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One Fish, Two Fish, Red Fish, Blue Fish: The Analysis of the Specter Cell Cycle Mutant in Zebrafish

Tetiana Petrachkova
Western Michigan University, tanya.petrachkova@gmail.com

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ONE FISH, TWO FISH, RED FISH, BLUE FISH: THE ANALYSIS OF THE
SPECTER CELL CYCLE MUTANT IN ZEBRAFISH

by

Tetiana Petrichkova

A thesis submitted to the Graduate College
in partial fulfillment of the requirements
for the degree of Master of Science
Biological Sciences
Western Michigan University
June 2015

Thesis Committee:
Don A. Kane, Ph. D., Chair
Pamela Hoppe, Ph. D.
Todd Barkman, Ph.D.
ONE FISH, TWO FISH, RED FISH, BLUE FISH: THE ANALYSIS OF THE SPECTER CELL CYCLE MUTANT IN ZEBRAFISH

Tetiana Petrachkova, M.S.
Western Michigan University, 2015

Cell division is controlled by genes that regulate the cell cycle. Here we show that the zebrafish specter (spr) mutant is mutation in the cyclin B1 gene, a gene necessary for the G2 to M transition of the cell cycle.

The spr mutation mapped to the cyclin B1 gene. Sequencing showed a transition (C139→T) that caused a nonsense mutation in exon 2 of the cyclin B1 gene. In situ hybridization of cyclin B1 revealed that the mRNA is absent in the mutant embryo by gastrulation. We found that the earliest visible mutant phenotype was a darkening of the head caused by the appearance of apoptotic cells in the brain. In situ hybridization of markers expressed in neural precursors and blood cells showed that, in general, mutant embryos have bigger and fewer cells. In situ hybridization of notch1b and deltaa also showed fewer neural stem cells that give rise to fewer but bigger neuronal precursors.

Phospho-histone-3 antibody staining suggested that the cell cycle is delayed in the mutant embryo. The expression of the Dual FUCCI transgene in the live spr mutant embryos confirmed that cells are delayed in the S/G2/early M phase of the cell cycle.

CRISPR/Cas9 mediated germline mutagenesis of the wild-type cyclin B1 allele showed that the spr mutant phenotype is a result of a nonfunctional Cyclin B1 gene. We conclude that the spr mutant phenotype is caused by the mutation in the cell cycle gene cyclin B1, an essential regulator of the cell cycle progression from the G2 to M phase of the cell cycle. This then leads to mitotic abnormalities and finally developmental arrest.
I take this opportunity to thank Dr. Kane for teaching me all the secrets of how to become a successful Developmental Geneticist. I want to express my gratitude to Dr. Warga for being so encouraging and supportive, and for sharing with me so many hours teaching me everything I have learnt in the past two years. I am also thankful to the former Kane lab members, Laura Bakke, Amber Bark and Jyotika Sighn for providing me the background for the current research, because, as I believe, the hardest are the very first steps. I want to thank Sarah Richards, Mark Webster and Peyton Johnson for being so helpful at the imaging center. These people have become my second family, whom I own my achievements at Western Michigan University. I am thankful to my Graduate committee, Dr. Barkman and Dr. Hoppe for their guidance with my project. I thank Dr. Kimelman, who shared the Dual FUCCI transgene with our laboratory. Without this transgene, one of the best part my work and this beautiful title would not have existed. I want to acknowledge the Fulbright Grant, Institute of International Education and the Department of State for giving the opportunity to pursue my Master’s Degree in the United States. Graduate college, who awarded me with the Research assistantship to be able to finish this project. And of course, I want to thank my family in Ukraine, who has been extremely supportive even being thousands miles away.

Tetiana Petrachkova
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CHAPTER I
INTRODUCTION

Background

Genes involved in pathways that regulate the cell cycle in eukaryotes are being actively studied. Genetic screens in yeast and *Drosophila* have made important contributions to understanding pathways regulating cell proliferation. It has been found that the expression of the human orthologues of the cell cycle genes identified in model organisms is disrupted in human tumors. The zebrafish model allows us to study cell cycle gene regulation to understand the cell cycle progression: its role in early development and cancerogenesis (Krause et al., 2000; Weaver et al., 2005).

We examined a cell cycle mutation to answer questions as to how some cell cycle genes are important for the growth and development of the cell and the whole organism. We now assume that the *spr* mutation in zebrafish is a mutation in the cell cycle gene *cyclin B1*. The product of the *cyclin B1* gene is essential for the control of the cell cycle at the G2 to M transition. There have been studies done on the role of Cyclin B in oocyte maturation, particularly in *Xenopus*, but these studies did not continue to see the role of this protein in tissue development (Gong & Ferrel, 2010; Frederick and Andrews, 1994). In mice the knock-out of Cyclin B1, causes homozygous embryos to die *in utero* (Brandeis et al., 1998). Since there has not been any study done on Cyclin B1 null protein in zebrafish, there are several significant questions that we ask in this proposal: 1) is the *spr* phenotype caused by a mutation in *cyclin B1* gene and 2) what are
the characteristics of the spr phenotype that affect the cell cycle and development in zebrafish. Knowing different aspects of how Cyclin B1 is essential for cell cycle progression will be another link in understanding how cell division is regulated.

Cell-Cycle Progression During the Maternal-Zygotic Transition

Cell cycle is comprised of the interphase and the actual cell division. Interphase consist of two Gap phases, G1 and G2, and a Synthesis phase, S-phase. In early development of many organisms, the cell cycle is very short and synchronous. This period in development is called the cleavage stage and is characterized by the absence of the G1 and G2 cell cycle stages (Edgar & Schubiger, 1986). The cleavage stage lasts until the midblastula transition (MBT). In zebrafish, the duration of mitosis and interphase is the same during cleavage stage. The MBT begins during cell cycle 10 (512-cell stage), which is marked by a slowing of the cell cycle. During this period the embryo begins the transition from maternal to zygotic control of development (Tadros et al., 2009). The MBT is accompanied by loss of division synchrony, increased cell cycle duration, activation of zygotic transcription, and an onset of cellular motility (Kane and Kimmel, 1993). The loss of synchrony is similar to that at cycle 12 in Xenopus (Newport and Kirschner, 1982) and at cycle 10 in Drosophila (Edgar and Schubiger, 1986). During the MBT the cell cycle length increases and, as in Xenopus and Drosophila, this change correlates with the nucleocytoplasmatic ratio: this ratio increases exponentially as a function of the cycle number, since
the total volume of the cytoplasm remains constant while genomic DNA replicates during each cell cycle (Kane and Kimmel, 1993). After inhibiting transcription by α-amanitin injection into *Xenopus* embryos Newport and Kirschner (1982) suggested that the lengthening of the cell cycle is independent of transcriptional activation. In *Drosophila*, the elongation of the cell cycle is required for transcription complexes to be established. The transcription comes after cell cycle lengthening, therefore it is independent of transcription (Edgar and Schubiger, 1986).

With the onset of the MBT, the G2 phase first appears in *Drosophila* cell cycle 14, whereas G1 phase appears in cell cycle 16 in the precursors for the neural and imaginal discs (Edgar and O'Farrell, 1990). The G1 phase in *Xenopus* is detected 1 hour after MBT, while the G2 phase appears much later, at the gastrula stage (Fredrick and Andrews, 1994). Zamir et al. (1997) have demonstrated by flow cytometry that in zebrafish the G1 phase of the cell cycle is initially detected at the MBT. Kane and Kimmel (1993) suggested that the elongation of the cell cycle in zebrafish, caused by the increase of the interphase duration, particularly the lengthening of the S phase. However, Nogare et al. (2009) have shown, that it is not G1, but G2 phase that is added during the MBT, and G2 contributes to the cell cycle lengthening in the deep cells. After cell cycle 10, novel zygotic transcripts might inhibit the immediate entry into S phase, and thereby induce appearance of G1 phase. One class of such transcripts might be cyclin-dependent kinase (Cdk) inhibitors. Induced transcription of Cdk inhibitors
might contribute to the S phase restriction, allowing the appearance of G1 phase after MBT (Krämer et al., 2004; Sherr and Roberts, 1995).

During cycles 11 and 12, the blastoderm separates into the yolk syncytial layer (YSL), the enveloping layer (EVL), and the deep cells of the blastoderm. Yolk cells are characterized by a short cell cycle, the EVL cells have long cell cycle, and the deep cells have a cell cycle of intermediate length (Kane et al., 1992). Zamir et al. (1997) suggest that the cell layers located away from the yolk, where the maternally derived transcriptional repressors are located, may contain less putative repressors. That maybe why G1 phase appears earlier in the cells located further from the repressors, e.g. EVL cells (Zamir et al., 1997).

G2 phase appears in cycles 11-13, to ensure the completion of DNA replication in S phase and prevent premature entry into mitosis. Norage et al. (2009) determined that a G2 phase is introduced shortly after the MBT in zebrafish. The major reason for the G2 phase addition, similar to Xenopus and Drosophila, is establishment of another checkpoint (Pietenpol and Vallier, 2013). The limitation of maternally provided cyclin mRNA is the reason for the cell cycle lengthening during blastoderm cellularization in Drosophila. Destruction of maternally derived Cdc25 (Cell division cycle 25) phosphatase homologs string and twine mRNAs, (Alphey et al., 1992; Courtot et al., 1992), and activation of mitotic kinase inhibitor Wee1, which inhibits Cdk1 (Cell division kinase 1), (Stumpff et al., 2004) results in hyperphosphorylation of Cdk1 and introduction of a G2/M arrest. Similarly, destruction of maternal Cdc25a occurs in Xenopus during MBT (Shimuta et al., 2002; Nogare et al., 2009) have shown that the
inhibition of Cdc25a and Cdk1 activity is essential for cell cycle lengthening and asynchrony cycle 9 through 12. This process begins with the onset of a G2 phase by a transcription-independent mechanism, and contributes to the cell cycle lengthening in deep cells (Concha and Adams, 1998; Nogare et al., 2009).

