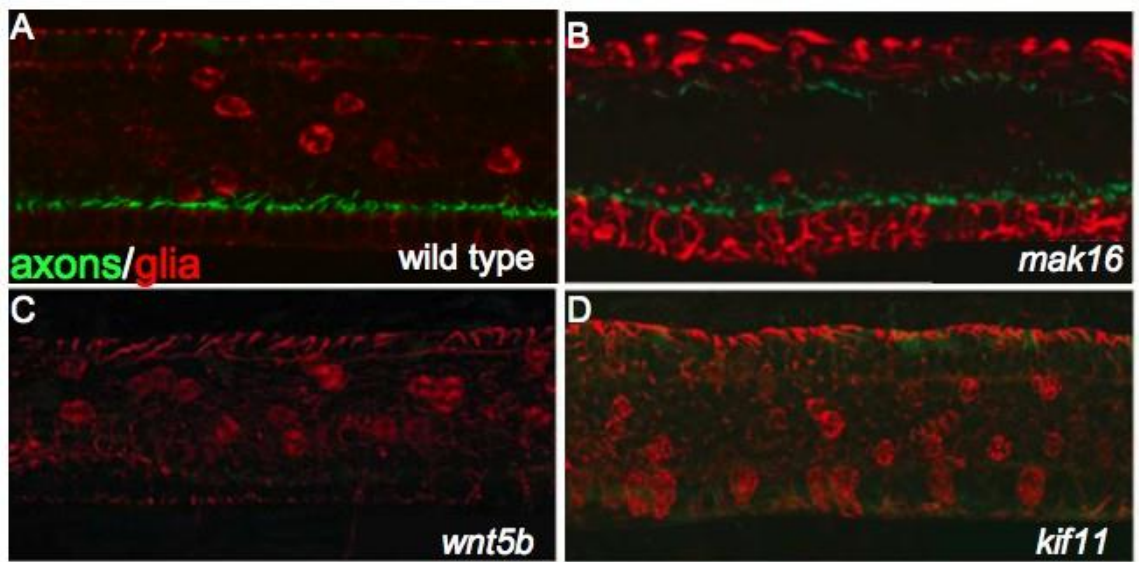


Figure 4: Radial glia class mutants. All images are maximum intensity projections of the spinal cord, centered on the ventricular zone, anterior to the left. Comparison of wild type (A) to radial glia class mutants discovered in our screen with either a decrease (B) or an increase (C,D) of radial glial number.

hi1780b is a *wnt5b* mutant

The hi1780b mutant was identified in our screen for axonal and glial defects as having disorganized forebrain commissure formation and an increased number of Gfap+ cell bodies in the spinal cord (Barresi et al., 2010). As for gross morphology, the hi1780b mutant has a shorter trunk than wild type, with a tail that is noticeably curved ventrally and a smaller head (figure 6).

Since hi1780b was produced in the Hopkins screen, an insertional mutagenesis screen, it was easily identified as to what gene contained the viral insertion. Since the sequence of the viral insert is known, PCR was performed for the region containing the insert, which was then sequenced and compared to the zebrafish genome. This method identified the hi1780b insert as being in exon 4 of the *wnt5b* gene (Figure 7).

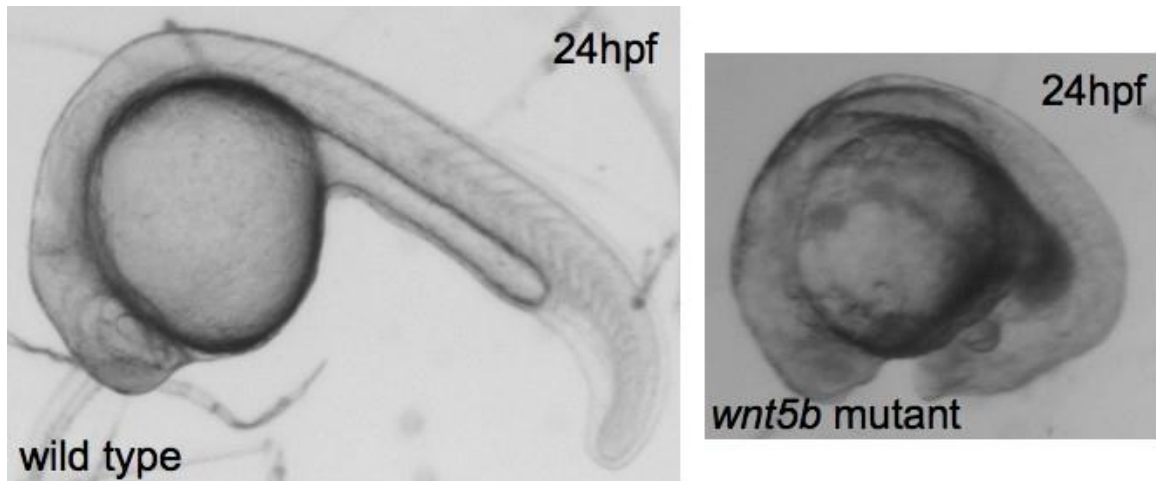


Figure 5: Comparison of wild type and *wnt5b* mutant gross morphology. In comparison to wild type embryos, *wnt5b* mutant embryos have a drastically truncated tail and a smaller head. This mutant phenotype is only appears in homozygous mutants, and

is lethal by 5 days post fertilization, although relatively few embryos survive to 4 days.

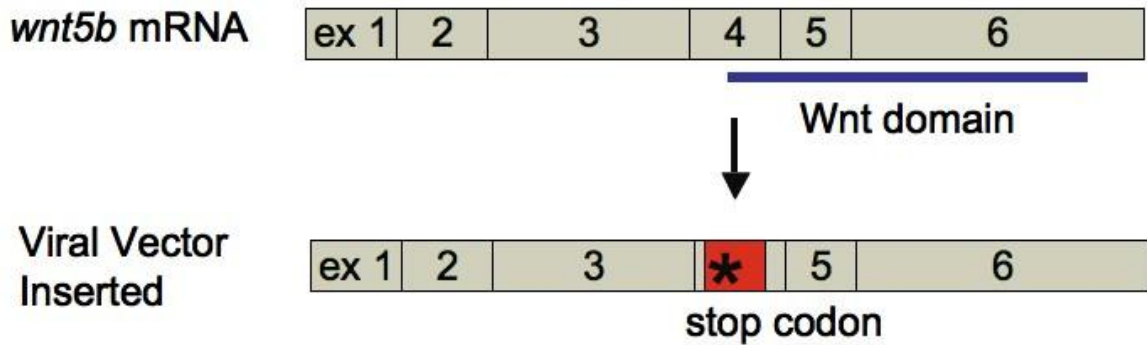


Figure 6: The viral insert in *wnt5b* should lead to a truncated protein. The hi1780b insert from the Hopkins screen is in exon 4 of *wnt5b*. It is predicted that this insert causes a premature stop codon and results in a severely truncated protein that lacks most of the Wnt domain; the domain that is responsible for wnt signaling transduction to wnt receptors.

CHAPTER 3

RESULTS

A loss of *wnt5b* function leads to an increased number of large Gfap+ cell bodies in the spinal cord.

The first step to take in the characterization of this mutant was to determine whether the increase in the number of large Gfap+ cell bodies in the spinal cord is significant. These cells bodies could belong to a few different cell types: radial glia, transit amplifying progenitors (TAPs) or astrocytes, but I will discuss the determination of these cell's identities later in my paper. First, to determine whether a loss of *wnt5b* function significantly increases the number of the large Gfap+ cell bodies that we observed in the *wnt5b* mutant spinal cord, wild type and *wnt5b* mutant embryos were labeled for Gfap using fluorescent immunohistochemistry, and comparisons were made between groups at three time points: 24, 36 and 48hpf. The number of large Gfap+ cell bodies were counted within the length of 3 somites, in lateral view images of the spinal cord. Mutants at displayed a 2-fold increase in the number of large Gfap+ cell bodies (mean=19, sd=4.5, n= 24) when compared to wild type (mean=9.7, sd=2.6, n=26) (figure 8). These numbers were compared using Welch's T-test , $t=-7.3$, $df=25.6$, $p= 1.029e-07$. Comparisons over the time points 24, 36 and 48hpf, are shown in figure 9.

