Is the Zebrafish speed bump Mutant a Mutation in wee1? And Is It a Complete Loss of Function?

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Is the Zebrafish *speed bump* Mutant a Mutation in *weel*? And Is It a Complete Loss of Function?

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Submitted in Requirement for Lee Honors College Graduation

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2022
Acknowledgments

To begin, I would like to thank Dr. Kane and Dr. Warga. Thank you for allowing me to join the lab and gain invaluable experience and knowledge. Dr. Kane’s class was challenging but at the same time is very exciting and interesting. He is a very fun professor who never failed to make everybody laugh and kept the atmosphere light and enjoyable during class or lab meetings. I would also like to thank Dr. Warga for everything she did for me. Thank you for never giving up on me and constantly pushing me to my best. Thank you for the vast amount of knowledge and guidance. Thanks for giving me the experience and knowledge that I could not get in my classes. Finally, Dr. Barkman, whom I just met in my last semester in Western. Thanks for being one of the best professors I’ve ever had. Thank you for always prioritizing your students, and thank you for always thinking and giving what is best for them. Thank you for always being patient and despite your business, thank you for agreeing to be my thesis member and helping me with the editing process of writing this thesis.

To my family, I would thank my mom and dad for convincing me to join the honors college and thank you for supporting till the very end. Thank you for putting up with my stressed self. Without you guys, I would never acquire all these invaluable experiences. Thank you so much for that. I would also like to thank my boyfriend, Matthew for giving me endless support throughout my final semesters. Thank you for always taking care of me and for always trying to make me happy whenever I feel stressed.

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Abstract

Every cell has to undergo the cell cycle process before it can divide or differentiate. The cell cycle is an error-prone complex process where mutations could happen. This study aimed to characterize the nervous system and blood cells of the speed bump mutant in zebrafish. This study also aimed to do gene characterization of the wee1 CRISPR mutant. The speed bump mutant is due to a mutation in the wee1 gene. Lack of the wee1 gene will cause no Wee1 Kinase, which inhibits the MPF, causing cells to go into mitosis too early. To fulfill these objectives, this study used in situ hybridization with neural probes and blood probes, antibody staining, as well as sanger sequencing. It is found that, like other cell cycle mutants, neural cells are differentiated in spbti279 mutants, but, unlike other cell cycle mutants, spbti279 mutants are still able to make neuroblasts (stem cells that have decided to become neurons) even at the later stages of development. It is also found that these spbti279 mutants do not produce red blood cells. This study also found that the wee1 CRISPR mutants all have more severe phenotypes compared to the spbti279 mutant and that frameshift mutations cause the wee1 CRISPR mutants to lose the Wee1 protein kinase domain. In spbti279 mutants, the Wee1 protein kinase domain still exists though it lost the last ten amino acids. These results indicate that the spbti279 mutants are less likely to have a loss of function mutation and more likely to have hypomorph mutation. The wee1 CRISPR mutants are likely to have a complete loss of function. The information gathered in this study will allow a better understanding about the wee1 gene as well as the Wee1 Kinase domain during development.
Introduction

All organisms in this world start from single living cells that undergo division to reproduce, such as bacteria and yeast, or to create multicellular organisms such as metazoans. This process, where cells divide, is tightly regulated and is known as the cell cycle. The cell cycle can be broken down into four different phases: G1 (Gap 1), S (Synthesis), G2 (Gap 2), and M (Mitosis), as seen in Fig. 1. In G1, cells grow in size and synthesize cell organelles and proteins, serving as the building block for division. G1 is also the phase where the cell may leave the cell cycle. Next is the S-phase, where DNA is duplicated. During G2, an important checkpoint includes *wee1* to determine if DNA replication is complete; here, the cell also produces more organelles and proteins to prepare for division. Cell division happens in the M-phase. Here, the cell and its’ DNA are divided into two new daughter cells, segregating organelles and proteins equally. If the newly divided cells do not enter another round of cell division, they next enter an inactive state known as G0 instead of G1.
The cell cycle is divided into four main phases: G1, S, G2, and M. G0 is a phase in the cell cycle where cells rest. The complex Cyclin B and CDK1, inhibited by Wee1, is also shown in this diagram to give an overview on how they affect the cell cycle.

The cell cycle is a complicated process; therefore, damage may occur to either individual cells or the organism as a whole when it goes wrong. In some cases, this causes cells to undergo programmed death through a process known as apoptosis (Elmore, 2007), or a mutation is introduced through the gain or loss of a chromosome (Levine & Holland, 2018). There are checkpoints at each phase of the cell cycle to ensure everything goes well. These checkpoints are composed of complexes made of a number of different cyclin proteins and cyclin-dependent kinases. Together they perform specific biochemical functions (Malumbres, 2014).

Zebrafish have become a popular model organism as 70% of all human genes are found in their genomes (Howe et al., 2013). Because zebrafish embryos are optically clear this has allowed scientists to follow live development and the cell cycle under a microscope from the
onset of fertilization (Kane & Kimmel, 1993). Furthermore, zebrafish lay many eggs (50-300) that are fertilized externally, making it easier for researchers to manipulate their genes. For example, one cell stage embryos can be easily injected with DNA or RNA to modify their genetic makeup and because these eggs are born externally, the researcher does not have to harm or kill the parents (D’Costa & Shepherd, 2009).

In the 1990s, a screen was carried out in Tübingen, Germany, by the Nusslein-Volhard lab to identify mutations needed for the early development of the embryo. A category of mutants was found in which embryos were arrested in the first 24 hours of development. This category came to be known as the early arrest group. Embryos in this early arrest group were further divided into two different groups: Class I and Class II. Class I mutants showed cellular abnormalities before gross morphological defects arose in the embryo, while Class II mutants exhibited gross morphological defects before cellular abnormalities appeared (Kane et al., 1996). Included in the Class I mutants was speed bump (spbti279), named so due to its only normal feature, the notochord causing the embryo to resemble a speed bump on the road.