Many cells leave the cell cycle due to the onset of differentiation during division 15, with the rise of notochord cells and a few somatic muscle cells. Division 16 produces the large primary motoneurons of the spinal cord and more somatic muscle cells (Lewis and Eisen, 2003). Cell cycle mutants that arrest early in development, such as zombie, speed bump, and ogre, first show their phenotypes in these stages (Kane et al., 1996), suggesting that maternal mRNA might be depleted and there is a lack of zygotic mRNA necessary for normal development after MBT (Mathavan et al., 2005).

The Role of Cyclin B1 in Cell Cycle Progression

The cyclin family of proteins is known to activate specific Cyclin-dependent kinases (Cdk) required for progression through the cell cycle (Pines, 1995; Santamaria et al., 2007) (Fig. 1). The Cyclin B1/Cdk1 complex is also involved in mitotic chromosome condensation (Kimura et al., 1998) and control of microtubule dynamics during mitosis (Vasquez et al., 1999).

The entry of eukaryotic cells into mitosis is regulated by the activation of Cdk1 at the G2 to M transition. This transition requires Cyclin B1 to bind with Cdk1 to form a protein kinase holoenzyme complex, also known as the
maturation promoting complex (Maller et al., 1989) or mitosis-promoting factor (MPF) (Draetta et al., 1989; Katsu et al., 1993). Normally, Cyclin B1/Cdk1 remains inactive until enough Cyclin B1 accumulates during S/G2 phase (Allan & Clarke, 2007). Phosphorylation of Cdk1 at Thr161 by Cdk-activating kinase (CAK) and dephosphorylation of Cdk1 at Thr14/Tyr15 by Cdc25 proteins are important steps in activation of the CyclinB1/Cdk1 complex (Timofeev et al., 2010). The active Cyclin B1/Cdk1 complex must translocate to the nucleus to begin phosphorylating nuclear substrates, such as caspases to protect mitotic cells against apoptosis, which is a necessary step for mitosis (Ikegami et al., 1999; Krause et al., 2000; Porter & Donoqhue, 2003). The Cyclin B1/Cdk1 complex also phosphorylates and disassembles the nuclear lamina in early prometaphase to promote nuclear envelope breakdown (Li et al., 1997; Nigg, 2001). At the end of mitosis, Cyclin B1 is degraded by the anaphase-promoting complex (APC/C), promoting the cell cycle progression; cdk1 is constitutively present (Clijsters et al., 2013; Zachariae and Nasmyth, 1999).

Figure 1. Schematic drawing of the cell cycle.
A critical target of Cyclin B/Cdk1 activity is the G2 checkpoint (Kastan and Bartek, 2004; Johnson et al., 2012). There are two possible pathways for a cell at the G2 checkpoint: cell cycle progression or activation of apoptosis. To prevent apoptosis, the Cyclin B1/Cdk1 complex phosphorylates caspase-9 at Thr125, which leads to a block of caspase-9 and caspase-3 activity (Allan & Clarke, 2007; Plaster et al., 2005), and prevents the cell from undergoing apoptosis. Without this modification, cells exhibit increased sensitivity to apoptosis. A delay in mitosis for a prolonged period increases the predisposition of the cells to undergo apoptosis (Allan & Clarke, 2007). Moreover, arresting human HeLa mitotic cells using microtubule poisons leads to the inability of the cells to maintain the CyclinB1/Cdk1 levels needed to block caspases, which leads to activation of apoptosis after a prolonged mitotic arrest (Allan & Clarke, 2007 Xie et al., 2005).

Therefore, CyclinB1/Cdk1 levels determine one aspect of the fate of the cells during the cell cycle, to live or to die (Brito and Rieder, 2006). Another study of Cyclin B1 importance in HeLa cells culture showed that depletion of Cyclin B1 affects its ability to localize at microtubules. Improper attachment of the Cyclin B1/Cdk1 complex results in lagging chromosomes. However, HeLa cells depleted of Cyclin B1 can enter anaphase eventually. Omitting chromosome segregation, cells lacking Cyclin B1 could still enter G1 phase, resulting in multinuclear cells (Chen et al., 2008; Chen et al., 2013). It is possible that there are different pathways and players that coordinate the cell cycle progression, and they remain not fully understood.
In mammals and zebrafish two types of Cyclins B (B1 and B2) have been identified (Minshull et al, 1989; Pines & Hunter, 1989). In chicken, frogs, flies and worms a third type of Cyclin B, called B3 also exists, essential for germline development (Gallant and Nigg, 1994; Kreutzer et al., 1995).

Mammalian B-type cyclins differ in their NH$_2$ termini and exhibit 57% of the rest of the protein (Chapman and Wolgemuth 1993; Jackman et al. 2003). Mice lacking Cyclin B1 die in utero, whereas mice lacking Cyclin B2 are smaller than normal and have reduced litter sizes. This indicates that Cyclin B1 is essential for viability, but Cyclin B2 is not (Brandeis et al., 1998). Both B cyclins are detectable in G1, rise through S phase peaking in late G2 or early M phase, and degrade after metaphase (Huang et al., 2013). However, differences exist in their cellular location. Cyclin B1 is primarily cytoplasmic, but constantly shuttles between the nucleus and the cytoplasm in interphase, whereas Cyclin B2 is primarily bound to the Golgi apparatus during both interphase and mitosis (Jackman et al., 2003) and may play a role in segregation of the Golgi apparatus. Recently, however, it was demonstrated that Cyclin B2 also enters the nucleus during mitosis in HeLa cells (Huang et al., 2013).

There are four types of cyclins required for proper cell cycle transition. D-type cyclins (Cyclins D1, D2, and D3) and they are associated with the kinases Cdk4 and Cdk6 (Duffy et al., 2005). The Cyclin D1/Cdk4 complex phosphorylates and inhibits members of the retinoblastoma (Rb) protein family and regulates the cell cycle during the G1 to S phase transition. E-type cyclins (Cyclin E1 and E2) associate with Cdk2 and are also required for the G1 to S phase transition.
Cyclin D expression is mostly dependent on extracellular signals and signaling cascades, while Cyclin E is regulated by transcriptional activity (reviewed by Coqueret, 2002). Cyclin A is required to promote DNA replication (Gong et al., 2010; Nieduszynski et al., 2002).

In *Drosophila* double mutant embryos that lack both zygotic Cyclin A and Cyclin B, cell cycle progression has been blocked just before maternally supplied Cyclins A and B are depleted. The cell cycle is blocked in the G2 phase prior to entry into the cell division 15 in these mutants. Using heat-shock expression of Cyclin A or Cyclin B is sufficient for the arrested cells to overcome the block. The Cyclin B mutants have abnormal spindles and delayed progression through mitosis. Heat-shock induction of Cyclin B expression completely rescues spindle abnormalities and the mitotic delay (Knoblich and Lehner, 1993).

Cyclin B1 consists of the destruction box (DB), cytoplasmic retention signal domain (CRS), N-terminal helix, cyclin box, and C-terminal domain (Fig. 2).

The first 107 residues of cyclin B1 are necessary and sufficient for chromatin localization, providing a mechanism to concentrate CDK1 near chromatin-associated substrates (Bentley et al., 2007). Domain mapping studies suggest that the N-terminal helix is required for proper protein localization. Disrupted N-terminal helix of Cyclin B is still able to promote normal Golgi disassembly, but cannot induce chromosome condensation, nuclear lamina solubilization and mitotic aster assembly (Draviam et al., 2001). The N-terminal helix is required for chromatin localization, and localization to kinetochores,
centrosomes and microtubules within residues 166-433 (Bentley et al., 2007; Draviam et al., 2001).

\[ \text{Figure 2. Cyclin B1 significant sequence domains.} \]

The destruction box (DB), cytoplasmic retention signal domain (CRS), N-terminal helix (NTH), and cyclin box. Asterisks indicate MRAIL motif (Bentley et al., 2007).

Bentley et al. (2007) have defined the potential importance of Cyclin B localization sequences that mediate protein’s localization to specific structures during mitosis. The DB is required for Cyclin B1 degradation by the APC/C during the metaphase to anaphase transition. Cyclin B1 with disrupted DB is localized in the cytoplasm during interphase and localized on chromatin, centrosomes, spindle microtubules, and kinetochores during mitosis; it delocalizes from kinetochores during metaphase. This indicates that localization of Cyclin B1 with disrupted DB is not changed; but APC/C complex cannot degrade Cyclin B1 with mutation in destruction box domain.

The CRS domain appears to be responsible for the cytoplasmic localization of Cyclin B and is sufficient to bind and activate Cdk1 (Nugent et al., 1991). Disruption of the CRS domain results in CyclinB/Cdk1 dissociation, however no changes in the localization of Cyclin B1 to centrosomes, nor the
dissociation of Cyclin B1 from kinetochores after microtubule attachment, or degradation of Cyclin B1 during the metaphase to anaphase transition occur (Bentley et al., 2007).

The MRAIL (Met-Arg-Ala-Ile-Leu) motif is required for the substrate proteins, that contain a hydrophobic Cy motif, binding and located at the opposite surface of the Cyclin B/Cdk1 catalytic site. Mutants for this motif (P-box mutants) are able to bind, but not activate Cdc25 (Bentley et al., 2007).

The *Specter* Mutant

In 1996 the Christine Nüsslein-Volhard group published the Tübingen mutant screen for mutants that affect embryonic development in zebrafish. The effect of zygotic genes that are necessary for embryonic survival during the first day of development still remains unclear. Mutagenesis was performed as described in Haffter et al. (1996): male zebrafish were mutagenized with ethyl nitrosourea and outcrossed. Embryos in F3 were screened for mutant phenotypes that affect morphology in the first 5 days of development.