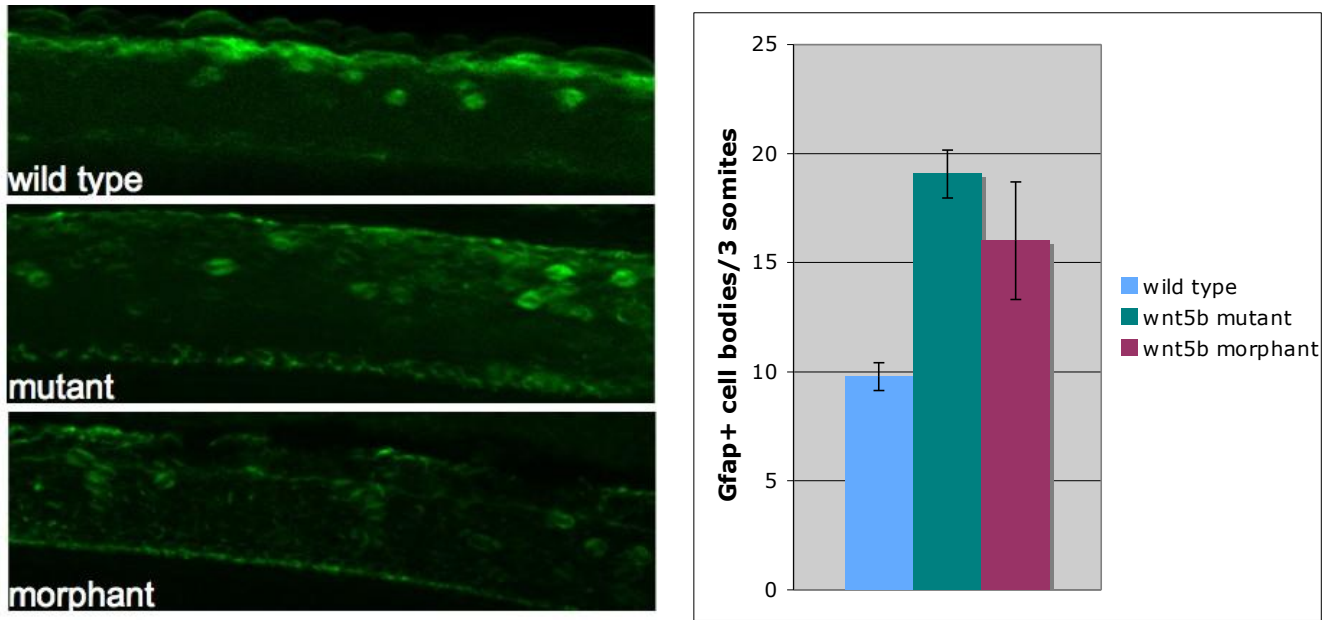


Figure 7: Mutation in *wnt5b* leads to an increase in the number of large Gfap+ cell bodies in the spinal cord. Fluorescent antibody labeling of Gfap in the spinal cord shows an increased number of large Gfap+ cell bodies in the *wnt5b* mutant in comparison to wild type. Embryos injected with 5ng *wnt5b* MO have a similar number of cell bodies to the mutant. Fluorescent images are lateral views of the spinal cord, anterior left.

I next asked whether this increase in large Gfap+ cell bodies was due to a loss of function of *wnt5b* rather than the possibility that the mutation creates a hypomorph, which would still generate a Wnt5b protein but with decreased function. The *wnt5b* transcript contains 6 exons with the translation start site in exon 3 and stop codon in the middle of exon 6; producing a 363aa product. The hi1780b insert is in exon 4, and results in a predicted truncated protein that should lack signaling function; therefore, a loss of function is expected, but needed to be verified. Embryos were injected at the 1-4 cell stage with a translation blocking morpholino oligonucleotide (MO) that should

provide a loss of function, which we can compare to *wnt5b* mutants. Comparing cell body numbers of the MO injected embryos (mean=16, sd=3.6, n=8) with wild type and *wnt5b* mutants revealed a similar number to the mutant (figure 8). Welch's T-tests between MO injected embryos and wild type revealed a significant difference ($p=0.002$), and a non-significant difference between MOs and mutants ($p=0.07$). This suggested to us that the mutation had a similar end result to that of the morpholino; that is, a loss of function, rather than a hypomorph.

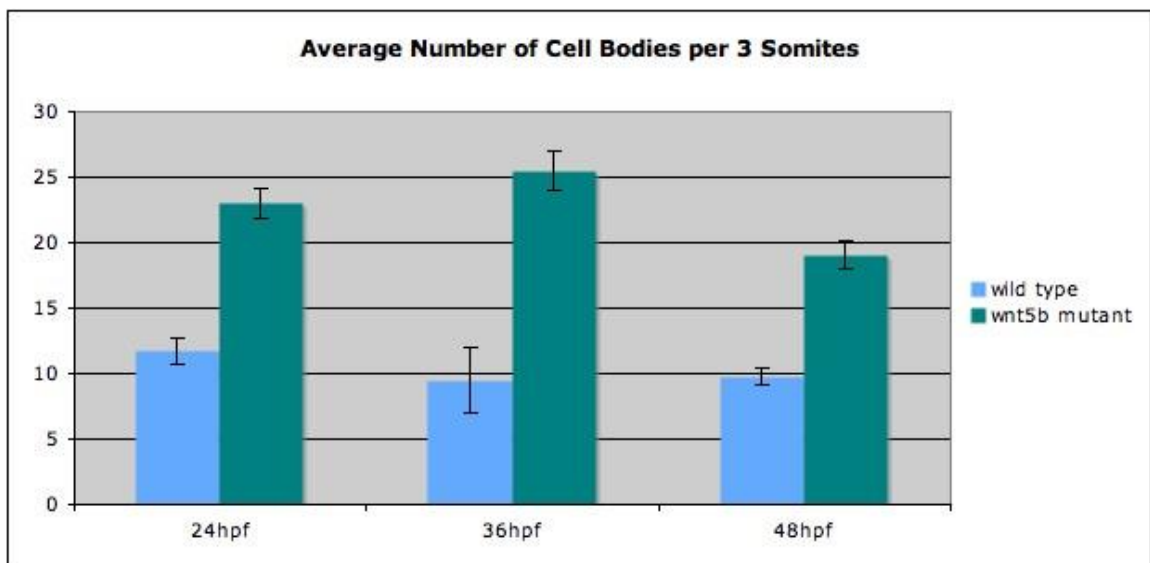


Figure 8: Average number of large Gfap+ cell bodies per 3 somites over time.

Differences between wild type and *wnt5b* mutants were significant at every time point.

Injection of *wnt5b* RNA decreases the number of large Gfap+ cell bodies in the spinal cord.

If loss of *wnt5b* function is sufficient to increase the number of large Gfap+ cell bodies seen in the spinal cord, the next question is whether or not a *wnt5b* gain of function is sufficient to decrease this number. To examine this, I injected wild type embryos with either 5 or 10pg of *wnt5b* RNA and performed the same assay to quantify large Gfap+ cell body numbers described above. Embryos that were injected with 5pg *wnt5b* RNA had approximately half the number of large Gfap+ cell bodies (mean=5, sd=1, n=6) that control embryos had (mean=12, sd=2, n=6) (figure 10). A Welch's a T-test yielded $p=0.002$, a significant decrease in number in RNA injected embryos. This data suggest that *wnt5b* plays a direct role in the regulation of the number of these cells. Embryos that were injected with 10pg *wnt5b* RNA did not survive long enough to be assayed.