The spbti279 mutant was one of the earliest mutants in the early arrest groups to express a phenotype and was represented by a single recessive allele (Kane et al., 1996). Its phenotype can be easily observed as early as 80% epiboly stage (8.5 hours post-fertilization) by a dark area on either side of the midline that becomes more evident over time (Fig. 2A-D). spbti279 mutant cells also appear to have difficulty with division (Kane et al., 1996). Since isolating the spbti279 mutant, the Kane lab mapped the spbti279 mutant to chromosome 18, near the vicinity of wee1 (D. A. Kane, unpublished). wee1 is a good candidate gene for spbti279 because it plays an important role in the cell cycle, and when wee1 is knocked out, it shows the same phenotype as the spbti279 mutants. The Kane lab sequenced wee1 in the spbti279 mutant and found that a
cytosine in exon 9 is changed into an adenine (R. M. Warga, Unpublished) (Fig. 2 E-F). This converts a serine at position 524 into a stop codon and prematurely truncates the protein (R. M. Warga, Unpublished). This premature termination could result in a null allele or loss of function mutation in \textit{wee1}. Nonsense mutations generally cause decay of the mRNA; therefore, no protein can be translated (Smith, 2022). However, not all nonsense mutations result in loss of function, in some cases, if the mutation is late in the gene, it leads to production of a truncated protein (Smith, 2022). Studies by Emily Johnston in the Kane lab found that \textit{spbti279} mutants have gradual depletion of \textit{wee1} mRNA but not complete loss of these transcripts (unpublished). For this reason, Rachel Warga in the Kane lab, generated mutations in exon 2 of \textit{wee1} using CRISPR/Cas9 mediated germline mutagenesis to create new alleles. CRISPR/Cas9 is a form of gene-editing technology that allows scientists to make targeted double-stranded breaks in the DNA. The CRISPR/Cas9 system consists of two main ingredients: the Cas9 enzyme that cuts through DNA and makes a double-stranded break and a small RNA molecule, known as the guide RNA that directs Cas9 to the specific sequence of DNA. Our DNA machinery will try to fix the double-stranded break. Unfortunately, the process is error-prone and often introduces mutations that can modify the genes (Arazoe & Mizutani, 2020). These \textit{wee1} CRISPR mutants have a more severe phenotype and fail to complement the original \textit{spbti279} mutant allele (R. M. Warga, unpublished). This indicates that the \textit{spbti279} mutant is truly a mutation in \textit{wee1}, but that it may not be a complete loss of function but rather a hypomorphic mutation, which is a mutation that cause partial loss of gene function.
Fig. 2 Overview of the spb<sup>ti279</sup> mutant  

A) A wild type zebrafish embryo developed normally at 15 hours after fertilization.  
B) A wild type zebrafish embryo developed normally at 24 hours after fertilization.  
C) spb<sup>ti279</sup> mutant embryo at 15 hours after fertilization with dark region around the neural keel and shorter length.  
D) spb<sup>ti279</sup> mutant at 24 hours after fertilization with dark region throughout the embryos (Kane et al., 1996)  
E) Wee1 sequence data for wild type zebrafish has serine at position 524.  
F) Wee1 sequence data for spb<sup>ti279</sup> mutant zebrafish has a mutation where cytosine (C) is replaced with adenine (A) causing a serine to be changed to a stop codon (R. M. Warga, unpublished).  

*wee1* is a gene first discovered in yeast by Paul Nurse in 1975 while working at the University of Edinburgh in Scotland. Nurse found smaller colonies than typical yeast colonies, hence the name *wee1*. Further study found that *wee1* mutants have a shorter G2 phase (Nurse, 1975; Russell & Nurse, 1987). Eventually, Nurse and members of his lab found that *wee1* encodes a Kinase that regulates cell entry into mitosis by inhibiting the activity of the Maturation Promoting Factor (MPF) (Murray, 2016).

The MPF is a complex composed of Cyclin-Dependent Kinase 1 (CDK1) and Cyclin B1 (Tyson & Novak, 2015). It is responsible for the breakdown of the nuclear envelope, stimulating spindle formation, and condensation of the chromatin within the cell (Blow & Laskey, 1988; Gautier et al., 1990; Labbé et al., 1989). MPF complex activity is also regulated by several other
factors such as CDC25, a dual specificity phosphatase (Perry & Kornbluth, 2007), and the CDK activation kinase (CAK). For cells to enter mitosis, the MPF must be activated (Fig. 3). First, CAK phosphorylates the MPF at threonine 161 while at the same time, Wee1 inhibits this activation by phosphorylating threonine 14 and tyrosine 15 on the MPF (Fesquet et al., 1993). This complex regulation prevents a cell from entering mitosis too early, giving the cell enough time to proofread all the DNA. Once the cell is ready to enter mitosis, CDC25 dephosphorylates threonine 14 and tyrosine 15, releasing the inhibition placed on the MPF by Wee1 (Fig. 3) (Berry & Gould, 1996; Den Haese, Walworth, Carr, & Gould, 2013). Thus CDC25 and Wee1 act in the opposite, one as the gas and the other as the brake pedal on the MPF; dephosphorylation by CDC25 actives the MPF, whereas phosphorylation by Wee1 inhibits the MPF. As a result, a wee1 mutation will lack of inhibition on the MPF, and cells will go into mitosis too early before DNA replication or DNA proofreading is complete (Perry & Kornbluth, 2007).

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Fig. 3 MPF, CDC25, and Wee1 interaction. Active MPF is necessary for the cell to enter mitosis. CAK will phosphorylate threonine 161 (T161) followed by Wee1 inhibiting the activation of MPF by phosphorylating threonine 14 (T14) and tyrosine 15 (Y15), keeping cells from entering mitosis prematurely. CDC25 dephosphorylates threonine 14 and tyrosine 15 to active the MPF and allowing cell to enter mitosis. CDC25 and Wee1 acts as both gas pedal and brake pedal respectively on MPF. The mutation on Wee1 will remove the brake pedal and will result in continually active MPF thus cells entering mitosis prematurely (Figure from E. Johnston, unpublished, 2019)
Cell cycle mutants generally have problems with cell types that maintain actively dividing cell populations, such as neural cells and blood cells. The Kane lab studies the cell cycle and throughout the years has worked with a number of cell cycle mutants including: harpy, ogre, and spectre (Riley et al., 2010; Warga et al., 2016; Petrachkova et al., 2019). These studies all examined the development of various tissues and organs and found a general trend that the initial patterning of the nervous system and blood is mostly normal, and only later differentiating cell types are affected. This is because these are the cells that continue to divide. This motivates a similar study with the spb\textsuperscript{279} mutant.

Here, I characterize the development of the spb\textsuperscript{279} mutant concentrating on the nervous system and blood using in situ hybridization and antibody staining. Unlike all other cell cycle mutants, there is no gradual reduction of the neuroblasts, which are neural stem cells progenitors. spb\textsuperscript{279} mutants appear able to constantly produce neuroblasts, even at later stages. In addition, while spb\textsuperscript{279} mutants produce endothelial cells, a cell type that shares a common lineage with blood, spb\textsuperscript{279} mutants have no red blood cells. I also characterized the development of the CRISPR/Cas9 wee1 generated mutants made by targeting exon 2 on wee1, from here on referred to as the wee1 CRISPR mutants, concentrating only on the nervous system. wee1 CRISPR mutants have a far more severe phenotype compared to spb\textsuperscript{279}, in that they have almost no neural precursors. We also analysed the sequence data of several of the wee1 CRISPR mutants, and based on their putative amino acid sequence, we show that all are missing the region responsible for Wee1 kinase activity. From all the collected data, I conclude that the spb\textsuperscript{279} mutant is not a true loss of function mutation and is more likely to be a hypomorph mutation which is a partial gene mutation, while the wee1 CRISPR mutants are likely the true loss of function mutation.
2. Materials and Method

2.1 Zebrafish strains or Identification of Speed bump heterozygotes

The \( sbp^{4279} \) mutants were outcrossed to wild-type strains to make heterozygote fish. For this study, outcrossed fish were set up weekly to identify carriers of the genetically recessive lethal mutation. Cells were collected, sorted, and cleaned. Then embryos were grown on E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2 2H2O, 0.33 mM MgSO4 7H2O) at 32°C for 24 hours. After 24 hours, if \( sbp^{4279} \) mutants embryos were identified from the crosses, the parent fish were transferred to a new tank.