The *spr* mutant (alleles ta214 and tu21) was isolated in the Tübingen screen and identified as mutants that display gross cellular abnormalities in the first 24 hours of development (Kane et al., 1996). The *spr* mutant phenotype becomes visible by the 7-somite stage (12 ½ hours, middle segmentation): an observed difference occurs in head-to-tail ratio, and the body is underdeveloped body – it corresponds to the phenotype of a normal 5-somite stage embryo. Initial studies showed that the *spr* mutant arrests with a body shape at roughly
midsegmentation (24-30 hrs) and eventually exhibits signs of massive degradation of the nervous system (Kane et al., 1996) (Fig. 3). DAPI staining of mutant embryos did not show a significant change in nuclear phenotype, but transplantation studies revealed that >90% cells transplanted from a mutant embryo into a wild type embryo did not survive if they were transplanted into the nervous system (Kane et al., 1996).

Mapping of the spr mutation linked it to chromosome 5 (D.A. Kane, unpublished). Many gene candidates are available within the region, one of which was the neurotrophic tyrosine kinase receptor (ntrk2b) (Laura Bakke, unpublished). Trk receptors is a family of tyrosine kinases that regulates synaptic strength and plasticity in the nervous system. Trk receptors affect neuronal survival, differentiation, synaptic plasticity, cell morphology and calcium influx through signal cascades. TrkB binds to the brain-derived neurotrophic factor (BDNF), important for survival and function of neurons in the central nervous system. Null trkB mice exhibit similar phenotype to spr: in that
Figure 3. Morphological characterization of the specter mutant phenotype. Morphology of (A-G) wild type embryos, (B-H) spr mutant embryos at the 9, 15, 20, and 25-somite stage. Side view.

many trigeminal neurons die during the early stages (Piñon et al., 1996).

However, after Rachel Warga (personal communication) saw that the spr mutant embryos have enlarged neurons and blood cells, which is a common characteristic of the cell cycle mutants in our laboratory, the candidate gene was revised. Further fine mapping revealed the nearby candidate gene cyclin B1 (L. Bakke, unpublished), a gene that regulates the G2 to M transition in the cell cycle. Sequencing of mutant cDNA revealed a nonsense mutation (C139T) in exon 2 (R.M.Warga, unpublished), that presumably results in a non-functional gene (Fig. 4).
Figure 4. DNA sequencing chromatogram of the cyclin B1 gene in the specter mutant embryo.

Sequencing of the cyclin B1 gene reveals a nonsense mutation (C139T) in the mutant embryo in exon 2 (Rachel Warga, unpublished).

Amber Bard demonstrated by in situ hybridization that the expression of cyclin B1 is absent by 10-somite stage, which supports our hypothesis that the spr mutation can be linked to a null Cyclin B1 protein (Fig. 5). Further studies revealed that the expression of the phosphorylated form of histone H3, which is the marker for cells undergoing mitosis, was downregulated in the spr mutant embryos compared to the wild type (J. Singh, unpublished). This study also revealed that “putative” neural stem cells in the mutant embryo did not divide at the midline as they do in the wild-type control.
Figure 5. The specter mutant embryo lacks Cyclin B1 mRNA expression. In situ hybridization for the Cyclin B1 mRNA in a wild-type embryo (A-E) and the spr mutant embryo (A’, D’, E’) (Amber Bard, unpublished).

Jyotika Singh also showed by in situ hybridization that the expression of deltaa, a marker for neural precursors that are still dividing (Appel & Eisen, 1998), was weaker and that these cells were larger in spr mutant embryos compared to wild-type embryos.

Summary

By characterizing spr mutant embryos we can elucidate the role of Cyclin B in the cell cycle and early development in zebrafish. In this work I describe the spr mutant phenotype as the effect of a nonsense mutation in the cyclin B1 gene. Using in situ hybridization and immunostaining to analyze markers of specification and differentiation, I show that the spr mutation is indeed a cell cycle mutation. By mutagenizing embryos in cyclin B1 gene, I show that the spr mutant phenotype is caused by lack of functional Cyclin B1 protein.
CHAPTER II

CHARACTERIZATION OF THE *SPECTER* MUTANT PHENOTYPE

As described in Chapter I, some characterization of the *spr* mutant phenotype had been done, but it was not clear precisely when the morphological and cellular phenotypes in mutants first appeared and what was the pattern of those changes. In this chapter I focus on a detailed description of the morphological and cellular changes during early development of mutants. First, I will point out alterations in the vascular system, alterations in the precursors of nervous tissue and alterations in the precursors of the muscular systems. Second, I will focus on changes at the cellular level, in order to reveal any cell cycle alterations in mutant embryos. Finally, I will describe cell death pattern during early development in mutant embryos.

Results

Characterization of the Morphological *specter* Mutant Phenotype

Experiments in this section were conducted on the embryos derived from zebrafish heterozygous for *spr*, therefore on average 25% of embryos were homozygous for the lethal recessive allele and have the *spr* mutant phenotype. Wild-type and mutant embryos were sorted and staged during the first few hours after fertilization in order to synchronize their development. Later, they were fixed at designated stages of development.
The spr mutant morphological phenotype is first distinguishable at the 6-somites stage. At this stage the main differences between wild-type and mutant siblings are smaller head and tail in mutant embryos, and clear boundaries only in the central somites of the mutants had clear boundaries. At the 10-somite stage, mutant embryos showed shorter body axes, an opaque head and central nervous system. Darkening of the head continues to spread along the body axes. By 24 hours zebrafish wild-type embryos showed motility; in mutant embryos, the motility pattern is altered and described as an uncontrolled “twitching” of the tail, which happens more often than the movement in the wild-type embryos.

To describe morphological changes more accurately we examined the mRNA in different tissues. Cells in vascular and nervous tissues undergo many rounds of stem cell divisions, producing many cells that commit to different cell fates within those tissues (Kimmel and Warga, 1986, Kimmel et al., 1994; blood: Warga et al., 2009). Using in situ hybridization, I determined the pattern of erythrocytes (hhbe) (Brownlie et al., 1998), macrophages (lplast or lcp1) (Herbonel et al., 1999) and neutrophils (mpx) (Lieschke 2001; Bennett et al., 2001) in spr mutant embryos. Erythrocytes and neutrophils are derived from a common lineage that undergoes frequent rounds of cell division (Warga et al., 2009). In earlier observation of the mRNA expression of blood markers R. Warga (unpublished) observed bigger blood cells in mutant embryos. This observation helped to distinguish the possible functional origin of the defects observed in mutants: disruption of the cell cycle, because the pattern of bigger and fewer cells is seen in some zebrafish cell cycle mutants such as zombie and harpy. We
determined that the mutant embryos had all three types of blood cells (Fig. 6). And by 24 and 26 hours they were fewer in number and bigger in size.

Figure 6. *In situ* hybridization of the blood cells markers in the wild-type and the *specter* mutant embryo.

*In situ* hybridization of blood cells markers in wild type (A, C, E) and *spr* mutant embryos (B, D, F) at 24 and 26 hours of development. A-B, expression of *hhbe*, erythrocytes; C,D, expression of *lplast*, macrophages; E,F, expression of *mpx*, neutrophils. Side view.

In wild-type embryos it is possible to see the blood flow by the second day of development. Due to the darkness of the mutant body, it is difficult to see the stream of erythrocytes in *spr* mutant embryos. I saw the heartbeat in mutant embryos, but erythrocytes lacked red pigment by the 4th day. *In situ* hybridization for blood markers at 24 and 26 hours showed fewer cells. Because this experiment was so late, one possible explanation for this pattern is a cell death in the mutant embryos. Another possible explanation for the reduced number of blood cells is that due to changes in the cell cycle, there could have been fewer blood cell precursors that could give rise to blood cells.
*pax2a* mRNA is expressed in cells in the midbrain-hindbrain boundary, spinal interneurons, otic placode and kidneys (Krauss et al., 1991). Embryos at the 10, 15, and 19-somite stage were sorted for the wild-type and the *spr* mutant phenotype and fixed. There are transparency changes in mutant embryos, therefore this marker could confirm if there are any alterations in some parts of nervous system and some organs. Expression of *pax2a* mRNA was indeed decreased in the otic placode and the midbrain-hindbrain boundary (Fig 7). Spinal interneurons had lower expression of *pax2a*, they were bigger in size and were absent posteriorly. Expression in kidneys remained the same compared to wild-type controls.

*islet 1* is expressed in primary motor neurons, branchiomotor neurons, neurectoderm, hatching gland, epiphysis, nuclei of the telencephalon and the diencephalon and Rohan-Beard sensory cells (Korzh et al., 1993). Embryos at 16.50, 19 and 24 hours were sorted for the wild-type and the *spr* mutant phenotype and fixed. Embryos at 12 hours were not sorted for mutant phenotype, because it was too early to distinguish it morphologically. At the 15-somite stage (16.50h) we observed changes in the branchio-motor nuclei of the mutant embryos, as they do not express *islet 1* as strongly as the wild-type controls (Fig. 8 C, D).

No changes of *islet 1* expression were detected in the pancreas at all stages in the mutant embryos. Expression in neurectoderm, epiphysis, nuclei of the telencephalon and the diencephalon was also decreased in the mutant embryo.
Figure 7 In situ hybridization of pax2a mRNA in the wild-type and specter mutant embryos.

(A, C) the wild-type embryo; (B, D) the mutant embryo at the 15 and 19-somite stage, respectively. Legend: mhb, midbrain-hindbrain border; os, optic stalk; ov, otic vesicle; pd, pronephric duct; scn, spinal cord interneurons. Side view.