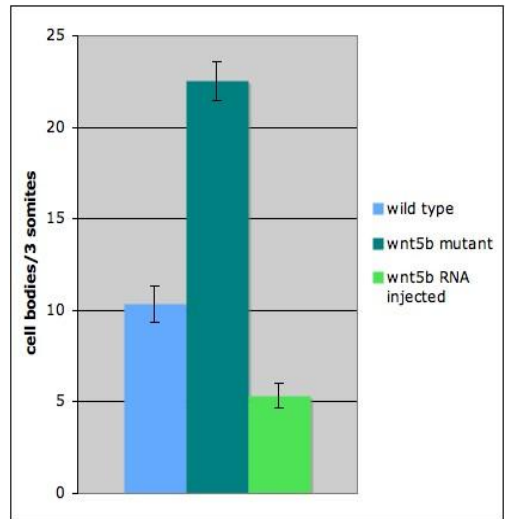
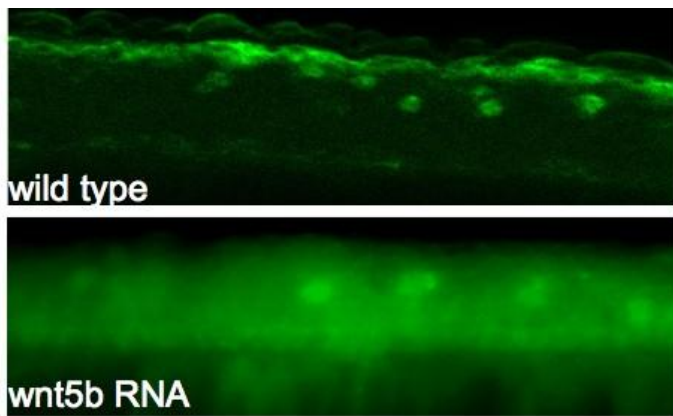


Figure 9: Ectopic expression of *wnt5b* decreases the number of large Gfap+ cell bodies in the spinal cord. Lateral views of a wild type embryo and an embryo that was injected with 5pg *wnt5b* RNA labeled for Gfap. Ectopic expression of *wnt5b* decreased the number of Gfap+ cells by fifty percent.

The large Gfap+ cell bodies observed in the spinal cord are radial glia.

To determine the identity of these cells, the first thing I did was to perform Gfap labeling on spinal cord cross sections from embryonic zebrafish to discern their morphology. Figure 11 shows cross sections of the spinal cord from wild type and *wnt5b* mutant embryos. These cell bodies lie near the ventricular zone with apical processes contacting the ventricular surface and have longer Gfap+ processes that extend toward the basal surface. These morphological traits identify these cell bodies as radial glia (reviewed in (Kriegstein & Alvarez-Buylla, 2009). However, radial glial cell bodies present in the embryonic spinal cord are only easily identified when they are in the m-phase of mitosis, when the cell body is much larger than in the other phases of the cell cycle, and is positioned nearest to the ventricular surface.

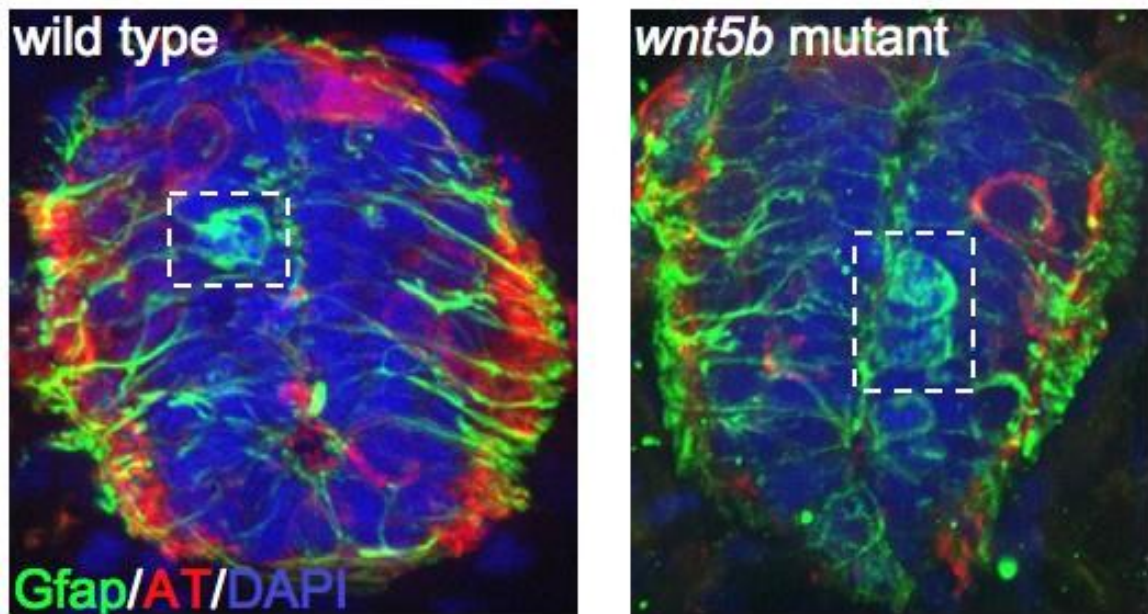


Figure 10: Spinal cord cross sections showing radial glia. Images show cross sections of wild type and *wnt5b* mutant spinal cords (dorsal up) at 48hpf. Glia are labeled in green, axons (acetylated tubulin) in red, and nuclei (DAPI) in blue. The large Gfap+ cell bodies (white boxes) in the spinal cord belong to radial glia. They have large, dual-nucleated somas that are next to the ventricle (medial) and long processes that extend to the basal surface (laterally).

The large radial glial cell bodies observed in the spinal cord are in M-phase.

I observed an increase in the number of radial glial cell bodies in the mutant spinal cord, and their size is indicative of radial glia in the m-phase of mitosis. This implies that *wnt5b* is involved in the cell cycle of these cells. To determine whether these cells are in m-phase, embryos were co-labeled for Gfap and phospho-histone H3 (PH3), a marker for cell nuclei in m-phase (Hendzel et al., 1997) and are shown in figure 12.

Radial glia in the spinal cord co-label for Gfap and PH3 in both wild type and *wnt5b* mutant embryos. Of the total number of radial glial cell bodies in the spinal cord, 86.71% in wild type and 88.46% in *wnt5b* mutants were co-labeled with PH3, a non-significant difference ($p=0.393$). This data verifies that these radial glia are in m-phase and seem to be the same cells in both wild type and mutants.

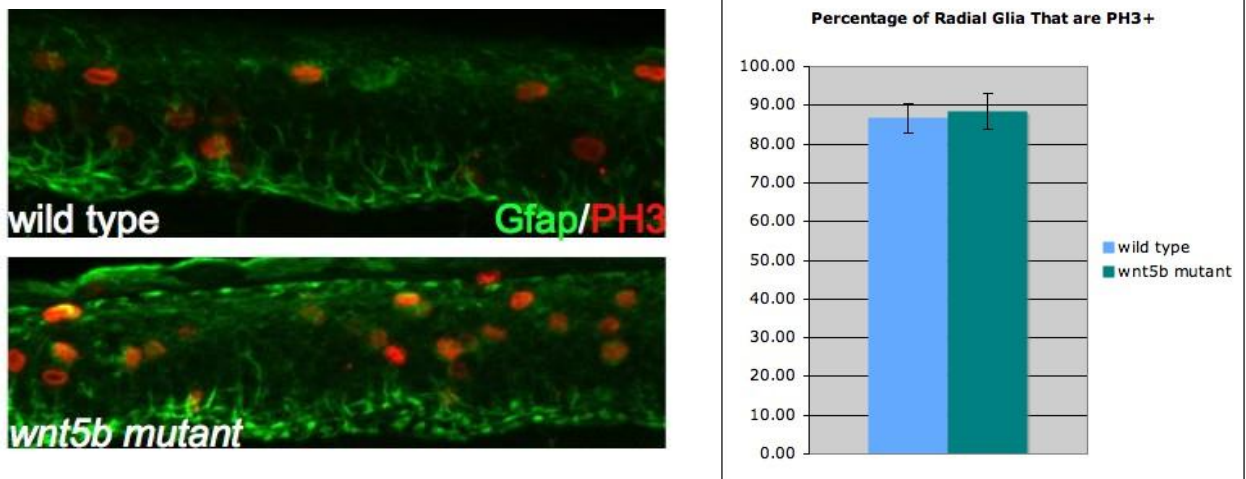


Figure 11: The large radial glia cell bodies in the spinal cord are in m-phase. Double labeling for Gfap (green) and PH3 (red) shows co-labeling in radial glia cell bodies. While the number of radial glia in the spinal cord are increased in the *wnt5b* mutant, the percentage of radial glia that are in m-phase is similar to wild type.