Trans-heterozygous fish stocks were made by crossing \( sbp^{4279} \) with wee1 CRISPR mutants. The embryos from these crosses were collected, sorted and cleaned. The embryos were then grown on E3 media at 32°C until it reached the right stage.

Wee1 CRISPR mutants that we subjected to in situ hybridization were made by crossing two different kinds wee1 CRISPR mutants (cr24xcr25, cr20xcr22, and cr50xcr22). The embryos from these crosses were collected, sorted, and cleaned. The embryos were then grown on E3 media at 32°C until it reached the right stage.

2.2 Embryos staging

Heterozygous fishes that were positively identified were crossed weekly to acquired wee1 CRISPR mutant embryos. Embryos were sorted, staged, and stored in E3 medium. By manipulating the temperature, the growth speed of the embryos can be controlled. The embryos were studied at these exact stages: tailbud, 4-somites, 5-somites, 8-somites, 12-somites, 16-somites, 20-somites, and 24 hours.
2.3 Embryo Fixation and Dechorionation

Embryos were fixed in 4% paraformaldehyde (4% PFA) in 1X Phosphate Buffer Saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4 7H2O, 1.4 mM KH2PO4). Embryos younger than 8-somites were fixed with intact chorions by placing them into a 2 mL Eppendorf tube containing 1 mL of 4% paraformaldehyde in 1X PBS. Embryos older than 8-somites were manually dechorionated first before placing them into a 2 mL Eppendorf tube containing 1 mL of 4% PFA. Embryos were fixed overnight at 4°C on a rocker. After the fixation period, embryos that haven’t been dechorionated, were manually dechorionated on a small petri dish.

Dechorionated embryos were then washed four times with 1x Phosphate Buffered Saline with Tween 20 (PBST; 137mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.76mM KH2PO4, 0.1% Tween 20, pH 7.4) with the solution changed every 10 minutes. Embryos were then stored methanol at -20°C in 1.5 mL Eppendorf tube.

2.4 Whole mount In situ hybridization

Stored embryos were rehydrated with sequential 5 minute, 200 uL washes of three to one, one to one, and one to three ratios of methanol to PBST solutions. Rehydrated embryos were washed four times with PBST sequentially for 10 minutes before being digested with Proteinase K (sigma) diluted in 1:1000 ratio. Figure 4 shows the times for which each stage was digested.

<table>
<thead>
<tr>
<th>Stage of Embryo Development</th>
<th>Ratio of Proteinase K to PBST</th>
<th>Time Required for Digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>tailbud</td>
<td>1:2000</td>
<td>50”</td>
</tr>
<tr>
<td>4-somites</td>
<td>1:2000</td>
<td>2’</td>
</tr>
<tr>
<td>8-somites</td>
<td>1:1000</td>
<td>1’</td>
</tr>
<tr>
<td>12-somites</td>
<td>1:1000</td>
<td>2’</td>
</tr>
</tbody>
</table>
Fig. 4 **Digestion times for different stages of zebrafish.** The figure illustrates the amount of time each stage should be digested for desired results based on the data collected in the lab.

Once the digestion is done, embryos were immediately refixed in 4% PFA for a minimum of 1.5 hours at room temperature on a rocker. Once the second fixation was done, embryos and solution handling were performed using gloves to reduce RNA contamination. The embryos were washed five times with PBST for five minutes each on a rocker to remove the 4% PFA completely. Embryos were then soaked in 200 uL prehybridization mixture (Hyb +; 60% Formamide, 5X SSC, 50mM Heparin, 500mM t(torula)RNA, 0.1% Tween 20, 1M citric acid) for two to five hours in a 70°C water bath. Prehybridization mix was then changed and replaced with 200 uL of hybridization mixture containing antisense RNA probe (deltaA, Notch1b, gata1, Pax2A, Islet1, tal1, and hbbe2) and held at 70°C overnight.

RNA probes were taken out and restored the next morning. A quick wash in 200 uL 100% hybridization solution (Hyb -) was done as soon as the probes were taken out. Then the embryos were sequentially washed with 200 uL of three to one, one to one, and one to three ratios of hybridization solution to 2X saline sodium citrate (SSC; 0.3M NaCl, 30mM Na3C6H5O7) for 15 minutes at 70°C. Embryos were subjected to 15 minute washes with 2X SSC at 70°C before washing twice with 0.2X SSC for 30 minutes at 70°C. After that, embryos were removed from the water bath and wash sequentially with three to one, one to one, and one to three ratios of 0.2X SSC to PBST for 10 minutes at room temperature. The embryos were then washed with 100% PBST for 10 minutes at room temperature on a rocker. After that, the

<table>
<thead>
<tr>
<th>Stage</th>
<th>Dilution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-somites</td>
<td>1:1000</td>
<td>2’30”</td>
</tr>
<tr>
<td>20-somites</td>
<td>1:1000</td>
<td>3’</td>
</tr>
<tr>
<td>24 hours</td>
<td>1:1000</td>
<td>4’</td>
</tr>
</tbody>
</table>
embryos were put into a 1 mL block solution (PBST, 2% goat serum, 2mg/ml BSA) for a minimum of two hours at room temperature. Block solution was then replaced and embryos were incubated at 4°C overnight on a rocker with anti-DIG diluted in 1 to 5000 ratio.

The next day, anti-DIG antibody was taken out and restored. Embryos were washed six times for 15 minutes with 1X PBST at room temperature on a rocker. Embryos then were washed in staining buffer (100mM Tris HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20) for 5 minutes at room temperature before being washed in staining solution (solution buffer, 50 mg/mL NBT, 50 mg/mL BCIP) in the dark until staining became visible. Staining was observed on a Nikon compound scope (MVI, Avon, MA). Once the staining was complete, embryos were washed four times for 30 minutes in 1X PBST. Embryos were cleared in 70% glycerol and stored in the dark at 4°C (Thisse & Thisse, 2008)

2.5 Whole mount Antibody staining

Stored Embryos were rehydrated with sequential 5 minutes washes with three to one, one to one, and one to three methanol to PBST. The embryos were washed for another 5 minutes in 100% PBST. Rehydrated embryos were then permeabilized in spot plates by washing for 5 minutes in dH2O then 5 minutes in ice cold acetone and lastly 2 to 3 minutes wash in dH2O. Following the last wash, dH2O was replaced with PBST. Permeabilized embryos were then incubated in AB block solution (PBST, 2% goat serum, 2mg/ml BSA) for a minimum of 2 hours on a rocker at room temperature. Block solution was replaced with 1 antibody and the embryos were incubated overnight at 4°C on a rocker. The next day, primary antibody was saved and stored and the embryos were subjected to four 30-60 minutes washes with PBST at room temperature. PBST was then replaced with secondary antibody and the embryos were incubated
overnight at 4°C on a rocker. After the incubation period, the 2 antibody was stored and then four 30-60 minutes washes with PBST were done with the embryos. After the PBST washes, the embryos were washed three times for 10 minutes each with staining buffer (100mM Tris HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20). Staining buffer was then replaced with staining solution (solution buffer, 50 mg/mL NBT, 50 mg/mL BCIP) and the embryos were stained in the dark until staining became visible. Once the staining is complete, embryos were wash four times for 30 minutes in 1X PBST before being cleared in 70% glycerol and stored in the dark at 4°C (Riley et al., 1999; Philips et al., 2006).