As for Rohan-Beard sensory neurons, at 16.50, 19 and 24 h, wild-type embryos had them aligned straight, while the mutant embryos had a different pattern (Fig. 8, C-E). Rohan-Beard sensory neurons in the embryos were missing in the posterior part of an embryo, looked bigger, compared to the wild-type embryo and did not align straight along the body. Same was true for the primary motor neurons: there were not as many in the mutant embryos and they were also bigger, compared to the wild-type. Our observations indicate that there were indeed fewer neurons, particularly sensory and motor in the mutant embryos. Fewer neurons supports our hypothesis that the possible darkening of the embryo might be caused by cell death in the nervous system. Also, the lack of
motor neurons could explain the abnormal motility in mutant embryos at later stages.

Figure 8. In situ hybridization of islet 1 mRNA in the wild-type and specter mutant embryos.

(A, C, E, G) wild-type embryos; (B, D, F, E) spr mutant embryos at 12, 16.50, 19 and 24 hours of development, respectively. Legend: hg – hatching gland; bmn, branchio-motor nuclei, ep, epiphysis; pcr, pancreas, pmn, primary motor neurons, rb, Rohon-Beard sensory neuron, tc, telencephalon. (A, B) dorsal view; (C-E) side view.

At 12 hours most of an observed group (~75%) had a distinct expression of islet 1 in the hatching gland, and weaker expression was seen in the remainder of the clutch. Therefore, we assumed that the remainder, about 25%, should be spr mutant embryos. The hatching gland stops dividing around cell cycle 14 (Kimmel et al., 1994; Kimmel and Warga, 1996), therefore we hypothesized that such a faint expression in the hatching gland in spr mutant embryos could indicate a delay of its development due to changes in the cell cycle, particularly in possible delay of mitosis (Fig. 8, A, B).
notch1b is expressed in the undifferentiated dividing neural stem cells (Bierkamp and Campos-Ortega, 1993). Embryos at 16.50, 19, 24 and 31 hours of development were sorted for the wild-type and the mutant phenotype, and fixed. Embryos at 12h hours were fixed without sorting, as the mutant phenotype was not clear.

Figure 9. In situ hybridization for notch1b mRNA in wild-type embryos and specter mutant embryos.

(A,C,E,G, I) Expression in wild type embryos; (B, D, F, H, J) expression in spr mutant embryos at 12, 16.50 and 19 hours of development, respectively. (A-F) Side view; (G-J) dorsal view.

At 12 hours, notch1b expression is fainter in mutant embryos and not as complete as in wild-type embryos (Fig. 9, A, B). At 16.50 hours there was no difference in notch1b expression between the control and the mutant embryos (Fig. 9, C, D). At 19 hours we observed a decrease in notch1b expression in the mutant embryos, which is possibly due to cell death (Fig. 9, E, F). The same pattern was true for 24 and 31 hours (Fig. 9, G-J). Increased expression of notch1b in mutant embryos at 16.50 and later stages, compared to the weaker expression at 12 hours, might indicate the delay in notch1b expression.
deltaa is expressed in the cells differentiating into neurons (Appel and Eisen, 1998). According to Jyotika Sigh, expression of deltaa was decreasing over time in older mutant embryos (24-30 h). To characterize the pattern of deltaa expression, I sorted and fixed embryos at 14, 16.50, and 24 hours (Fig. 10). Expression of deltaa was weaker in the mutant embryos, compared to the wild-type control at 14h (Fig. 10, A, B). deltaa positive cells were bigger in spr mutant embryos (Fig. 10, F, enlarged). At 16.50 and 24 hours deltaa expression became more intense, however not as bright and strong as in the wild-type embryos (Fig. 10, C-F).

Stem cells must support their own population and give rise to differentiated neurons. Mutations in cyclin B1 might prevent these stem cells from being able to renew and support notch-positive cells, or to differentiate through the Delta pathway. In the mutant embryos we might observe a situation when notch cells do not enter the Delta pathway and remain in the Notch pathway due to a delayed cell cycle, or do not exit the Delta pathway to give rise to neurons. This would allow us to make the proposal that the pattern of missing neurons that we observed with islet 1 and pax2a expression in mutant embryos is caused by the lack of stem cells choosing neural pathway of development.

The myoblast is the type of progenitor cell that differentiates to give rise to muscle cells. According to initial transplants results with the mutant embryos, most cells surviving would give rise to muscle (Kane et al., 1996). On the other hand, possible cell death, lack of cells to give rise to the nervous system and the delay in nervous system development, could also be true for the mesoderm
formation. MyoD is expressed in the adaxial muscle precursors and in segmental boundaries (Weinberg et al., 1996). To characterize possible changes in mesoderm development in the mutant embryos, embryos were sorted (except 12 hours) and fixed at 12, 16.50 and 24 hours. Embryos at 12 hours were fixed without prior sorting (Rachel Warga, unpublished).

![Figure 10](image)

Figure 10. In situ hybridization for deltaa mRNA in wild-type embryos and specter mutant embryos.

(A, C, E) Expression in wild-type embryos; (B, D, F) expression in spr mutant embryos at 14, 16.50 and 24 hours of development, respectively. Side view.

At 12 hours of development, wild-type embryos had expression of MyoD in the somites; the spr mutant embryos showed a delayed expression of MyoD at this point (Fig. 11, A, B). Later, at 16.50 hours MyoD expression returns to the wild-type strength, but it is disorganized and the somite borders are not clear. This result was also true for 24 hour stage (Fig. 11, C-F).

Delayed expression of MyoD is consistent with the delayed expression of islet 1 and notch1b. Interestingly, we did not see diminished expression at later stages, which might indicate that mesoderm is not affected by cell death as is nervous system, therefore it does not appear opaque.
Figure 11. In situ hybridization for MyoD mRNA in wild-type embryos and specter mutant embryos.

(A,C,E) Expression in wild-type embryos; (B, D, F) expression in spr mutant embryos at 12, 16.50 and 24 hours of development, respectively. Dorsal view.

It has been shown that MyoD upregulates p21 in muscle cells, and transactivation of p21 regulates the duration of the G2 phase of the cell cycle through repression of CyclinB/Cdk1. CyclinB/Cdk1 is responsible for MyoD phosphorylation, and further degradation of MyoD. Furthermore, if MyoD does not get phosphorylated, then there is a delay in mitosis entry (Tintignac et al., 2004). Therefore, this could explain why we see morphological defects in the somites of the spr mutant embryos.

Characterization of the Cellular specter Mutant Phenotype

I started this part of the work, by repeating a subset of Amber Bard’s (unpublished) experiments. She showed that the cell cycle in the spr mutant embryos is not normal, and some cells were bigger, compared to the wild type. Second, the expression of the phosphorylated form of histone H3, which reveals
cells undergoing mitosis, was downregulated in the mutant embryos compared to the wild type starting at the 15-somite stage (Amber Bard, unpublished).

Could I detect a similar pattern of downregulated expression of phosphorylated form of histone H3, which is as early as I can see the spr mutant phenotype, which is at 7 somites? Embryos were sorted by the wild-type and mutant phenotype, fixed at the 7, 15, 20 and 25-somite stage and then processed for whole-mount in situ hybridization for krox20 to determine rhombomeres three and five as described in Thisse at al. (1993). Subsequently, I performed whole-mount antibody staining for the phosphorylated form of histone H3 (Fig. 12).

At the 7-somite stage the mutant embryos had abnormal shape and showed fainter staining mitotic nuclei, compared to the wild type (Fig. 12 A, E). Later in development, wild-type embryos have a distinct alignment of mitotic cells at the midline. At the 15-somite stage and later, the mutant embryos showed the similar cellular phenotype as the mutants at the 7-somite stage (Fig.12, F-H). Also, “putative” neural cells in the mutant embryo did not divide at the midline starting at the 15-somite stage, as seen in the wild-type control (Fig. 12, B-D, F-H). At the 25-somite stage, mitotic nuclei in the mutant embryos were drastically fragmented, compared to either mutant embryos at earlier stages of development or wild type controls at the 25-somite stage (Fig. 12, D’-H’).

Absence of the cells dividing at the midline in the mutant embryos is consistent with previously shown data by Amber Bard (unpublished). Also, the
abnormal shape of the mitotic nuclei in the mutant embryos might be caused by the effects on normal mitotic progression.

Figure 12. The comparison of the mitotic phenotype in wild-type and specter mutant embryos.

Anti-phospho-histone H3 (pH3) staining with expression of krox20 mRNA in wild-type (A-D) and spr mutant embryos (E-H) 7- through the 25-somite stage. The arrow in B shows cell dividing at the midline. Red squares in D and H indicate areas in D’ and H’.

The second part of mitotic cells analysis consisted of counting nuclei positive for the phosphorylated form of histone H3 within krox20 stripes (Fig. 13). It was shown that the number of mitotic nuclei in the mutant embryos is the same as in wild-type embryos at the 7-somite stage. At the 15-somite stage the number of mitotic nuclei in spr mutant embryos was significantly lower compared to wild-type control at the same stage, and not statistically different from the mutant embryos at the 7-somite stage. At the 20-somite stage the number of mitotic
nuclei in the mutant embryos remained statistically lower compared to the wild type control, but was statistically greater than in mutant embryos at earlier stages. At the 25-somite stage the number of mitotic nuclei in the mutant embryos was significantly lower compared to either wild-type embryos at the 25-somite stage or mutant embryos at earlier stages.

![Bar graph showing the number of mitotic nuclei in wild-type (grey) and specter mutant (yellow) embryos between krox20 stripes.](image)

*Figure 13. Quantitative analysis of the mitotic cells in wild-type embryos and specter mutant embryos.*

The number of mitotic nuclei in wild-type (grey) and spr mutant embryos (yellow) between krox20 stripes. Error bars represent standard error.