The *wnt5b* mutant does not have a complete block in radial glial mitosis

Since the increased number of radial glia in the spinal cord of *wnt5b* are in m-phase, I now asked the question of whether or not this mutation blocks exit from mitosis. This would imply a role for *wnt5b* in regulating radial glial proliferation. Since these cells are in m-phase, I compared the *wnt5b* mutant with embryos treated with S-trityl-L-

cysteine (STLC), a known inhibitor of radial glial mitosis (Brier, Lemaire, DeBonis, Forest, & Kozielski, 2006).

Radial glial cells in the CNS of embryos treated with STLC have been shown to be locked in mitosis, as it inhibits the separation of the mitotic spindles by blocking function of *eg5*, a motor kinesin responsible for their separation.

Wild type embryos were treated with 0.875mM STLC from 5 to 24hpf, and then fixed for immunohistochemistry. Comparisons of MPRG cell counts in STLC treated embryos (mean=61, sd=14, n=7) and *wnt5b* mutant embryos (mean=23, sd=3, n=10) revealed a significantly increased number in the drug treated group ($p=1.130e-06$) (figure 12). This suggests that *wnt5b* mutants only have a partial loss of the total proliferation of the spinal cord radial glia. To determine how this may be the case, I first compared the location of the glial cell bodies in the *wnt5b* mutant with the location of the expression of *wnt5b* in the spinal cord. To do this, I first had to establish the expression pattern of *wnt5b* during the time points I was studying.

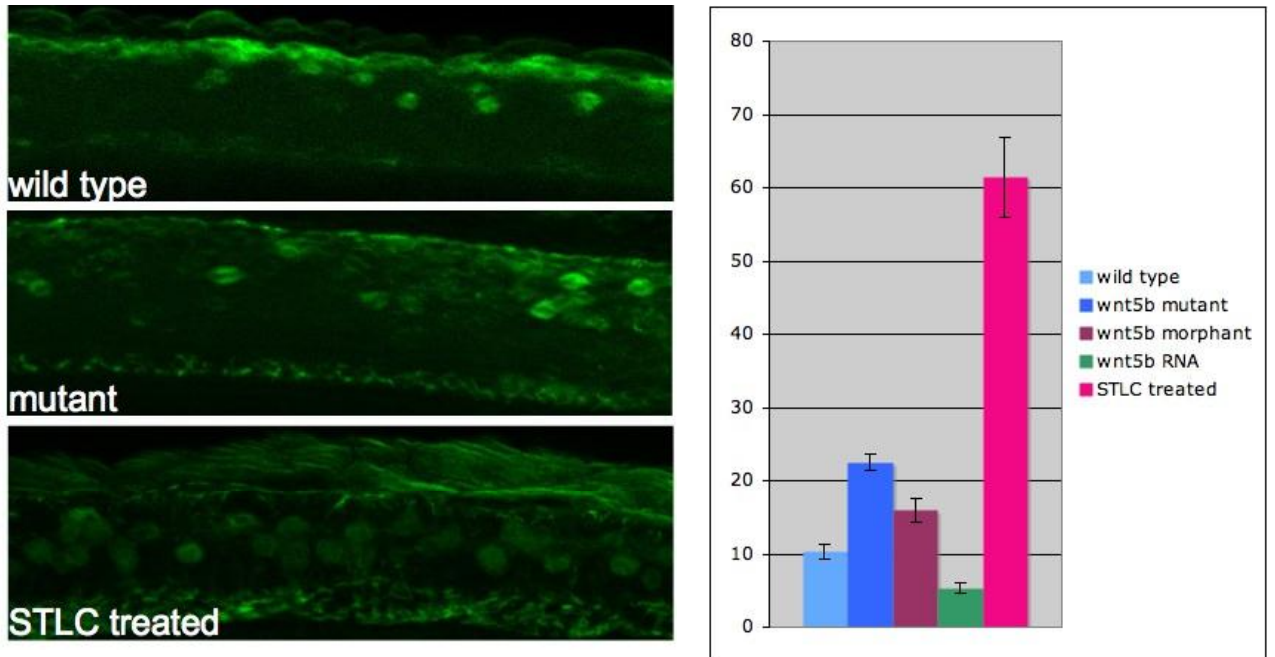


Figure 12: Comparison of STLC treated embryos to wild type and *wnt5b* mutants. Embryos were bathed in 0.875mM STLC from 5hpf to 24hpf, then fixed and processed for immunohistochemistry. Embryos that had been treated with STLC have a marked increase in the number of radial glia in m-phase over both wild type and *wnt5b* mutant embryos. This increase is statistically significant when compared to all other treatments.

Embryonic expression of *wnt5b*

To determine the location of *wnt5b* expression, *in situ* hybridization was performed on 24, 36 and 48hpf embryos. At 24hpf, *wnt5b* is expressed in the dorsal spinal cord, the ventral telencephalon, the dorsal diencephalon (figure 14), the tail bud, and the ventral midbrain and hindbrain (data not shown). At 36 and 48hpf, expression is similar, 36hpf forebrain and spinal cord (figure 14), and a 48hpf spinal cord cross section is shown in figure 15.

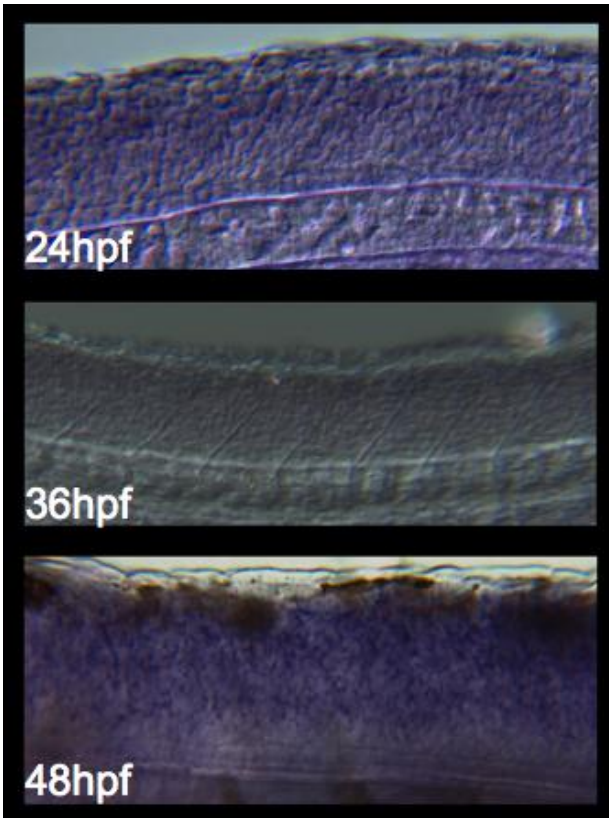


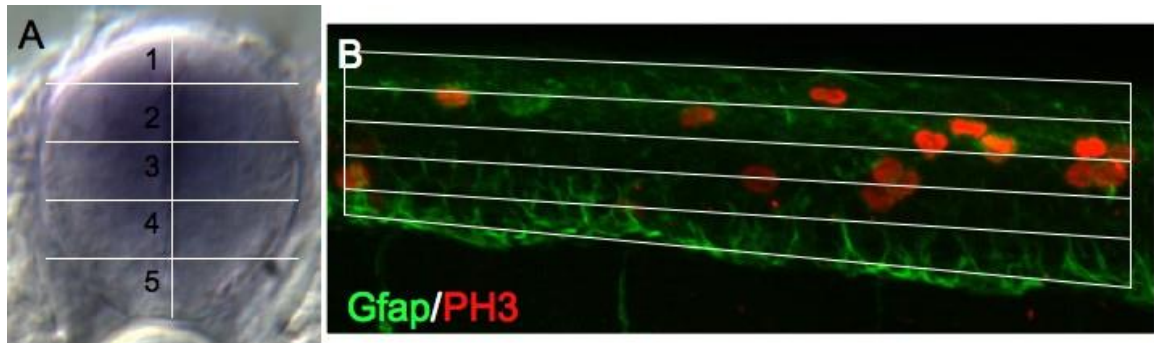
Figure 13: Expression pattern of *wnt5b* in the spinal cord at 24, 36 and 48hpf. (left) Lateral views of *wnt5b* in situ hybridization in whole mount embryo spinal cords. At these three time points *wnt5b* seems to be expressed in all but the most ventral regions of the spinal cord with the strongest expression appearing more dorsal.