2.6 PCR and Gel Agarose

Five-somites embryos were manually dechorionated, placed into 96 PCR wells containing 50 uL lysis buffer (10 mM Tris-HCl, pH8.3, 50 mM KCl, 1.5 mM MgCl2, 0.3% Tween 20, 0.3% Triton X-100) and covered with PCR tape. The sample was incubated for 94°C for 20 minutes in a PCR machine before 5 uL of Proteinase K (Sigma) was added to every well. Embryos were incubated again at 55°C for 90 minutes and then denatured for 20 minutes at 94°C. The products then were diluted to 1:20 ratio with ddH2O in a 200 uL new 96 well plate and stored at -20°C.

PCR solution was made by adding 5uL of the 1:20 dilution DNA Extract, PCR mix (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, 100 mM each dNTP, 100 mg/ml BSA). The PCR was carried out using 5 minute denaturation at 94°C followed by 40 cycles of 94°C denaturation for 30 seconds, followed by 45°C of annealing for 30 seconds, and
ended with 72°C extension for 1 minute in PCR machine. The PCR reactions were then analyzed using 1% agarose gel in 1X TBE buffer stained with ethidium bromide (EtBr).

2.7 Agarose Gel

Four grams of agarose powder was added to a 400 mL 1X TBE, then the solution was boiled using a microwave in order to melt the agarose completely. When the gel cooled down, 3uL EtBr was added and swirled before pouring it into a gel tray with three 19 well combs. The reservoir was filled with 1X TBE buffer and 8uL of EtBr was added throughout the reservoir. Once the gel hardened, it was loaded into the reservoir. Loading dye was mixed with PCR product before loading into the gel wells. 5 uL of 100 BP plus ladder was loaded into the wells as reference. The gel was run for 180 volts for about 1 hour before being photographed under UV light.

2.8 Photograph

Once the embryos were cleared, photographs were taken on a Nikon compound scope (MVI, Avon, MA) for wild type and mutants at every stage.
3. Results

3.1 *spb*<sup>n279</sup> mutants do not undergo gradual depletion of neuroblasts

The nervous system is a complex organ with many cell types, including an actively dividing stem cell population whose function is to provide a constant source of cells that become specified to a neural fate, leave the cell cycle, and differentiate. For this study, different kinds of neural-specific genes including *notch1b*, *deltaA*, *pax2A*, and *islet1* were used for in situ hybridization, a method that visualizes the mRNA in the cells. Notch and Delta control neural cell fate and proliferation in the nervous system. Notch is expressed in proliferating neural cells, believed to be the stem cells (Bierkamp & Campos-Ortega, 1993). Its ligand, Delta, is briefly expressed in newly specified neuroblasts or in newly post mitotic neurons, cells that have made the decision to become neuron. The expression of Delta is transient as once the cell begins to differentiate it turns off Delta (Appel & Eisen, 1998; Haddon et al., 1998). This study found that in wild type embryos, *notch1b* gene expression is robust throughout the nervous system growing stronger as the embryo gets older (Fig. 5A-E). Likewise, *deltaA* gene expression is constantly high and robust in the wild type embryos at all stages (Fig. 6A-E, K) with neuroblasts constantly being specified. In the *spb*<sup>n279</sup> mutant, *notch1b* gene is still expressed in the nervous system although its expression is somewhat delayed and severely reduced compared to the wild type counterpart (Fig. 5F-J) and there were far fewer *deltaA* positive cells in the nervous system. However, throughout all stages examined there was always a good amount of cells (Fig. 6F-J, L). Thus although neural stem cells are severely depleted in *spb*<sup>n279</sup> mutants, there appears to be a continual supply of neuroblasts leaving the cell cycle even at later stages of development.

*pax2A* and *islet1* are two post mitotic markers visualizing cells that have differentiated in the nervous system. *pax2A* is expressed in cells in the midbrain-hindbrain border and in the
spinal CoSA interneurons (Krauss et al., 1991). In wild type embryos, expression in the midbrain-hindbrain boundaries as well as in the CoSA interneurons are very clear at all stages of development (Fig. 7 A-C). In $spb^{i279}$ mutants, at the 12-somite stage in the $spb^{i279}$ mutant, the midbrain-hindbrain boundary looks almost normal however its expression decreases as embryos get older (Fig. 7 D-F). Decreasing expression in the mutant midbrain-hindbrain boundary suggests that this is not a differentiated cell population as a whole and that perhaps cells die as they divide. Additionally, in $spb^{i279}$ mutants, very few CoSA interneurons differentiate but their number does not seem to change over time (Fig. 7 D-F).

$isl1$ is expressed in the trigeminal ganglion and primary neurons cells (Korzh et al., 1993; Inoue et al., 1994). In wild type embryos, trigeminal ganglia expression is clearly seen throughout all stages, starting from 4-somites (Fig. 8A-C). Primary motor neuron expression first appears starting at 12-somites (Fig. 8C). In the $spb^{i279}$ mutant embryos, both trigeminal ganglia and primary motor neuron expression does not show up until the 12-somite stage (Fig. 8F). Hence, both $pax2A$ and $isl1$ suggest that neurons differentiate in $spb^{i279}$ mutants, but their number is not increased overtime even though neuroblasts continue to form.