*p>0.05; **p<0.05.

These data suggest that spr mutant embryos have an equal number of mitotic nuclei in the analyzed hind-brain area as the mitotic nuclei of wild-type embryos at the 7-somite stage, although nuclei in mutant embryos differ by shape and intensity of staining. We think that at the 7-somite stage no more cells enter mitosis and some cells might get arrested in M phase of the cell cycle. Later in development, the number of mitotic nuclei in the mutant embryos
remains almost the same until the 25-somite stage, when mutant embryos are characterized by vast apoptosis.

We hypothesize that nuclei positive for the phosphorylated form of histone H3 in mutant embryos do not exit mitosis beginning at the 7-somite stage, rather they are stopped by, presumably, lack of Cyclin B1. Also, we do not see an increase in mitotic nuclei with time because the predominant number of cells could be in G2, and therefore delayed in entering mitosis.

To understand cellular changes in the spr mutant embryos I used DAPI staining to analyze the nuclear phenotype of the mutant embryos at 7, 15 and 25 somites (Fig. 14). At the 7-somite stage there was no difference in nuclear morphology, as well as mitotic activity between mutant and wild-type embryos (Fig. 14, A, B). At the 15-somite stage nuclei of the mutant embryos were heterogeneous and did not have as many dividing cells, compared to the nuclei of wild-type embryos (Fig. 14, C-D) At the 25-somite stage, mutant cells did not show such high heterogeneity in size, and they were all at least twice as big as the wild-type cells at this stage (Fig. 14, C, F).

In the phospho-histone H3 analysis, I saw changes in the shape of the spr mutant cells beginning at the 7-somite stage, but in the DAPI analysis, no changes in cellular morphology were detected. In the mitotic analysis for later stages, I did not see cells in mitosis larger in size, compared to the wild-type control, unlike in the DAPI assay. The inconsistency in the results from the two assays might be explained by only a small portion of the cells analyzed in the pH3 assay and no complete picture is seen of how the cell cycle changes in the
mutant embryos. On the other hand, in the DAPI staining experiment I was looking at all the cells in the embryo.

**Figure 14.** The nuclear phenotype of wild-type and specter mutant embryos. DAPI staining of nuclei of wild type embryo (A-C) and the spr mutant embryo (D-F) at 7-, 15-, and the 25-somite stage, respectively. At the 7-somite stage nuclei appear to be equal in size, no abnormal nuclei are observed. At the 15- and the 25-somite stage nuclei of the spr mutant embryo are bigger, have abnormal shape. Bar = 20 µm.

In order to study this question more precisely, I used the Dual FUCCI (Fluorescnet Ubiquitination-based Cell-Cycle Indicator) technique (Abe et al., 2012; Bouldin et al., 2014; Sugiyama et al. 2009). Bouldin et al. (2014) recently created an improved version of the Dual FUCCI system, expressing both probes of zCdt1 and zGeminin. This construct is comprised of Flag-Cerulean-zGem1-100 followed by a viral 2A peptide and mCherry-zCdt1-190 (Fig. 15).

Instead of using the EF1α promoter, limited to early development (Sugiama et al., 2009), the Dual FUCCI system uses the zebrafish ubiquitin
promoter, which is expressed ubiquitously and can be visualized beginning at the 50% epiboly stage, with no further limitation in time (Bouldin et al., 2013).

Figure 15. Schematic representation of the dual FUCCI reporter.
zGeminin degron cloned downstream from a 3xFlag tag. Flag-Cerulean-zGeminin placed downstream of a viral 2A peptide. mCherry-zCdt1 fusion protein was placed downstream from the 2A peptide and inserted in a plasmid Tol2 elements and the ubiquitin promoter. Adapted from Bouldin et al., 2013. Construct is comprised of Flag-Cerullean-zGeminin, DNA-replication inhibitor; followed by a viral 2A peptide; and mCherry-cdt1, DNA replication factor. The 2A-viral peptide allows translation of a multiple protein products. This gives rise to two fluorophores produced as independent proteins from a single transcript. 2A region encodes a polycystronic sequence that mediates self-processing and allows a continuous translation. Therefore Dual FUCCI polypeptide gives rise to Cerulean-based S/G2/M reporter, and a Cherry-based reporter, detectable at the G1 stage. zGeminin is a DNA replication inhibition, which accumulates in the nuclei and cytoplasm, it reveals morphology of individual cell that have undergone DNA replication (Sugiyama et al. 2009). Geminin is absent during G1 phase and accumulates through S/G2/M phases of the cell cycle. At the metaphase to anaphase transition, APC/C degrades Geminin through
ubiquitination (McGarry and Kirschner, 1998). Thus, we can visualize cells in S/G2/early M stage of the cell cycle. Cdt1 (Chromatin licensing and DNA replication factor 1) – DNA replication factor, with maximum concentration in G1 phase. Thus, we can visualize cells in the G1 stage.

We hypothesized that wild-type embryos should have cells predominantly in the G1 phase of the cell cycle (Sugiyama et al. 2009; Pauklin & Vallier, 2013; Kimelman, 2014), and if the spr mutant embryos lack functional Cyclin B1, most of the cells should be arrested in G2/M phase, according to previous studies (Allan and Clarke, 2009; Chang et al., 2003; Shepard et al., 2005). Wild-type and mutant embryos carrying Dual FUCCI transgene were synchronized in development and sorted by the morphological phenotype at the 20-somite stage and then visualized by confocal microscopy (Fig.16). We found that cells in wild-type embryos were indeed in the G1 phase of the cell cycle with few cells in S/G2/M phase. Cells of the spr mutant embryos indeed had much more “blue cells”, than “red cells”, meaning that most of the cells were in S/G2/M phase. Considering that there is no nuclear envelope during mitosis, Dual FUCCI system does not allow us to visualize M-phase of the cell cycle (Pauklin & Vallier, 2013). Therefore, the blue cells in the spr mutant embryos are the cells in S and G2 phase. Cells in G1 phase have an altered morphology, compared to the wild-type cells in G1 phase. Cells in S/G2 phase are bigger, than in wild-type, which could suggest that mutant embryos might undergo some type of endoreplication.
Figure 16. Cell cycle visualized with the dual FUCCI reporter system in the live wild-type embryo and the specter mutant embryo.

(A-A") the wild-type embryos; (B-B") the spr mutant embryo at the 20-somite stage. (A-B) cells in G1 stage, Cdt1-mCherry (red); (A'-B') cells in S/G2/early M phase, zGem-Cer (blue); (A"-B") merged image. Bar = 20 µm.

Abnormal looking cells in the spr mutant embryos indicate that many cells die. Thus, I wanted to follow the changes in apoptosis pattern in mutant embryos.

Apoptosis in the specter Mutant Embryos

Because we observe darkening in the anterior part of the spr mutant embryos, first we hypothesized that apoptotic cells should first appear in this area. Darkening of the embryo in the rostral area might be due to the increased size of the cells. Also, Amber Bard’s data seems to show apoptosis started in the mutant embryos in the head. According to Cole and Ross (2001), apoptosis normally occurs in the nervous system of the zebrafish in a rostral to caudal manner by 12 hours. In their study apoptotic cells localized at the dorsal midline,
suggesting that these are neural precursors cells. Because development proceeds in a rostral to caudal manner and formation of the neural keel, which gives the rise to the brain and spinal cord, follows this pattern the authors suggest that apoptosis is a way of diminishing excess cells during development of the nervous system (Cole & Ross, 2001).

A critical target of Cyclin B/Cdk1 activity is the G2 checkpoint (Kastan & Bartek, 2004). There are two possible pathways for a cell after it enters G2 checkpoint: cell cycle progression or activation of apoptosis. To prevent apoptosis, the Cyclin B1/Cdk1 complex is responsible for the phosphorylation of caspase-9 at Thr125, which leads to a block of caspase-9 and caspase-3 activity (Allan & Clarke, 2007), and prevents the cell from undergoing apoptosis. Without this modification cells exhibit increased sensitivity to apoptosis. Delay in mitosis for a prolonged period increases the predisposition of the cells to undergo apoptosis (Allan & Clarke, 2007). Moreover, arresting human HeLa mitotic cells using microtubule poisons leads to inability of the cells to maintain CyclinB1/Cdk1 levels needed to block caspases, which leads to activation of apoptosis after a prolonged mitotic arrest (Allan & Clarke, 2007). Therefore, CyclinB1/Cdk1 levels determine one aspect of the fate of the cells during the cell cycle, to live or to die. The spr mutant embryos lack functional Cyclin B1, which possibly interferes with required levels of CyclinB1/Cdk1 resulting in activation of apoptosis pathways.

To analyze dynamics of apoptosis I first determined when apoptosis started using an antibody that detects the phosphorylated form of caspase-3,
which is normally only phosphorylated once the caspase cascade is initiated in
the apoptotic pathway (Negron & Lockshin, 2004). This revealed that apoptosis
was not detected until the 10-somite stage (Fig. 17). These data suggest that
morphological changes in the spr mutant embryo by the 7-somite stage, the
earliest stage the mutant phenotype is visible, are not due to apoptosis. Next, I
counted the number of apoptotic cells within the forebrain and the midbrain area.
To identify this region I used the mRNA expression of pax2a, which is expressed
in the optic stalk (forebrain) and in midbrain-hindbrain boundary. pax2a is also
expressed in the kidneys, otic vesicle and a spinal cord interneurons (Krauss et
al., 1991). Embryos were fixed at the 5, 7, 10, 15, 20 and 22-somite stage and
then processed for whole-mount in situ hybridization as described in Thisse et al.
(1993). Subsequently, I performed whole-mount antibody staining for the
phosphorylated form of caspase-3 (Fig. 17). Finally, I counted the number of cells
expressing the phosphorylated form of caspase-3 between the optic stalk and the
midbrain-hindbrain boundary. In all but the 10-somite stage, I was able to sort the
mutant embryos from the wild types before fixation.