Figure 14: *wnt5b* expression in spinal cord cross section. A cross section of the spinal cord of a 48hpf embryo. Here, *in situ* hybridization for *wnt5b* shows expression to be dorsal and centered medially around the ventricular surface. Since radial glia undergo mitosis at the ventricular surface this places *wnt5b* in the proper location for a role in the m-phase of their cell cycle.

Loss of *wnt5b* Function has a Regionally Restricted Effect on Radial Glial Mitosis

In situ hybridization reveals that *wnt5b* is expressed dorsally in the spinal cord, with expression at its strongest nearer the ventricle. To determine if *wnt5b* is directly regulating cell cycle progression of radial glia in proximity to its region of expression, Gfap labeled lateral spinal cord images were divided into 5 rows of equal height along the dorsal ventral axis (1=most dorsal, 5=most ventral) and the number of MPRG in each of those rows was recorded (figure 16). Whereas wild type embryos have a roughly even distribution of MPRG along the dorsal/ventral axis, *wnt5b* mutant embryos have an increased number of these cells in the 2nd and 3rd rows; a region that correlates with the portion of the spinal cord where *wnt5b* is expressed (mean data shown in figure 17).



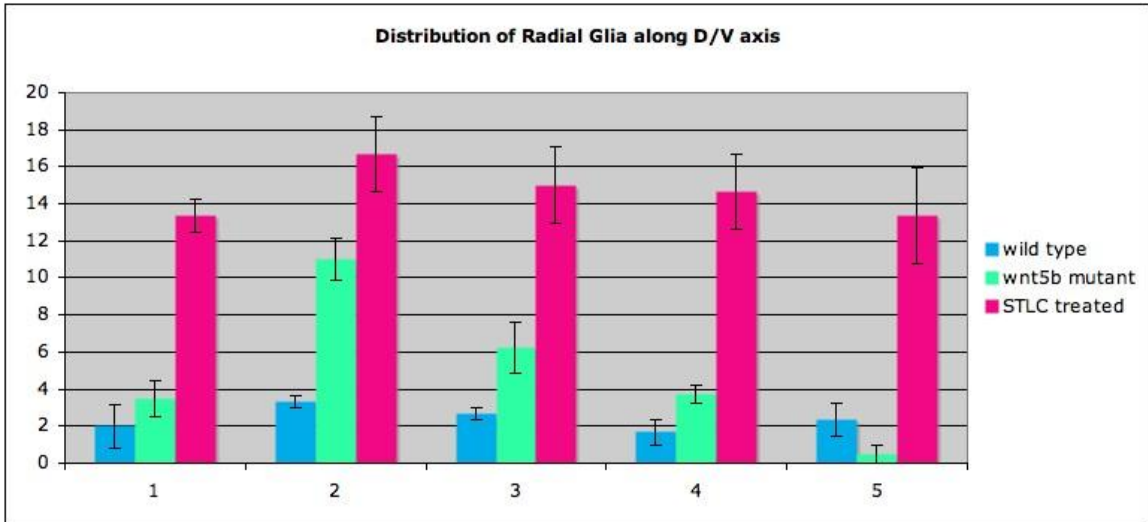


Figure 15: Distribution of MPRG cell bodies over the dorsal/ventral axis. (A)

Diagram of dorsal/ventral axis division into fifths for the purpose of describing radial glia cell body location, 1=most dorsal, 5=most ventral. (B) Dorsal/Ventral cell body counts were done on lateral views of the spinal cord from maximum intensity projections of ~40 μ m thick Z-stack Apotome images. Gfap and PH3 were co-labeled to best determine the location of the glial cell bodies. (Chart) Dorsal/Ventral distributions of cell bodies in both wild type and STLC treated embryos showed no difference along the axis. However, *wnt5b* mutant embryos showed a significant increase in the number of cells in the second row, which corresponds to the location of the strongest *wnt5b* expression. Row 3, with the next strongest expression, showed a very nearly significant increase as well (Tukey test, $p=0.06$).

Both wild type and STLC treated embryos show an even distribution of MPRG cells along the dorsal/ventral axis. However, the *wnt5b* mutant has a significantly increased number of MPRG cell bodies in row 2, the row that corresponds most to the expression of *wnt5b* along the dorsal/ventral axis. Interestingly, the only row that

showed a significant difference between wild type and mutants was row 2 ($p=0.0049$), although row 3, the row with the strongest *wnt5b* expression after row 2, was close ($p=0.06$), and with an increase in sample size, may also show a difference.

Row	wild type	<i>wnt5b</i> mutant	STLC treated
1	2	4	13
2	3	11	17
3	3	6	15
4	2	4	15
5	2	1	13

Figure 16: Average number of cell bodies by row, along

the dorsal/ventral axis. 1=most dorsal, 5=most ventral.

Discussion

A novel role for *wnt5b* as a regulator of Radial Glial Proliferation

The results described above reveal a role for *wnt5b* in regulating proliferation of spinal cord radial glia during CNS development. The finding that a loss of function mutation in *wnt5b* results in an increased number of radial glial cell bodies in the spinal compared to wild type embryos suggests that *wnt5b* negatively regulates proliferation of these radial glia. This increase in the number of radial glial cell bodies is seen across ages, at 24, 36, and 48 hours post fertilization. Unfortunately, *wnt5b* is a gene that is required for embryonic development, so that embryos with the hi1780b insertion in *wnt5b* do not survive through the embryonic stage, days 0-5. In fact, these homozygote mutant embryos rarely live as long as 72 hours post fertilization, making it difficult to collect data from ages older than 48 hours in any significant amount. Conversely, I have shown that, over expression of *wnt5b* RNA leads to a decrease in the number of large radial glial cell bodies. This suggests that *wnt5b* plays a direct role in regulating the proliferation of these radial glia.

The findings that a decrease in *wnt5b* function increases the number of radial glia and that an increase in *wnt5b* function decreases the number of radial glia is in direct agreement with other findings (Y. Lee et al., 2009; Stoick-Cooper et al., 2007), in which they found the same outcomes in regards to blastemal cells of the fin in fin regeneration studies. Thus, it should not be surprising then to find similar results in regards to proliferation of radial glia. However, the question still remains as to how *wnt5b* regulates the proliferation of radial glial.

Proposed model for the regulation of proliferation in radial glia by *wnt5b*

While the work presented above reveals a novel role for *wnt5b* in regulation of radial glial proliferation, there still remains many questions as to how *wnt5b* regulates this process. I propose the following model to describe the way in which *wnt5b* regulates the proliferation of radial glia (figure 18).

Radial glia in the neural tube/spinal cord are originally derived from neural epithelial cells. These radial glia will begin to proliferate, generating both neural and glial precursors. When the radial glia are in the m-phase, they reach one of the cell cycle check points in which proliferating cells must meet certain requirements, such as a lack of DNA damage, in order to continue the cell cycle. Failure to pass checkpoints in either of the g-phases of the cell cycle results in p53 activation and apoptosis (reviewed in (Chiarugi, Magnelli, Cinelli, & Basi, 1994)). I propose that *wnt5b* function is necessary to pass through a similar checkpoint for the radial glia in m-phase. Without this *wnt5b* signal, the m-phase checkpoint requirements are not met, and the cell undergoes a process called mitotic catastrophe (Portugal, Mansilla, & Bataller, 2010). Mitotic catastrophe is a relatively uncharacterized means of cell death, as compared to apoptosis and necrosis. While molecular markers for mitotic catastrophe are not yet identified, cells undergoing this process have a multi-nucleated cell body before death. The radial glia that were found to be increased in number in my study have a similar morphology to the cells described as undergoing mitotic catastrophe. This could be the reason why I did not observe an accumulation of radial glia over time, rather a similar increase at all time points. This would also account for the similarity in radial glial cell number in the *wn5b*

mutants and embryos co-injected with *wnt5b* and *p53* morpholino, as mitotic catastrophe is not dependent on p53 activation (Portugal et al., 2010).