Finally, I asked if these differentiated neurons in $spb^{i279}$ mutants are functional. The anti-acetylated tubulin antibody labels mature neurons and their axons (Riley et al., 2010). At 24 hours in wild-type embryos, a vast number of neurons express anti-acetylated tubulin throughout the nervous system including cells of the trigeminal ganglion (Fig. 9A). In $spb^{i279}$ mutant embryos, we found three different categories of expression: $spb^{i279}$ mutants with a high number of positive cells with axons (Fig. 9B; 6 embryos), $spb^{i279}$ mutants with moderate amount of positive cells (Fig. 9C; 3 embryos) and $spb^{i279}$ mutants with almost no positive cells (data not shown; 2 embryos). This indicates that even at older stages of development, some $spb^{i279}$
mutants have neural cells that survive quite well and are able to make axons. Thus like other cell cycle mutants, \textit{spb}^{1279} mutants can make some differentiated neurons. Because their number does not appear to change over time, these for the most part do not die once they differentiate. However, unlike other cell cycle mutants, such as \textit{ogre} and \textit{specter}, both neural stem cells and neuroblast cells do not completely disappear over time in \textit{spb}^{1279} mutants.
Fig. 5 *notch1b* expression of neural cells in wild-type and *spb*<sup>ii279</sup> mutant embryos. A) Notch 1B expression in wild type embryos at tailbud (10 hpf). B) Notch 1B expression in wild type embryos at 4-somites (11.5 hpf). C) Notch 1B expression in wild type embryos at 8-somites (13 hpf). D) Notch 1B expression in wild type embryos at 12-somites (15 hpf). E) Notch 1B expression in wild type embryos at 16-somites (16 hpf). F) Notch 1B expression in *spb*<sup>ii279</sup> mutant embryos at tailbud (10 hpf). G) Notch 1B expression in *spb*<sup>ii279</sup> mutant embryos at 4-somites (11.5 hpf). H) Notch 1B expression in *spb*<sup>ii279</sup> mutant embryos at 8-somites (13 hpf). I) Notch 1B expression in *spb*<sup>ii279</sup> mutant embryos at 12-somites (15 hpf). Neural plate width expression can be seen clearly (arrowheads) J) Notch 1B expression in *spb*<sup>ii279</sup> mutant embryos at 16-somites (16 hpf). Neural plate width expression can be seen clearly (arrowheads). smt, somatic; ntc, notochord; arrow heads, neural plate width
Fig. 6 deltaA expression of neural cells in wild-type and spb<sup>6279</sup> mutant embryos. A) delta A expression in wild type embryos at tailbud (10 hpf). B) delta A expression in wild type embryos at 4-somites (11.5 hpf). C) delta A expression in wild type embryos at 8-somites (13 hpf). D) delta A expression in wild type embryos at 12-somites (15 hpf). E) delta A expression in wild type embryos at 16-somites (16 hpf). F) delta A expression in spb<sup>6279</sup> mutant embryos at tailbud (10 hpf). G) delta A expression in spb<sup>6279</sup> mutant embryos at 4-somites (11.5 hpf). H) delta A expression in spb<sup>6279</sup> mutant embryos at 8-somites (13 hpf). I) delta A expression in spb<sup>6279</sup> mutant embryos at 12-somites (15 hpf). J) delta A expression in spb<sup>6279</sup> mutant embryos at 16-somites (16 hpf). K) delta A expression in wild type embryos at 24 hours. L) delta A expression in spb<sup>6279</sup> mutant at 24 hours.
Fig. 7 *pax2A* expression of neural cells in wild-type and *spb*<sup>tl279</sup> mutant embryos. A) *pax2A* expression in wild type embryos at 12-somites (15 hpf). B) *pax2A* expression in wild type embryos at 16-somites (16 hpf). C) *pax2A* expression in wild type embryos at 20-somites (17.5 hpf) D) *pax2A* expression in *spb*<sup>tl279</sup> mutant embryos at 12-somites (15 hpf). Neuron cells are clearly visible (arrow) E) *pax2A* expression in *spb*<sup>tl279</sup> mutant embryos at 16-somites (16 hpf). F) *pax2A* expression in *spb*<sup>tl279</sup> mutant embryos at 20-somites (17.5 hpf). mhb, midbrain-hindbrain; CoSA, CoSA interneurons.
Fig. 8 islet1 expression of neural cells in wild-type and spb^{t279} mutant embryos. A) islet1 expression in wild type embryos at 4-somites (11.5 hpf). B) islet1 expression in wild type embryos at 8-somites (13 hpf). C) islet1 expression in wild type embryos at 12-somites (15 hpf). D) islet1 expression in spb^{t279} mutant embryos at 4-somites (11.5 hpf). E) islet1 expression in spb^{t279} mutant embryos at 8-somites (13 hpf). F) islet1 expression in spb^{t279} mutant embryos at 12-somites (15 hpf). trg, trigeminal ganglia; pmn, primary motor neuron
Fig. 9 Anti-acetylated tubulin staining in wild-type and $spb^{t279}$ mutant embryos. A) acetylated tubulin staining in wild-type embryos at 24 hours post fertilization with trigeminal ganglion clearly visible (arrow). B) Acetylated tubulin staining in $spb^{t279}$ mutant embryos with lots of mature axon expression at 24 hours. C) Acetylated tubulin staining in $spb^{t279}$ mutant embryos with fewer axon expression at 24 hours. trg; trigeminal ganglia
3.2 \(spb^{d279}\) mutants do not produce red blood cells

Blood cells are another example where proliferation of less differentiated stem cells are necessary. Blood cells have short life spans and once they fully differentiate, do not divide (Cooper, 2000). This study used several kinds of blood specific genes: \(gata1\) encodes a transcription factor specific only to red blood cells (Brownlie et al., 2003), \(hbbe2\) that encodes hemoglobin, differentiated red blood cells (Detrich et al., 1995), and \(tal1\) encodes a transcriptional regulator for cells that become either blood or endothelium (Liao et al., 1998). In wild-type embryos, robust expression from all probes can be observed (Fig. 10 A-E). Contrasting that, red blood cell expression was undetectable in \(spb^{d279}\) mutants as neither \(gata1\) nor \(hbbe2\) expression was visible (Fig. 10 F-G). The lack of blood cell expression in \(spb^{d279}\) mutants suggested that we look at more upstream markers. While wild type embryos have lots of blood and endothelial progenitors, there were far fewer in \(spb^{d279}\) mutants (Fig. 10 C-E, H-J). It is plausible that these cells expressing \(tal1\) were either endothelial in nature or cells whose fate has not been decided. Additionally, in \(spb^{d279}\) mutants \(tal1\) expression is delayed which indicated by more robust expression in 16-somites rather than at 8-somites or 12-somites (Fig. 10 H-J). Thus, \(spb^{d279}\) mutants do not have red blood cells.
Fig. 10 **Blood probes expression in wild-type and spb<sup>4279</sup> mutant embryos.**

3.3 *weel* CRIPSR mutants have a more severe phenotype compared to the *spb*^{l279} mutants

Because we have identified new alleles of speed bump generated by targeting exon 2 of *weel* using CRISPR/Cas9 we examined its phenotype as well. This was done by crossing two different alleles of CRISPR mutants to each other. Examination of the nervous system of these *weel* CRISPR mutants revealed that neural cell expression was severely reduced in the *weel* CRISPR compared to *spb*^{l279} mutants as only very few or almost no cells were observed (Fig. 11 A-F, Fig. 12 A-F, Fig 13 A-C). In *notch1b*, at 4-somites, expression in the brain and spinal cord of *weel* CRISPR mutant was almost similar to *spb*^{l279} mutants (Fig. 11B, C). By 12-somites, very little expression remained in either area (Fig. 11E, F). In *dla*, at 4-somites and 12-somites, *weel* CRISPR mutants have far less *dla* expression compared to *spb*^{l279} mutants (Fig. 12 B-C, E-F).