Analysis at later stages (15-, 20- and 22-somite stage with animals of
three clutches for each stage) revealed that all mutant embryos had significantly
more apoptotic cells than the wild type, and that the number of apoptotic cells in
the mutant embryo increased over time from a maximum of 106±13 by the 15-
somite stage to 177.97±5.77 by the 20-somite stage (Fig. 17, B’, C’; Fig. 18).
Figure 17. The analysis of an apoptosis in the wild-type control and *specter* mutant embryos.

*In situ* hybridization for *pax2a* mRNA and antibody staining for the phospho-cas-3 (A-C) the wild-type control; (A’-C’) *spr* mutant embryos at the 10-, 15-, and 20-somite stage, respectively. Panels to the right: enlarged view of the head, where apoptotic nuclei were counted. (A”, B”, C”) the number of apoptotic nuclei at the 10-, 15-, and 20-somite stage in the wild-type control (black frame) and the mutant embryos (red frame). Legend: ar – anterior retina; os, optic stalk; mhb, midbrain-hindbrain border; ov, otic vesicle; pd, pronephric duct; scn, spinal cord interneurons.
Figure 18. The mean number of apoptotic cells in the wild-type control and *specter* mutant embryos.

Grey bars – wild-type; yellow bars – mutant embryos at 15- and 20-somite stage. Data adapted from Fig. 17, B” and C”. Bars indicate standard deviation.

At the 10-somite stage the number of apoptotic cell varied. There was a group of animals (approx. 1/3) that had many apoptotic cells (from 50 to 102 apoptotic cells) (Fig. 17, A’). There was a group of animals (approx. over 1/3) with few apoptotic cells (from 2 to 25 apoptotic cells), inferred as “wild-type”. However, there was also a group that could be a “dominant effect”, because I could not determine if these animals represent mutants with fewer apoptotic cells or wild type with an elevated apoptosis rate (from 33 to 55 apoptotic cells). This same phenotype was observed in two independent clutches. In the third clutch the “dominant effect” group was shifted to the right, suggesting that the “wild-type” phenotype corresponds to 2/3 of the clutch.

These results suggest that already by the 15-somite stage there is a high amount of apoptosis in the forebrain – midbrain area of the *spr* mutant embryo (Fig. 18). It also supports our hypothesis that the cells in the mutant embryo
eventually undergo apoptosis which gradually increases with time of development. This supports our proposal that opaque embryos can be explained by exceeding apoptotic cells and degeneration of tissues.

Summary

In this section I characterized the spr mutant phenotype in detail. The observation of morphological phenotype revealed specific mutant morphological characteristics beginning the 7-somite stage. Through in situ hybridizations I have showed that the mutant embryos have fewer and bigger cells in vasculature and nervous systems, which supports our proposal that the mutant embryos are cell cycle mutants. The cellular phenotype was characterized with antibody staining for the mitotic marker, which suggested that cells do not enter the next cell cycle after the 7-somite stage, possibly due to the lack of Cyclin B1 protein. By DAPI staining I showed that nuclei in the mutant embryos have abnormal shape and most of the cells are bigger in size. Also, I showed that the cell cycle progression in the mutant embryos is not normal, due to a majority of the cells being in S/G2/ early M stage, while the cells in the wild-type embryos remain in G1 stage at that time. The cellular phenotype also supports the hypothesis that the mutant embryos have a disruption in normal cell cycle progression. Loss of cells and the opaque body of the mutant embryos was explained by an active apoptosis. In the following chapters I will show that the mutant phenotype is caused by a mutation in the cyclin B1 gene.
CHAPTER III

ATTEMPTED RESCUE OF THE SPECTER MUTANT PHENOTYPE

Background

mRNA Rescue

As Don Kane, Laura Bakke and Rachel Warga (unpublished) showed, the spr locus mapped in the vicinity of the Cyclin B1 locus and the spr mutant embryos are characterized by a nonsense mutation in exon 2 of cyclin B1 gene. This mutation results in a premature stop codon in the transcribed RNA, and nonfunctional Cyclin B1 protein. To reduce errors in gene expression, mRNA transcripts that contain premature stop codon are eliminated through nonsense mediated mRNA decay (Chang et al., 2007). We assumed that the lack of functional Cyclin B1 disturbs the transition from G2 to M phase of the cell cycle. cyclin B1 mRNA is maternally supplied to the embryo, which remains sufficient through the first fifteen cell cycles. Considering that mutant embryos do not express endogenous zygotic cyclin B1, we hypothesized that by providing wild-type cyclin B1 mRNA using microinjections could rescue the spr mutant phenotype. “Rescuing” the mutant phenotype would demonstrate it is a result of the mutation in cyclin B1 gene. cyclin B1 mRNA is constantly degraded, therefore without continuous supply of wild-type cyclin B1 mRNA, mutant embryos are not provided with enough Cyclin B protein and cannot be fully rescued.
Injection of *in vitro* transcribed mRNA is the most common way to test our hypothesis (Hammerschmidt et al., 1999). Huang et al. (2013) have shown the transient rescue of the mitotic defect in *emi1* mutant embryos through injection of wild-type mRNA: the rescue effect was evident through gastrulation, it declined by the tailbud and was absent by the 18-somite stage. This provided the evidence that their candidate gene had been identified correctly. A purpose of the experiments in this chapter is to confirm that *spr* mutant phenotype is a result of a non-functional Cyclin B1 protein.

I have attempted to “rescue” mutant phenotype by injecting synthetic wild-type *cyclin B1* mRNA into embryos from a heterozygous cross at the 1-2-cell stage. I would not expect full rescue, because the amount of synthetic wild-type mRNA required to provide enough protein might be toxic to an embryo.

Results

**mRNA Rescue of the specter Phenotype**

To confirm that the *spr* mutant phenotype is a result of a mutation in the *cyclin B1* gene, I injected synthesized wild-type *cyclin B1* mRNA into embryos derived from a heterozygous cross. As a control, I injected *kaede* mRNA, originally isolated from the stony coral *Trachyphylia geoffroy*. Kaede is a non-toxic green fluorescent protein that does not interfere with early development of embryos (Hatta et al., 2006). First, I determined the lethal threshold for the wild-type *cyclin B1* mRNA. By performing injections of wild-type *cyclin B1* mRNA in different concentrations (30-70 pg). After a series of such experiments, I
determined that the concentration of >52pg was damaging and lethal for the embryos. To distinguish any developmental delay after mRNA injections, I injected kaede mRNA in the control embryos in the same concentration as cyclin B1 mRNA. kaede mRNA injected embryos had the same delay in development as cyclin B1 mRNA injected embryos, compared to uninjected group.

Most injection experiments I used on average 30-50 embryos in each control and test group, with the remainder of the embryos left uninjected. Because spr is a recessive Mendelian mutation, 25% of the embryos derived from a heterozygous cross will have the spr mutant phenotype. After a series of different mRNA concentration experiments, I was not able to distinguish the spr mutant phenotype at the 7-somite stage, unlike in control group, when I used 52 pg of cyclin B1 mRNA. This suggested that this was the right concentration to see a possible rescue phenotype. Not until the 15-somite stage was I able to determine mutants in the injected group, due to the length of the body axis, which were shorter possible due to the fewer cells.

To determine any changes in cellular phenotype, I have used DAPI staining of embryos injected with kaede and cyclin B1 mRNA. No significant changes were observed within the two injected groups.

I assayed the number of apoptotic calls in the “rescued” mutants using in situ hybridization and antibody staining methods, to determine the effect a wild-type cyclin B1 mRNA was having on the spr apoptosis phenotype. I preferred using injected embryos fixed at the 15-somite stage, because at that point it was easy to determine the phenotype of wild-type and spr mutant embryos, as
described in Chapter II. In addition, I also had extensive data on the apoptosis
dynamics and the pattern at the 15-somite stage in uninjected embryos (Fig.
17, B-B”). *Figure* 19 represents the data of two experiments and showing the
number of apoptotic cells in the midbrain-hindbrain area of embryos injected with
*kaede* and *cyclin B1* mRNA.

*Figure* 19. Number of apoptotic cells in embryos injected with the wild-type
cyclin B1 mRNA.

No rescue effect was detected after the wild-type *cyclin B1* mRNA injections.
The number of apoptotic nuclei in embryos injected with *kaede* (blue) and wild-
type *cyclin B1* mRNA (orange). Embryos were fixed at the 15-somite stage. A,
injected: 42-46 pg of wild-type *cyclin B1* mRNA; total number of embryos
injected with *kaede* n=33, with wild-type *cyclin B1* mRNA n=28. B, injected:
52 pg of wild-type *cyclin B1* mRNA; total number of embryos injected with
*kaede* n=46, with wild-type *cyclin B1* mRNA n=43.
The number of apoptotic cells in the *kaede* injected control did not differ from former experiments (Fig. 17, B”). This suggests that *kaede* mRNA did not interfere or disrupt the morphological, or cellular phenotype of the embryo. Wild-type *cyclin B1* mRNA did not cause a significant decrease of apoptotic cells in mutant embryos. According to these data, I did not confirm any rescue effect of *spr* mutant phenotype from wild-type *cyclin B1* injections.