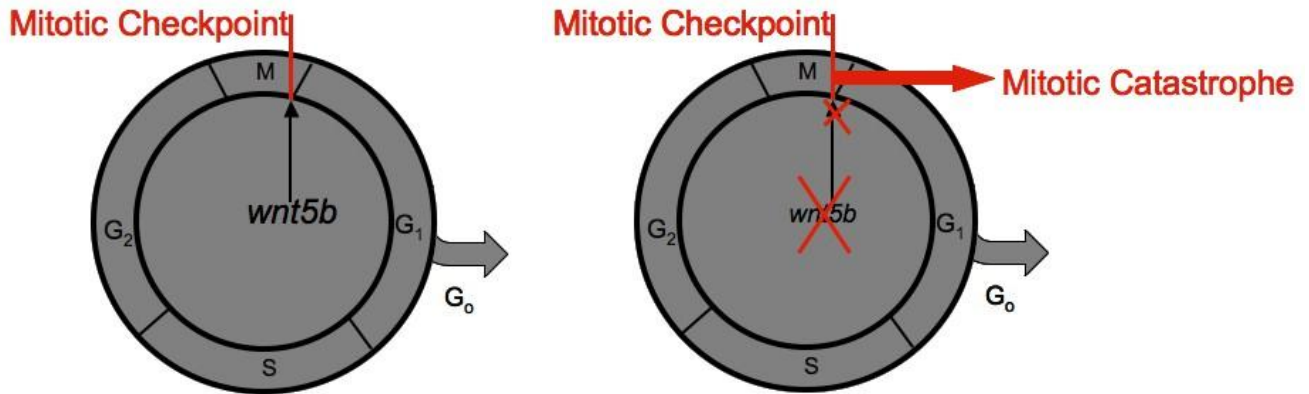


Figure 17: Model for *wnt5b* regulation of proliferation of radial glia. (Left) With functioning *wnt5b* radial glia will pass through m-phase and continue the cell cycle. (Right) Without *wnt5b*, mitotic radial glia will arrest in m-phase and undergo mitotic catastrophe.

Wnt5b may be an important target for cancer treatment

The work I have presented here suggests a novel role of *wnt5b* as a direct regulator of proliferation of the radial glial, which are neural stem cells, in the embryonic zebrafish spinal cord. As current thought in cancer research now regards cancer cells as mutated stem cells, this may imply a role for *wnt5b* in preventing tumorigenesis. This link between *wnt5b* and cancer has already been shown in other research directed at understanding cancer stem cells (see (Kuorelahti, Rulli, Huhtaniemi, & Poutanen, 2007; D. Lu et al., 2004; Mangioni et al., 2005; H. O. Sercan, Pehlivan, Simsek, Ates, & Z. Sercan, 2007)). As defects in *wnt5b* can lead to the inappropriate increase in neural stem cell number, this could imply an importance for further research of *wnt5b* as a target for cancer therapy

Materials and Methods

Fish Lines and Ethics Approval

Zebrafish lines used in all the described experiments were maintained at the University of Massachusetts, Amherst. Embryos were raised in Embryo Rearing Medium (ERM) and staged according to (C. B. Kimmel, Ballard, S. R. Kimmel, Ullmann, & Schilling, 1995)). Embryos were dechorionated with 10mg/ml Pronase (Sigma), fixed at the desired stage in 4% Paraformaldehyde (Ted Pella), and stored in 100% MeOH at -20°C. Mutant hi1780b embryos were identified by gross morphology. The Institutional Animal Care and Use Committee of the University of Massachusetts Amherst approved the housing and maintenance of animals and all experimental protocols.

Immunohistochemistry and In Situ Hybridization

Immunohistochemistry was performed as previously described (Barresi, Hutson, Chien, & Rolf O Karlstrom, 2005), with minor modifications. Briefly, embryos aged 48 hours post fertilization (hpf) were incubated in 100% acetone at -20°C for 10 minutes. Phosphate Buffered Saline (PBS) with 0.3% Triton X (PBS-Tx), was used for rehydration and blocking solution, as well as all washes with the exception of six 10-minute washes in PBS-0.1%Tw (Tween) performed after incubation in secondary antibody. Embryos were digested in 10µg/ml Proteinase K (Pro K) for varying durations depending upon age, 18hpf-0 min, 24hpf-5 min, 36hpf-10 min, and 48hpf-25 min. Blocking solution consisted of PBS with the addition of 0.3% Tx, 2% BSA, 2% DMSO and 5% NGS. All

antibodies were diluted in blocking solution; primary antibodies were incubated overnight at 4°C, secondary antibodies were incubated for 2 h at room temperature.

The anti-goldfish-Gfap antibody (generous donation from Sam Nona, University of Manchester, UK) was used at a dilution of 1:400 to label glial cell bodies and processes (Marcus & Easter, 1995). Radial glia were also labeled using Zrf-1 antibody (monoclonal IgG1, ZIRC) at a dilution of 1:10 (Trevarrow, Marks, & C. B. Kimmel, 1990). Acetylated tubulin antibody (monoclonal IgG2b, Sigma) was used at a concentration of 1:800 to label axons (Wilson, Ross, Parrett, & Easter, 1990). Phospho-Histone H3 antibody (monoclonal IgG1, 6G3 Cell Signaling) was used to label cells in the M-phase of the cell cycle (Kaitna, Pasierbek, Jantsch, Loidl, & Glotzer, 2002) at a dilution of 1:100. A BrdU antibody (monoclonal IgG1, G3G4 Hybridoma Bank) was used at a dilution of 1:10 to label cells that have passed through the S-phase of mitosis. PCNA antibody (polyclonal, Santa Cruz) was used to label cells that are in late G1 to S-phase of the cell cycle, at a dilution of 1:50.

Probes used for in situ hybridization were digoxigenin-labeled anti-sense mRNA probes, synthesized using SP6 or T7 Dig RNA Labeling Kits (Roche). Primers used to synthesize *axin2* and *vim* probes are as follows; *axin2* forward primer sequence GAGAGCGACCGACAAACCAAG, reverse primer sequence CATTGGCAGAACTGTGCAGTC, and *vim* forward primer sequence GCAGATCCAGATGCAGGAGC, reverse primer sequence CGTCTCTGGTCTCGATGGTC. Additional probes used in this study include *wnt5b*, *sfrp5* (Tendeng & Houart, 2006), *ascl1a* (Pogoda et al., 2006), *prl* and *pomc* (Herzog et

al., 2003). In situ hybridizations were performed as previously described (R O Karlstrom, Talbot, & Schier, 1999).

BrdU Treatments

BrdU stock solution (50mM) was thawed at 37°C for 30 minutes, added to ERM at a dilution of 1:5. Embryos were incubated in BrdU solution for 1, 2, 3, and 4 h, and then transferred to fresh ERM to wash out BrdU and then to a second dish of ERM to grow to the desired age. Embryos are fixed overnight at 4°C. BrdU antibody labeling protocol follows the above immunohistochemistry protocol with one exception. After Pro K has been washed out 3x5 minutes in PTx, all embryos are rinsed 2x in 2N HCl, incubated in 2N HCl for 1 hour incubation; followed by 2x rinse and 3x10 minutes wash in PTx.