Furthermore, *weel* CRISPR mutant had almost no cells in the midbrain-hindbrain boundary or in CoSA interneurons as visualized with *pax2A* (Fig. 13C). This severe reduction of neural cell types suggests that the newly generated *weel* CRISPR mutant is the true loss of function mutation while the original *spb*^{l279} allele is likely a hypomorph mutation.
Fig. 11 *notch1b* expression comparison in *wee1* CRISPR mutant, wild-type, and *spb*<sup>ii279</sup> mutant embryos. A) *notch1b* expression in *wee1* CRISPR mutant wild type embryos at 4-somites (11.5 hpf). B) *notch1b* expression in *spb*<sup>ii279</sup> mutant embryos at 4-somites (11.5 hpf). C) *notch1b* expression in *wee1* CRISPR mutant embryos at 4-somites (11.5 hpf). D) *notch1b* expression in *wee1* CRISPR mutant wild type embryos at 12-somites (15 hpf). E) *notch1b* expression in *spb*<sup>ii279</sup> mutant embryos at 12-somites (15 hpf). F) *notch1b* expression in *wee1* CRISPR mutant embryos at 12-somites (15 hpf). ntc, notochord; arrowheads, neural plate width.
Fig. 12 *deltaA* expression comparison in *wee1* CRISPR mutant, wild-type, and *spb^t279* mutant embryos. A) *dla* expression in *wee1* CRISPR mutant wild type embryos at 4-somites (11.5 hpf). B) *dla* expression in *spb^t279* mutant embryos at 4-somites (11.5 hpf). C) *dla* expression in *wee1* CRISPR mutant embryos at 4-somites (11.5 hpf). D) *dla* expression in *wee1* CRISPR mutant wild type embryos at 12-somites (15 hpf). E) *dla* expression in *spb^t279* mutant embryos at 12-somites (15 hpf). F) *dla* expression in *wee1* CRISPR mutant embryos at 12-somites (15 hpf).
Fig. 13 *pax2A* expression comparison in *wee1* CRISPR mutant, wild-type, and *spb*\textsuperscript{ti279} mutant embryos. **A)** *pax2A* expression in *wee1* CRISPR mutant wild type embryos at 12-somites (15 hpf). **B)** *pax2A* expression in *spb*\textsuperscript{ti279} mutant embryos at 12-somites (15 hpf). **C)** *pax2A* expression in *wee1* CRISPR mutant embryos at 12-somites (15 hpf).
3.4 Sequence analysis of the *wee1* CRISPR mutant

To determine what type of mutations were generated by targeting exon 2 of *wee1*, I assisted in gaining *wee1* sequence data from these *wee1* CRISPR mutants. Because we are working with the original generation and each founder fish has its own unique mutation, we had to cross a CRISPR mutant carrier to a *spb*<sup>ti279</sup> mutant carrier to create trans-heterozygous mutants embryos. We then amplified the DNA around exon 2 of these mutant embryos by PCR and sent them out to be sequenced. We then analyzed the chromatograms and decoded the CRISPR sequence from the *spb*<sup>ti279</sup> sequence which we already knew (Fig. 14A). Here we found that there is a 5 base deletion with 2 base mismatch, which cause it goes out of frame (Fig 14B). There was one exception, we had already generated a F1 generation from *wee1* Cr7, thus in this case we were able to create homozygous *wee1* Cr7 mutant from which we obtained sequence data that we show in Fig. 14 C and its derived mutant sequence shown in Fig. 14D that is 7 nucleotides longer due to insertions and deletions and goes out of frame. If we’re to translate the wild-type and *spb*<sup>ti279</sup> mutant nucleic acid to protein, we will see protein kinase domain for Wee1 in both embryos, with 10 amino acids being lost in the *spb*<sup>ti279</sup> mutants (Fig. 15A). In comparison, in all cases of all the *wee1* CRISPR mutant that we have sent for sequencing so far, the mutations which start in target 2 of exon 2, cause a frame shift resulting in premature termination of the gene product. Due to the frame shift, if encoded, none of these *wee1* CRISPR mutants would seem to have the protein kinase domain for Wee1 (Fig. 15B). It is notable that in all the *wee1* CRISPR mutants there are no example where the sequence remains in frame after the mutation site. We would expected these protein to be roughly 1/3 of full length, perhaps such mutants have no phenotype.
Fig. 14  *weel* CRISPR mutants sequence analysis  

A) Sequence chromatograms of *spb*<sup>ti279</sup> and *weel* Cr38 trans heterozygote.  

B) Nucleic acid sequence comparison between *weel* wild-type and *weel* Cr38 mutant.  

C) Sequence chromatograms of *weel* Cr7 wild type embryos.  

C’) Sequence chromatograms of *weel* Cr7 mutant embryos which were shown to be longer due to
insertion mutations. **B)** Wild type and mutant nucleic acid sequenced for *wee1* cr7 mutant. Purple highlight indicated target 2, red font indicated bases that get inserted, green font indicated bases that get replaced/ mismatched. Blue font (-) indicated bases that get deleted.

**A**

WT:

```
MSFGAGRHKGPKLVPQRKSLQFSSTGEEDEISIDANNSTGAEGFTEDLSVPLRSSLEKRPELGNSPLSRNDDDESHDEEG
FSGSRVKNFAMKSSPSRPSFKPSFAYDSPEISIIYHMECSSPISPDCPDTQPKFRKLPILFDTPTFKSLLSRPRVGRFL
VAFTNVDTSKACLDRKRAMTBFIVPNTFSDLSQVSTQLQNNRKSHWNDSCGEMDADAEIDEELIPSKRTVMENMM
SRYASELMKAIERKCSALRMLKIONEYCMGLSDVIAENNFRNHLESLEELKDLLQVSRLKVINSTALVMHDPVISFRKASVEFEDETDFTTNGVYKB
HVTVVNPQEEDSRFLANEMVQEDSNLKLADDIFALATVQAAGAEPLPTNGKWHKIRQGILPHIQVLSQFDSLILKINH
IFDPRSKVLPVTLDTSRMSADQLRVLENASEKFNALLQKELKKAQMAAAEERVLTTQDRVLTSTIQSSRSALSPIK
MNRSVSLTIY*
```

**A’** _spb_1279:

```
MSFGAGRHKGPKLVPQRKSLQFSSTGEEDEISIDANNSTGAEGFTEDLSVPLRSSLEKRPELGNSPLSRNDDDESHDEEG
FSGSRVKNFAMKSSPSRPSFKPSFAYDSPEISIIYHMECSSPISPDCPDTQPKFRKLPILFDTPTFKSLLSRPRVGRFL
VAFTNVDTSKACLDRKRAMTBFIVPNTFSDLSQVSTQLQNNRKSHWNDSCGEMDADAEIDEELIPSKRTVMENMM
SRYASELMKAIERKCSALRMLKIONEYCMGLSDVIAENNFRNHLESLEELKDLLQVSRLKVINSTALVMHDPVISFRKASVEFEDETDFTTNGVYKB
HVTVVNPQEEDSRFLANEMVQEDSNLKLADDIFALATVQAAGAEPLPTNGKWHKIRQGILPHIQVLSQFDSLILKINH
IFDPRSKVLPVTLDTSRMSADQLRVLENASEKFNALLQKELKKAQMAAAEERVLTTQDRVLTSTIQSSRSALSPIK
MNRSVSLTIY*
```