**Summary**

In this chapter I have shown that endogenous *cyclin B1* mRNA injected into the *spr* mutant embryos during early cleavage stage does not have any rescue effect. No partial or full rescue effect was noticed in morphological and cellular phenotypes, and no difference in apoptotic patterns. No significant phenotypic effects suggests that this experiment did not prove that Cyclin B1 is the right candidate for the mutants. However, in this case there might not be sufficient levels of injected *cyclin B1* mRNA, since the dynamic change in protein levels may require transcription at every cell cycle after cleavage divisions. Therefore, in the next chapter I will show what effect does the disruption of *cyclin B1* gene has on zebrafish embryos using CRISPR/Cas9 system.
CHAPTER IV
CRISPR/CAS9 SYSTEM

Background

CRISPR/Cas9 System as an Efficient System of Genome Editing

Clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) have recently been established as an efficient tool for gene disruptions (Auer et al., 2014; Gangnon et al., 2014; Hwang et al., 2013; Irion et al., 2014; Wiedenheft et al., 2012). Other editing tools, such as zinc-finger nucleases and transcription activator-like effector nucleases (TALENs) are also effective genome editing systems, however they are limited by the cost of methods and a requirement for substantial molecular cloning (Gaj et al., 2013; Gagnon et al., 2014).

Bacteria and archaea integrate short fragments of foreign nucleic acid into the host chromosome at one end of the CRISPR locus as an adaptive defense mechanism against invading conjugative plasmids, transposable elements, and viruses (Brouns et al., 2008). Originally, CRISPRs were described in Escherichia coli as an “unusual sequence element”, consisting of 29-nucleotide repeats separated by “spacer” sequences. These sequences function similarly to a genetic memory to defend a host from being infected by viruses containing this recognition sequence (Brouns et al., 2008).
There are three types of prokaryotic CRISPR immune adaptive systems. The type II CRISPR system from *Streptococcus pyogenes* consists of Cas endonucleases to target and cleave invading foreign DNA, CRISPR RNA (crRNA) and a trans-activating RNA (tracrRNA) for sufficient RNA-guided cleavage of foreign DNA by Cas proteins. Recent studies showed that crRNA and tracrRNA chimera guide RNA (sgRNA) can direct Cas9 endonuclease-mediated cleavage of target DNA (Jinek et al., 2012). Cas9 can introduce site-specific DNA double-stranded breaks *in vitro* (Jinek et al., 2012), in mammalian cell lines (Fu et al., 2013), bacteria (Brouns et al., 2008; Wiedenheft et al., 2012), yeast (DiCarlo et al., 2013), zebrafish (Auer et al., 2014; Hwang et al., 2013; Irion et al., 2014; Auer & Bene, 2014; Jao et al., 2013), nematodes (Chen et al., 2013) and mice (Wang et al., 2013). CRISPR/Cas9 system targets any sequence 5’-GG-N_{18}-NGG-3’. The range of available sequences is limited by T7 promoter, used to make sgRNA (GG at the 5’end of the transcript) and by the protospacer adjacent motif (PAM) sequence NGG in genomic DNA 3’ to the target site (Hwang et al., 2013; Wiedenheft et al., 2012).

In this chapter I describe how we used CRISPR/Cas9 system to mutagenize the *cyclin B1* associated locus. This method allows specific gene disruption, such as small insertions or deletions (indels) introduced during the repair of cleaved DNA by nonhomologous end joining and homology directed repair causing frameshifts and premature stop codons (Auer & Bene, 2014). I hypothesized that by editing the functional *cyclin B1* gene using CRISPR/Cas9 system, I could reproduce the *spr* mutant phenotype. To test this, I conducted
several experiments: mutagenizing the functional *cyclin B1* gene in somatic cells and germline clones; and a complementation test.

**Results**

Mutagenizing of the functional *cyclin B1* gene

By editing the functional *cyclin B1* gene using CRISPR/Cas9 construction that would recognize 20 nucleotides of the second exon of the *cyclin B1* gene on chromosome 5, a site upstream of the mutation that occurs in the *spr* mutant embryos, I tried to reproduce the *spr* mutant phenotype. sgRNA can direct Cas9 endonuclease activity to alter endogenous genes in zebrafish embryos. Dr. Kane (unpublished) constructed expression vectors that enable T7 RNA polymerase-mediated production of a capped, polyadenylated mRNA, encoding monomeric Cas9 endonuclease and sgRNA carrying specific 20 nucleotides – a targeting region complementary to a sequence in the *cyclin B1* gene (Fig. 20).

![Figure 20. Schematic representation of the CRISPR/Cas9 system, recognizing the target site within the exon 2 of the *cyclin B1* gene.](image)

*Figure 20.* Schematic representation of the CRISPR/Cas9 system, recognizing the target site within the exon 2 of the *cyclin B1* gene.

AG (first two nucleotides of the target site) is a donor site. Grey frame, exon 2 on chromosome 5; blue, guide RNA; green, target site; red, PAM site; yellow, nonsense mutation site in the *spr* mutant embryo (C139T). Ellipsis indicate omitted 84 and 6 nucleotides of the sequence.
To determine the optimal quantity of RNA for genome editing, we microinjected varying amounts of cyclin B1-targeted sgRNA and Cas9-encoding mRNA into 1-2 cell stage zebrafish embryos, derived from the wild-type fish crossed to heterozygous for spr fish. One half of the embryos from such cross must carry two wild-type alleles, and second half should be heterozygous and carry one wild-type allele. By injecting CRISPR/Cas9 mRNA into these embryos and disrupting one allele, we expected to see a mosaic spr phenotype in 50% of the embryos that originally were heterozygous for the spr mutation. As control, I had two additional groups: one injected with only Cas9 mRNA, and second group was left uninjected. After injection, embryos were left in the incubator (33°C) and monitored periodically throughout the progression of development.

We found that most of the injected embryos showed severe morphological defects and early death caused by high apoptosis rates. Therefore, we assumed that this could be an indication of off-target mutations caused by CRISPR/Cas9 system, as these mRNAs might be toxic for embryos. Optimal quantity of RNA, which did not cause as severe damage to embryos, was 153 pg. Morphological characteristics of CRISPR/Cas9 injected embryos did not reveal spr mutant phenotype, therefore I looked closer at the cellular phenotype.

To check if I was able to mutagenize some clones within CRISPR/Cas9 injected embryos, I performed whole-mount antibody staining for the phosphorylated form of caspase-3 (Fig. 21). I described this method in Chapter II, as apoptosis is an essential characteristic of the spr mutant phenotype, and can help us to determine whether injected embryos developed any apoptosis.
pattern similar to the in the mutant embryos. Apoptosis pattern in embryos injected with CRISPR/Cas9 and Cas9 was almost the same: embryos had groups of apoptotic cells in the yolk and around the midbrain area. However, embryos injected with only Cas9 usually showed apoptosis in the whole embryo, rather than in some parts of it, than in CRISPR/Cas9 injected group.

Figure 21. Somatic mutagenesis of the specter heterozygote embryo using the CRISPR/Cas9 system.

Antibody staining for the phosphorylated form of caspase-3 of CRISPR/Cas9 injected embryos at the 18-somite stage.

Since the possible mutant phenotype should be mosaic, I could not concentrate on apoptotic cells only in one area, as it was described in Chapter II. I counted the number of apoptotic cells in the whole embryo in the uninjected control group, Cas9-injected group and CRISP/Cas9 injected group at the 15-somite stage. I hypothesized, that if we are able to create a mosaic spr mutant phenotype using CRISPR/Cas9 system, we would expect higher rates of apoptosis in 50% of the embryos (heterozygous embryos) in the test group (injected with CRISPR/Cas9), and lower rates in all embryos in 2 control groups. Results of the experiment are presented in Fig. 22.
Unfortunately, I did not see the expected 1:1 ratio in CRISPR/Cas9 injected group, probably because of the Cas9 (1:1) destructive effect. The apoptosis level was elevated in both CRISPR/Cas9 and Cas9 injected groups, unlike in the uninjected group. Therefore, we assumed that the Cas9 indeed had a toxic effect on zebrafish embryos and the resulting phenotype cannot be considered as an effect of a direct CRISPR/Cas9 mutation in cyclin B1 gene.
Figure 22. The analysis of apoptosis in embryos mutagenized with the CRISPR/Cas9 system.

The number of apoptotic nuclei in uninjected embryos (grey), injected with Cas9 mRNA (orange), and with the CRISPR/Cas9 mRNA (blue). (A) The number of apoptotic cells in the embryos from a heterozygous cross, where 25% of embryos (framed) reveal the spr mutant phenotype. Red points in (E) indicate >400 apoptotic cells.
Phenotype of non-complementation in \( spr \) heterozygous embryos injected with the CRISPR/Cas9 mRNA

In a second experiment, we mutagenized wild-type zebrafish embryos with no \( spr \) mutant background using the same CRISPR/Cas9 mRNAs for injections. We disrupted a wild-type allele of the \textit{cyclin B1} gene, creating a heterozygous mutant. We grew up putative founders from this cross and crossed them with known \( spr \) heterozygotes. We tested whether the mutagenized allele fails to complement the original gene, in the progeny of such cross, and is therefore allelic. Therefore, this method could reveal and confirm whether the \( spr \) mutant phenotype is caused by the lack of functional \textit{cyclin B1}.

To mutagenize fish, we injected wild-type embryos with CRISPR/Cas9 mRNA at concentrations from 8.5 to 13.5 pg of CRISPR/Cas9. The total number of embryos injected with CRISPR/Cas9 mRNA was about 1300. On average, in every experiment, I injected around 300 embryos at 1-16 cell stage. Embryos tend to die during the first few days of development (Table 1). The most lethal were the first two experiments probably because of high mRNA concentrations injected. According to the data on apoptosis that was conducted with injecting heterozygous embryos for the mutant \( spr \) allele, this might explain high lethality rates in this experiment as well. For this reason, we lowered the concentration of injected CRISPR/Cas9 mRNA to 6.76pg. Further experiments had more success, as measured at the first week after injection. However, many fish were lost, and after 3 months we were left with five fish.
Table 1. Survival Rates of Embryos Injected with CRISPR/Cas9 mRNA at 2-16 Cell Stage.