Cloning *wnt5b*

The *wnt5b* coding sequence (CDS) was cut from its original vector (unknown vector, with only ~1000bp 5' and 3' of the *wnt5b* CDS known) in a digest reaction using EcoRV and SpeI for 2 h at 37°C, followed by 20 min incubation at 65°C to inactivate the enzymes. To blunt the ends of the *wnt5b* fragment, 0.3µl of dNTPs and 0.5µl Klenow were added to the reaction and incubated at room temperature for 15 min, followed by 20 min incubation at 75°C with 1µl 0.5M EDTA, to inactivate the Klenow. The *wnt5b* CDS were then ligated into a pCS2+ vector to gain a poly(A) tail. The pCS2+ was cut in the same digest reaction described above, using StuI. The reaction was then treated with 0.3µl calf intestinal alkaline phosphatase (CIP) for 1 hour at 37°C to prevent self-ligation of the vector.

Both the *wnt5b* insert and the pCS2+ vector were run out on a 1.5% agarose gel for 1 h at 85 V, in separate wells. The gel was visualized using a Gel Doc 2000 (Bio Rad) and the two bands excised for purification using ZR-96 Zymoclean™ Gel DNA Recovery Kit (Zymo Research). 10µl ligation reactions (0.5µl NEB ligase, 1µl NEB ligase buffer, 2µl insert, 1µl vector and 5.5µl Sigma water) were incubated overnight at 16°C.

Ligated product was then transformed in *E. coli*. A 4µl reaction volume was combined with 100µl *E. coli* cells, and placed on ice for 30 min. Cells were then heat shocked at 42°C for 1 min. and then placed on ice for 2 min. Finally, 500µl SOC medium was added to the cells, which were incubated at 37°C for 1.5 h. Cells were then plated on carbenicillin plates and left to incubate at 37°C overnight. Eight samples were taken from a plate and prepared using a QIAprep Spin Miniprep Kit (Qiagen). To test directionality of the insertion, digest reactions were performed using BamHI, and checked by gel electrophoresis.

RNA and Morpholino Injections

The *wnt5b*/pCS2+ plasmid was linearized using APAI, and *wnt5b* RNA was transcribed using SP6 polymerase (Message Machine-SP6 kit, Ambion). RNA was diluted in 1x *Danio* solution, as described (Devine et al., 2009). Embryos were injected with 5pg (Jopling & den Hertog, 2005) or 10pg of *wnt5b* RNA at the 1-4 cell stages. Injected embryos were raised at 28°C until the desired age and fixed in 4% PFA overnight at 4°C.

Pilot morpholino injection experiments were performed using a sample of the *wnt5b* morpholino (Jopling & den Hertog, 2005), generously provided by Jeroen den

Hertog. Experimental data presented here used the *wnt5b* translation blocking MO (Open Biosystems) with sequence: CGCCTCCTCAGATGGAAGTTCTTGG, targets a site 32 bases 5' of the start site. MO injection followed the same process as RNA injection described above. MO was injected at doses of 1, 2, 3, 4, 5 and 6ng. The *wnt5b* morpholino was either injected alone, or co-injected with a p53 morpholino at 4ng; no difference in gross morphology nor radial glia cell number was detected between the two treatments.

Cryosections

Embryos used for obtaining spinal cord cross sections were not treated with MeOH after fixation, instead they were stored in PBS-Tw at 4°C. Embryos were embedded in 1.5% agar, 5% sucrose blocks, which were then equilibrated in 30% sucrose overnight at 4°C. Coronal sections of zebrafish embryos were made using a Leica CM 1950 cryostat. Sections were cut 40µm thick to capture cell bodies in the spinal cord, and mounted on slides. These sections were antibody labeled using the above protocol with some minor modifications. Briefly, 0.1% PTw was used in place of PTx in all cases. Washes and rinses were performed in slide jars (Scienceware); while incubations in antibodies were performed in slide boxes (Evergreen) with slide covers applied after antibody solution. These covers were dissociated from the slides during washes and discarded. After washing out the secondary antibodies, ProLong® Gold antifade reagent with DAPI (Invitrogen) was applied, and a slide cover was again added to the slides.

APPENDIX

EXTRA MATERIAL

Wnt signaling

The wnt family of secreted signaling molecules consists of a large number of different growth factor proteins, with approximately 19 members of the family in humans and 24 members in zebrafish (Rao & Kuhl, 2010; Sprague et al., 2006). They are responsible for a variety of functions both during embryonic development and adulthood including proliferation, cellular polarity, cell fate determination and differentiation, and cell death (Jeffrey R Miller, 2002; Logan & Roel Nusse, 2004; Rao & Kuhl, 2010). Before Wnt proteins are secreted from the cells within which they are generated, they undergo post-translational modification in the form of glycosylation, a modification that seems to be necessary for functional wnt signaling (Gavin, J. A. McMahon, & A. P. McMahon, 1990; Logan & Roel Nusse, 2004). In *Drosophila*, the Wnt homolog Wingless is glycosylated by the transmembrane protein Porcupine, without which wnts are not secreted, but remain in the endoplasmic reticulum (Cadigan & R Nusse, 1996; T Kadowaki, Wilder, Klingensmith, Zachary, & Perrimon, 1996; Logan & Roel Nusse, 2004). Zebrafish have a porcupine homolog, *zgc:55392*, which is predicted to play a role similar to porcupine in wnt signaling (Strausberg et al., 2002), but is relatively uncharacterized. Once the wnt molecules have been successfully glycosylated, they are secreted from the cell of origin and are transported between cells by interacting with heparin-sulfated forms of proteoglycans (G H Baeg, X Lin, Khare, Baumgartner, &

Perrimon, 2001; X Lin, Buff, Perrimon, & Michelson, 1999; X Lin & Perrimon, 1999; Tsuda et al., 1999).

Once the secreted wnt molecule reaches the receiving cell, it binds to a member of the Frizzled (Fzd) family of transmembrane receptors (Bhanot et al., 1996). The Fzd receptors have seven transmembrane domains and a long N-terminal region that contains a cysteine-rich domain; it is this domain to which the wnt molecule binds (Bhanot et al., 1996; Dann et al., 2001; J. C. Hsieh, Rattner, Smallwood, & Nathans, 1999). The wnts also bind to a number of co-receptors, including the low density lipoprotein receptor-related protein (LRP) family of single transmembrane domain co-receptors, without which, Fzd receptors are unable to transduce the wnt signal (Pinson, Brennan, Monkley, Avery, & Skarnes, 2000; Tamai et al., 2000). The extracellular portion of the wnt signaling pathway is conserved between wnt family members. However, when the signal reaches the receiving cell, one of multiple signaling cascades may be activated.

Within the receiving cell, wnt signaling is divided into three categories: the Wnt/ β -catenin, or canonical, pathway, the Wnt/JNK pathway, and the Wnt-Calcium pathway (figure 5), the latter two were formerly grouped together under the umbrella category of non-canonical (Elston & Clifton-Bligh, 2010). Which pathway is initiated at this point is dependent upon several factors, for example, which wnt member is involved and which receptor it binds to (Kawano & Kypta, 2003).