**B**

*wee1* _Cr7_ mutant: 3 base replace/mismatch, 7 base insertion, 1 base replace/mismatch

```
MSFGAGRHKGPKLVPQRKSLQFSSTGEEDEISIDANNSTGAEGFTEDLSVPLRSSLEKRPELGNSPLSRNDDDESHDEEG
FSGSRVKNFAMKSSPSRPSFKPSFAYDSPEISIIYHMECSSPISPDCPDTQPKFRKLPILFDTPTFKSLLSRPRVGRFL
VAFTNVDTSKACLDRKRAMTBFIVPNTFSDLSQVSTQLQNNRKSHWNDSCGEMDADAEIDEELIPSKRTVMENMM
SRYASELMKAIERKCSALRMLKIONEYCMGLSDVIAENNFRNHLESLEELKDLLQVSRLKVINSTALVMHDPVISFRKASVEFEDETDFTTNGVYKB
HVTVVNPQEEDSRFLANEMVQEDSNLKLADDIFALATVQAAGAEPLPTNGKWHKIRQGILPHIQVLSQFDSLILKINH
IFDPRSKVLPVTLDTSRMSADQLRVLENASEKFNALLQKELKKAQMAAAEERVLTTQDRVLTSTIQSSRSALSPIK
MNRSVSLTIY*
```

*wee1* _Cr20_ mutant: 25 base deletion

```
MSFGAGRHKGPKLVPQRKSLQFSSTGEEDEISIDANNSTGAEGFTEDLSVPLRSSLEKRPELGNSPLSRNDDDESHDEEG
FSGSRVKNFAMKSSPSRPSFKPSFAYDSPEISIIYHMECSSPISPDCPDTQPKFRKLPILFDTPTFKSLLSRPRVGRFL
VAFTNVDTSKACLDRKRAMTBFIVPNTFSDLSQVSTQLQNNRKSHWNDSCGEMDADAEIDEELIPSKRTVMENMM
SRYASELMKAIERKCSALRMLKIONEYCMGLSDVIAENNFRNHLESLEELKDLLQVSRLKVINSTALVMHDPVISFRKASVEFEDETDFTTNGVYKB
HVTVVNPQEEDSRFLANEMVQEDSNLKLADDIFALATVQAAGAEPLPTNGKWHKIRQGILPHIQVLSQFDSLILKINH
IFDPRSKVLPVTLDTSRMSADQLRVLENASEKFNALLQKELKKAQMAAAEERVLTTQDRVLTSTIQSSRSALSPIK
MNRSVSLTIY*
```

*wee1* _Cr22_ mutant: 5 base deletion, 16 base insertion resulting in: 11 extra bases and 4 mismatches.

```
MSFGAGRHKGPKLVPQRKSLQFSSTGEEDEISIDANNSTGAEGFTEDLSVPLRSSLEKRPELGNSPLSRNDDDESHDEEG
FSGSRVKNFAMKSSPSRPSFKPSFAYDSPEISIIYHMECSSPISPDCPDTQPKFRKLPILFDTPTFKSLLSRPRVGRFL
VAFTNVDTSKACLDRKRAMTBFIVPNTFSDLSQVSTQLQNNRKSHWNDSCGEMDADAEIDEELIPSKRTVMENMM
SRYASELMKAIERKCSALRMLKIONEYCMGLSDVIAENNFRNHLESLEELKDLLQVSRLKVINSTALVMHDPVISFRKASVEFEDETDFTTNGVYKB
HVTVVNPQEEDSRFLANEMVQEDSNLKLADDIFALATVQAAGAEPLPTNGKWHKIRQGILPHIQVLSQFDSLILKINH
IFDPRSKVLPVTLDTSRMSADQLRVLENASEKFNALLQKELKKAQMAAAEERVLTTQDRVLTSTIQSSRSALSPIK
MNRSVSLTIY*
```

*wee1* _Cr24_ mutant: 5 base deletion, 10 base replace/mismatch

```
MSFGAGRHKGPKLVPQRKSLQFSSTGEEDEISIDANNSTGAEGFTEDLSVPLRSSLEKRPELGNSPLSRNDDDESHDEEG
FSGSRVKNFAMKSSPSRPSFKPSFAYDSPEISIIYHMECSSPISPDCPDTQPKFRKLPILFDTPTFKSLLSRPRVGRFL
VAFTNVDTSKACLDRKRAMTBFIVPNTFSDLSQVSTQLQNNRKSHWNDSCGEMDADAEIDEELIPSKRTVMENMM
SRYASELMKAIERKCSALRMLKIONEYCMGLSDVIAENNFRNHLESLEELKDLLQVSRLKVINSTALVMHDPVISFRKASVEFEDETDFTTNGVYKB
HVTVVNPQEEDSRFLANEMVQEDSNLKLADDIFALATVQAAGAEPLPTNGKWHKIRQGILPHIQVLSQFDSLILKINH
IFDPRSKVLPVTLDTSRMSADQLRVLENASEKFNALLQKELKKAQMAAAEERVLTTQDRVLTSTIQSSRSALSPIK
MNRSVSLTIY*
```

*wee1* _Cr25_ mutant: 41 base deletion and 7 base insertion/mismatch

```
MSFGAGRHKGPKLVPQRKSLQFSSTGEEDEISIDANNSTGAEGFTEDLSVPLRSSLEKRPELGNSPLSRNDDDESHDEEG
FSGSRVKNFAMKSSPSRPSFKPSFAYDSPEISIIYHMECSSPISPDCPDTQPKFRKLPILFDTPTFKSLLSRPRVGRFL
VAFTNVDTSKACLDRKRAMTBFIVPNTFSDLSQVSTQLQNNRKSHWNDSCGEMDADAEIDEELIPSKRTVMENMM
SRYASELMKAIERKCSALRMLKIONEYCMGLSDVIAENNFRNHLESLEELKDLLQVSRLKVINSTALVMHDPVISFRKASVEFEDETDFTTNGVYKB
HVTVVNPQEEDSRFLANEMVQEDSNLKLADDIFALATVQAAGAEPLPTNGKWHKIRQGILPHIQVLSQFDSLILKINH
IFDPRSKVLPVTLDTSRMSADQLRVLENASEKFNALLQKELKKAQMAAAEERVLTTQDRVLTSTIQSSRSALSPIK
MNRSVSLTIY*
```

*wee1* _Cr27_ mutant: 14 base deletion, some mismatches follow
weeICr38 mutant: 5 base deletion

weeICr42 mutant: 50 base deletion causing abrupt truncation

Fig. 15 Wild-type, spb^{4279} mutants, and wee1 CRISPR mutants amino acid sequence analysis. A) spb^{4279} wild type amino acid sequences. A’) spb^{4279} mutant amino acid sequences. B) Amino acids sequences for wee1 CRISPR mutant that have been sequenced so far. Purple highlight indicated target 2; green highlight indicated protein kinase domain for Wee1; Blue highlight indicated frameshift to frame +3; yellow highlight indicated frameshift mutation to frame +2; asterisk (*) indicated stop codon.
4. Discussion

This study aimed to characterize the development of the *spb* mutant which is believed to be a mutation in the *wee1* gene as well as to provide further analysis that the *spb*\textsuperscript{d279} mutant is truly a mutation in *wee1*. This study compared development of the nervous system and blood between the *spb*\textsuperscript{d279} mutant and the new *wee1* CRISPR mutants. Furthermore, this study also provided sequence analysis data to show that the new *wee1* CRISPR mutants are indeed mutations in exon 2 that all result in premature termination before the *wee1* kinase domain. Hence because this study shows *spb*\textsuperscript{d279} has a less severe phenotype than the *wee1* CRISPR mutants and because *wee1* CRISPR mutants lack a kinase domain and the *spb*\textsuperscript{d279} mutants does not, this study indicates that *spb*\textsuperscript{d279} mutants are not likely to be a true loss of *wee1* function.

*The *spb*\textsuperscript{d279} mutation likely retains some activity of Wee1 protein suggesting that is a hypomorph mutation rather than a loss of function mutation*

Like any other cell cycle mutant, stem cells and neural percussors cells would be depleted gradually as *spb*\textsuperscript{d279} mutants embryos get older (Riley et al., 2010; Warga et al., 2016; Petrachkova et al., 2019) and that neural cells differentiate and production of functioning axons do not increase. However, unlike other cell cycle mutants, neuroblasts in *spb*\textsuperscript{d279} mutants keep being produced even at later stages of development. For example, we can see that there are high numbers of *dla* expressing cells even at 24 hours. It is unusual to see constant production of neuroblasts while at the same time not seeing increases in the number of differentiated *pax2A* and *islet1* positive cells. A possible explanation for this is because as neuroblast divide, some of them just die before differentiation due to the replication mistakes; therefore no *pax2A* or *islet1* positive cells could be observed. Additionally, it is also unusual to see that even though there is a
slight decrease on *notch1b* positive cell, *dla* positive cells remain high. Notch is associated with proliferating stem cells that will keep stem cell pools while Delta is associated with neuroblasts (Appel et al., 2001). It is possible that the reason why there is a decrease in *notch1b* positive cells but high number of *dla* positive cells as *spb^d279* mutants get older, is because as neural stem cells divide, there are too many replication mistakes which cause cells to die. Meanwhile, neuroblast cells do not undergo as many divisions as neural stem cells therefore they exhibit less cell death. Previous study by the Kane lab also found that there are a lot of cells that undergo apoptosis in *spb^d279* mutants (E. Johnston E, unpublished; J. Kelly, unpublished). Naturally, as there are less and less stem cells, we would expect to have less neuroblasts as they come from the same pool; yet, this is not observed in *spb^d279* mutants. Another possible explanation is that the *dla* expressing cells we see at later stages are the same cells we see at younger stages. This may be possible if *dla* is not a transient marker as it is thought (Appel & Eisen, 1998). *dla* labels cells that have decided to become neurons and once the cell differentiated it will turn off *dla*. If the cells do not turn off *dla* even at later stages, this would explain why we found high number of *dla* expressing cells. As to why *spb^d279* mutants are able to produce neuroblasts cells even at later stage is likely because the protein kinase domain of the *wee1* gene in *spb^d279* mutants embryos is never completely obliterated. *wee1* mRNA in situ data collected by the Kane lab showed that even though *wee1* mRNA undergo gradual depletion, *wee1* mRNA was never completely gone in *spb^d279* mutants (E. Johnston, unpublished). Based on the *spb^d279* mutant putative amino acid sequence data (Fig. 15 A’), we can see that the Wee1 protein kinase only lacks the last 10 amino acids due to a nonsense mutation. We speculate that this Wee1 protein kinase domain, can still perform some or most of its function. This is also backed up by comparison between *spb^d279* mutant in situ hybridization with *wee1* CRISPR mutants in situ hybridization data which showed
that the *wee1* CRISPR mutants have more severe phenotype compared to *spb*\(^{\text{it279}}\) mutants, This would mean that the *spb*\(^{\text{it279}}\) mutant is likely a hypomorphic mutation.

*spb*\(^{\text{it279}}\) mutants do not produce red blood cells

Just like with the nervous system, I found that blood cells in *spb*\(^{\text{it279}}\) mutants undergo gradual depletion, like other cell cycle mutants (Riley et al., 2010; Warga et al., 2016; Petrachkova et al., 2019). This study found that in *spb*\(^{\text{it279}}\) mutants there are no *gata1* and *hbbe2* positive cells. Since we know that both *gata1* and *hbbe2* label red blood cells (Brownlie et al., 2003; Detrich et al., 1995), we can infer that the *spb*\(^{\text{it279}}\) mutants do not produce red blood cells. We do see some *tal1* positive cells but since *tal1* encodes for both endothelium cells and blood cells (Liao et al., 1998), it is more likely that the positive cells that observed are endothelial cells.

One possible explanation to why *spb*\(^{\text{it279}}\) mutants do not produce blood cells is that because all the stem cells prefer to become endothelial cells instead of blood cells due to shortening time of both G1 and G2. We know that *spb*\(^{\text{it279}}\) mutants have shorter G2 phase. A study found that *wee1* can extend G1 phase by suppressing the activity of CDK1 and CDK2 (Moiseeva et al., 2019), which mean that *spb*\(^{\text{it279}}\) mutants will have shorter G1 phase as well. Blood cells and stem cells come from the same stem cell pool. A study had found that the duration of G1 phase could have effect on cell fate determination and that a longer G1 phase could lead to formation of pancreatic cells instead of liver cells or duct cells (Yang et al., 2021). With a shorter G2 and possibly G1 phase, cells from the stem cell pools might prefer to form endothelial cells instead of blood cells.

Another possible explanation why no red blood cells can be observed in *spb*\(^{\text{it279}}\) mutants is that *tal1* is expression is usually early while in *spb*\(^{\text{it279}}\) mutants, *tal1* expression is so delayed that it
may have changed cell fates from blood to endothelial instead. This would help explain why no red blood cells can be observed in \textit{spb}^{\text{nil279}} mutants.

\textit{Wee1 CRISPR mutants is the true loss of function mutation.}

In situ hybridization with neural markers indicate that \textit{wee1} CRISPR mutants have a more severe phenotype compared to \textit{spb}^{\text{nil279}} mutants. In \textit{wee1} CRISPR mutants, there was very little, or almost no neuroblasts produced at either early or late stages. Furthermore, analysis of the sequenced \textit{wee1} CRISPR mutants show that thus far, all have lost the Wee1 protein kinase domain due to frame shift mutations. Without a protein kinase domain Wee1 would be unable to perform its function which is to inhibit the MPF. Thus cells would go into mitosis too soon and likely to have replication mistakes. Therefore, we argue that the \textit{wee1} CRISPR mutants are the true loss of function mutation. The most common \textit{wee1} CRISPR mutation was a deletion, however there were also insertion and mismatched mutation. Due to deletion, insertion, and mismatches, frame shifts mutation occured which causes \textit{wee1} CRISPR mutants to terminate before its Wee1 protein kinase domain. This likely explains its more severe phenotype. Therefore, the \textit{wee1} CRISPR mutants are likely the true loss of function mutation and \textit{spb}^{\text{nil279}} mutants are hypomorph mutation.

Present and Future Studies

In the future, further in situ hybridization and antibody staining can be performed with \textit{wee1} CRISPR mutation at different stages to gain more data. Since we are still unable to get CRISPR homozygous mutants and had been working with trans-heterozygote, experiments with CRISPR homozygous mutants should be performed to give more insight about \textit{wee1} CRISPR
homozygotes. Furthermore, in situ hybridization with different kind of blood probes should also be performed to see if $spb^{hl279}$ truly does not produce red blood cells.
References