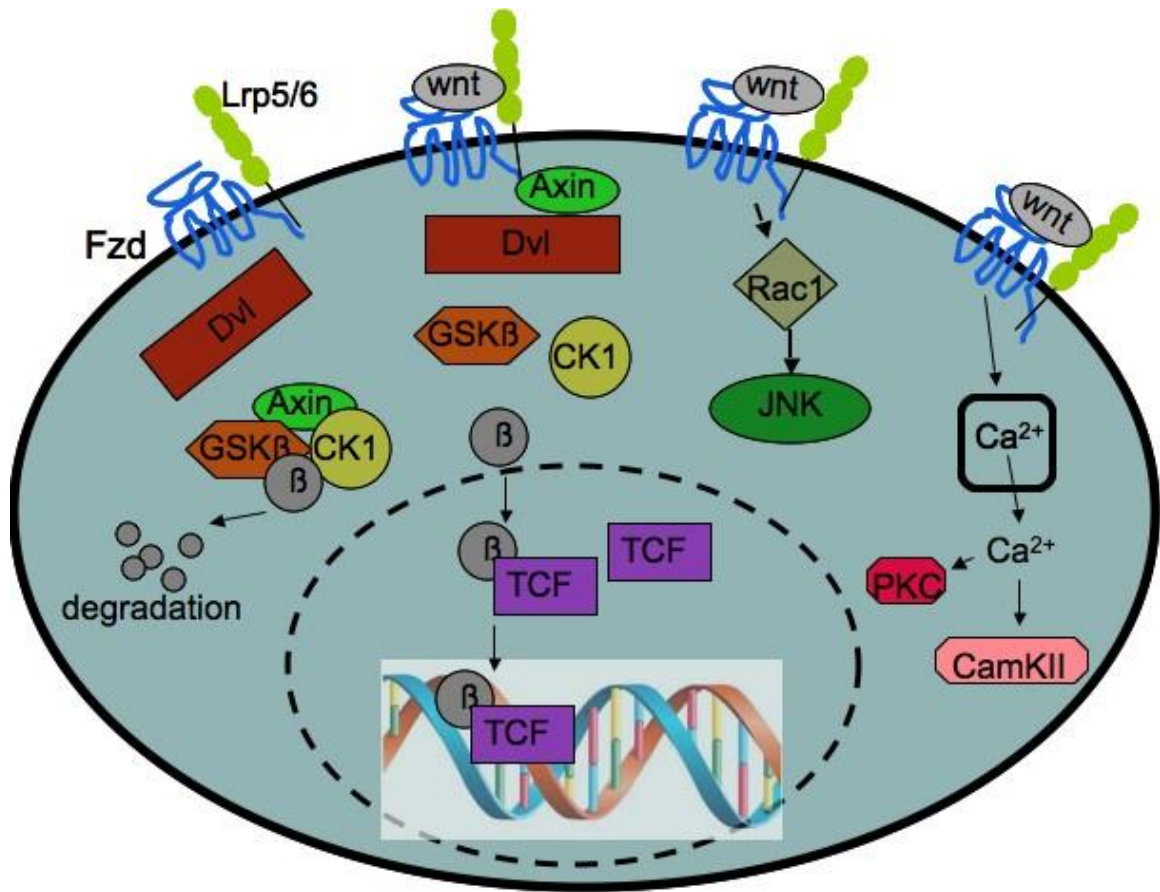


Figure A1: Intracellular components of the Wnt signaling pathways. Wnt signaling can take one of three primary paths once it reaches the receiving signal. The canonical pathway is illustrated (left) in both the inactivated (β -catenin degradation) and activated (β -catenin stabilization) forms. The non-canonical pathways (the JNK and Ca pathways) are illustrated on the right. Much less is known about these two pathways in comparison to the canonical pathway.

Canonical wnt signaling begins with the activated Fzd receptor interacting with the scaffolding protein, Disheveled (Dvl) (Wallingford & Habas, 2005; Wong et al., 2003). Dvl then interacts with Axin, causing Axin to bind to the cytoplasmic domain of

LRP, and this process disrupts a complex consisting of Axin, Adenomatous Polyposis Coli (APC), glycogen synthase kinase-3 β (GSK3 β) and casein kinase 1 (CK1) (Amit et al., 2002; Hart, de los Santos, Albert, Rubinfeld, & Polakis, 1998; S. Kishida et al., 1998; Chunming Liu et al., 2002; Yanagawa et al., 2002; Yost et al., 1996). This Axin complex normally binds β -catenin and phosphorylates it so that it is ubiquitinated by E3 ubiquitin ligase and then degraded by the proteasome (Aberle, Bauer, Stappert, Kispert, & Kemler, 1997; Latres, Chiaur, & Pagano, 1999; C Liu et al., 1999). When Dvl disrupts this complex, it allows β -catenin to avoid phosphorylation and subsequent degradation and instead accumulate in the nucleus, where it binds as a co-activator to T cell factor/lymphoid enhancer factor (TCF/LEF) to activate canonical wnt target genes (Behrens et al., 1996; Cox et al., 1999; J R Miller & R T Moon, 1997; Molenaar et al., 1996; Tolwinski & Wieschaus, 2004; van de Wetering et al., 1997). This canonical wnt signaling pathway is thus also referred to as the β -catenin dependent pathway, as compared to the non-canonical/ β -catenin independent pathways.

Compared to the well studied canonical pathway, the wnt/JNK and wnt/Calcium pathways are less well characterized. These pathways are both activated by certain wnts, such as wnt5a and wnt11, that have been described as non-canonical wnts (Elston & Clifton-Bligh, 2010). Despite these inherent difficulties, it has been shown that wnt5b, which signals primarily through the JNK pathway, is capable of inhibiting the wnt/ β -catenin pathway (Kanazawa et al., 2005; Masuko Katoh & Masaru Katoh, 2007)

The role of wnts in CNS development

Extensive research has been done on many members of the Wnt signaling family in various model organisms. Much of this research has focused on the roles that various Wnts play in development. In mouse, for example, studies on mutants with loss of function mutations in several Wnt genes have produced defects in CNS development including loss of midbrain and cerebellum development (Ikeya, S. M. Lee, Johnson, A. P. McMahon, & Takada, 1997; Mastick et al., 1996; A. P. McMahon & Bradley, 1990; A. P. McMahon, Gavin, Parr, Bradley, & J. A. McMahon, 1992; Thomas, Musci, Neumann, & Capecchi, 1991), decrease in the number of dorsal neural tube derivatives (Ikeya et al., 1997), loss of the hippocampus (S. M. Lee, Tole, Grove, & A. P. McMahon, 2000), and a reduction of the number of proliferating cells (Yamaguchi, Bradley, A. P. McMahon, & S Jones, 1999).

Furthermore, it has also been shown in humans that wnt signaling plays an important role in tumorigenesis (J R Miller, Hocking, Brown, & R T Moon, 1999; Polakis, 2000). More specifically, mutations in components of the canonical wnt signaling pathway, such as APC, Axin and β -catenin, can lead to an increased accumulation of β -catenin in the nucleus, which has been highly correlated with a number of types of human cancers (Polakis, 2000; Satoh et al., 2000). This implies an importance for researching the functions of wnts as a route to discovering and improving methods of treating cancer.

Human Diseases and *wnt5b*

WNT5B has been identified as playing a role in several human diseases. Changes in *WNT5B* expression have been found in association with both osteoarthritis and Type II diabetes mellitus (Hopwood, Tsykin, Findlay, & Fazzalari, 2007; Kanazawa et al., 2005, 2004). Of even greater interest to my work is the close connection found between changes in *WNT5B* expression and cancer.

Wnt5b is over expressed in several types of cancers including mammary tumors, chronic lymphocytic leukemia, and uterine leiomyoma (Kuorelahti et al., 2007; D. Lu et al., 2004; Mangioni et al., 2005). Moreover, it has also been found to be over expressed in chronic myeloid leukemia cells that are undergoing drug-induced apoptosis (H. O. Sercan et al., 2007). In all, these findings suggest an importance in better characterizing the functions of *wnt5b*, with implications for formulating treatments for several types of cancer.

The spinal cord radial glia mutants that we identified in our screen have changes in the number of large Gfap+ cell bodies observed in the spinal cord. Previous work (Alexandre, Reugels, Barker, Blanc, & Clarke, 2010) has shown that the large size and ventricular location of these cell bodies suggests that they are radial glia in the M-phase of the cell cycle. This is in part due to a phenomenon that these radial glia undergo, referred to as interkinetic nuclear migration, which is the movement of the radial glial nucleus over the course of the cell cycle (Taverna & Wieland B Huttner, 2010). During M-phase the radial glial nucleus is near the ventricular surface and during S-phase it is located more basally within ventricular zone (Alexandre et al., 2010; Taverna & Wieland

B Huttner, 2010) (figure 4). However, because of the location of Gfap protein in relation to the cell body, the radial glial cell body is only able to be visualized with Gfap labeling during M-phase, when Gfap accumulates near in the cell body (März et al., 2010). If it is the case that the *wnt5b* mutation affects the number of radial glia in M-phase, it may be the case that *wnt5b* plays a role in the cell cycle of these radial glia.

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