<table>
<thead>
<tr>
<th>Concentration of CRISPR/Cas9 mRNA injected, pg</th>
<th>Number of embryos injected</th>
<th>Number of injected embryos, 1 day old</th>
<th>Number of injected embryos, 1 week old</th>
<th>Grown to adult fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.45</td>
<td>242</td>
<td>44</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>13.52</td>
<td>52</td>
<td>22</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>6.76</td>
<td>313</td>
<td>265</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>5.07-8.45</td>
<td>293</td>
<td>113</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>6.76</td>
<td>281</td>
<td>176</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>6.76</td>
<td>119</td>
<td>70</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td><strong>Total number</strong></td>
<td><strong>1300</strong></td>
<td><strong>690</strong></td>
<td><strong>250</strong></td>
<td></td>
</tr>
</tbody>
</table>

Similar fish losses occurred with normal fish growing at that time, although other off-target mutations might have disrupted normal development as well, and the fish have been weaker. During 3 months of development many fish were lost, due to unknown reasons, and we were left with only 5 fish.

To determine if the founders were carrying spr mutations, we crossed these 5 fish with spr heterozygotes and looked for mutants via lack of complementation. Out of 5 fish, only one male crossed to a spr heterozygote gave progeny that resembled the spr mutant phenotype (Fig. 23, C): shorter body axes, different head shape, smaller head, opaque anterior axes.
Figure 23. CRISPR-mediated cyclin B1 mutagenesis.

(A) The morphological phenotype of a wild type embryo, (B) the spr homozygote and (C) the transheterozygote at the 18-somite stage.

Cellular phenotype of the clones I characterized using Dual FUCCI reporter system (Fig. 24) that was described in Chapter II (Fig. 10).

Embryos from the same cross were sorted for wild-type and putative spr mutant phenotype. The Dual FUCCI reporter gene allowed visualization of the cell cycle in the live embryos at the 20-somite stage. The majority of the cells in wild-type embryos were in G1 stage, as Cdt1-mCherry accumulates during G1 stage, with several cells in S/G2/early M phase (zGem-Cer) (Fig. 24, A-A\textsuperscript{*}). The majority of the cells in spr mutant embryos, as was shown in Chapter II, reveal that most of the cells were in S/G2/early M phase (Fig. 24, B-B\textsuperscript{*}). Cells in G1 stage of spr mutant embryos had an irregular, abnormal shape and showed signs of apoptosis, cells in S/G2/early M phase were enlarged with no changes in the cell shape, compared to the cells in the wild type embryos. The majority, but not as many as in spr mutant embryos, of the cells in embryos derived from the non-complementation test also were in S/G2/early M phase (Fig. 24, C-C\textsuperscript{*}). All the cells were enlarged, and cells in G1 phase also had an irregular shape.
A clone of mutant progeny represents a mutation in a germ line cell. By injecting CRISPR/Cas9 system into embryos during zygote and early cleavage stage we can mutagenize progenitor germline cells. Having 10% clone size means that 40% of the cells in the germline contains the mutation.

Figure 24. The cell cycle of the CRISPR-mediated cyclin B1 mutagenized embryo.

(A-A") Live wild-type embryo; (B-B") the spr mutant embryo; (C-C") the transheterozygote embryo. (A-C) cells in G1 stage, Cdt1-mCherry (red); (A'-C') cells in S/G2/early M phase, zGem-Cer (blue); (A"-C") merged image.

Bar = 50 µm.

To determine what kind of indel mutation was caused by the CRISPR/Cas9 mutagenesis, R. Warga (unpublished) used a PCR amplification method to extract DNA from 16 clones to sequence. Sequencing showed that the CRISPR/Cas9 mRNA injection resulted in a splice site mutation with deletion of
the AG donor splicing site and insertion of 4 nucleotides GGAT (Fig. 25), which might lead to the translation of an abnormal Cyclin B1 protein.

We showed that the mutation caused by CRISPR/Cas9 system indeed failed to complement, therefore the mutation caused in cyclin B1 gene should be the cause of the spr mutant phenotype.

![DNA sequencing chromatogram of cyclin B1 gene of the wild-type embryo and the transheterozygote.](image)

**Figure 25.** DNA sequencing chromatogram of cyclin B1 gene of the wild-type embryo and the transheterozygote.

Sequencing of the cyclin B1 gene reveals a splice-site mutation in transheterozygotes (B) in exon 2 (Rachel Warga, unpublished. (A) Donor site AG in the wild-type embryo (black frame). (B) Detelion of an AG donor site and insertion of GGAT in the mutant (red frame).

**Summary**

In this chapter I showed that by mutagenizing cyclin B1 gene using CRISPR/Cas9 system, we caused changes in cell cycle progression similar to those of the spr mutant phenotype. We successfully showed that embryos
derived from the germline experiments failed to complement, which supported our hypothesis that the mutant phenotype is a result of disruption in the *cyclin B1* gene. Mutation caused by CRISPR/Cas9 mRNA is a splice site mutation that presumably disrupted the production of the normal Cyclin B1 protein, required for the cell cycle progression.
CHAPTER V

DOUBLE MUTANT PHENOTYPE

Background

The Harpy Mutant Phenotype

*harpy (hrp)* is a Mendelian recessive mutation; early arrest mutants were isolated from the Tübingen screen (Kane et al., 1996). The *hrp* mutant phenotype is caused by mutation of the coding region of *early mitotic inhibitor1 (emi1)* also called RCA1 in *Drosophila*, resulting in premature stop codons. *hrp* is characterized by a very distinct morphological and cellular phenotype: abnormally “bumpy head”, shortened body axes, no apparent cell death, big nuclei due to lack of cell divisions that occur after 6 hours of development (Riley et al., 2010). During S and G2 phases the Anaphase Promoting Complex (APC/C), which promotes degradation of cyclins, is inhibited by Emi1 in order to accumulate Cyclin A and B and to prevent re-replication (Moshe et al., 2011). We would predicted that *hrp* would be epistatic to *spr* because Emi1 acts upstream of Cyclin B1.

The Zombie Mutant Phenotype

The *zombie* early arrest mutants were isolated from the Tübingen screen. *zombie* mutant embryos segregate as simple Mendelian recessives. The phenotype displays gross cellular abnormalities before morphological changes in
tissue and body shape are obvious (Kane et al., 1996). This phenotype is visible as early as the 4-somite stage: embryos tend to be opaque, cells are larger and rounded (Kane et al., 1996). Mutants with the zombie phenotype were mapped to linkage group 2 (T. Dams, unpublished) in the vicinity of the cdc20 gene. Sequencing data showed that zombie mutant embryos have a nonsense mutation in the cdc20 gene, resulting in a prematurely terminated protein and nonsense mediated degradation of cdc20 mRNA. As in spr mutant embryos, zombie mutant embryos have only maternal cdc20 mRNA supplies, which deplete after the midblastula transition (Kane lab, unpublished). Cdc20 activates the Anaphase Promoting Complex (APC), which in turn ubiquitinates Securin and releases Separase to degrade Cohesins that hold sister chromatids together during the metaphase-anaphase transition. Another important role of the APC/C is to degrade all the S and M cyclins (Zachariae & Nasmyth, 1999; Harper et al., 2002).

The cellular zombie mutant phenotype is characterized by arrest of the cell cycle during the metaphase/anaphase transition, as was shown with DAPI staining (Kane et al., 1996). This phenotype was confirmed by antibody staining for a mitosis marker (phosphorylated form of histone H3): metaphase/anaphase arrest can be seen as early as the 3-somite stage, and the number of cells arrested in mitosis is higher than in wild-type embryos (Kane lab, unpublished).

In this chapter I would like to concentrate on Cyclin B1, Emi1 and Cdc20 as essential cell cycle players, their interaction and possible outcomes for double mutant phenotypes. By this analysis, I would reveal how known cell cycle
proteins influence the progression of the cell cycle in zebrafish embryos during early development.

Results

Morphological and Cellular Phenotype of the specter/harpy Double Mutant

To examine the effect of hrp/spr double mutation, I crossed fish heterozygous for hrp with fish heterozygous for a spr mutation. I expected to see double mutant phenotype in the F2 in the ratio 9:3:3:1. In the F2 I got the following numbers: 296 wild type, 120 spr, 95 hrp, 41 spr/hrp. Morphologically I was able to distinguish 4 groups of embryos in the F2 generation. The first group, the biggest, was wild-type embryos; second – the spr mutant embryos; third – the hrp mutant embryos; and forth – double mutant embryos with aspects of the hrp and spr mutant phenotype.

The double mutant morphological phenotype had aspects of both parents: body length was like in the hrp mutant embryos, however, the somites were more organized and better formed, which resembled the spr mutant phenotype (Fig. 26, A-D). Double mutant embryos had extruded eyes like the hrp mutant embryos. Double mutants also had opaque head, which is not normal for the hrp mutant embryos, suggesting that these embryos might undergo apoptosis like the spr mutant embryos.

To examine the nuclear phenotype I performed DAPI staining of the same embryos at 20 hours (Fig. 26, A’-D’). Wild-type embryos had normal size and shape nuclei. spr mutant embryos had abnormal and enlarged nuclei with signs of apoptosis. hrp mutant embryos had very big nuclei, typical for this mutation.
Double mutant embryos had a heterogeneous population of nuclei: some nuclei were as big as in hrp mutant embryos, some had an abnormal shape and as big as in spr mutant embryos with signs of programmed death. Interestingly only in double mutant embryos I was able to clearly see cells in early mitosis; these mitotic stages were not easily observed in either single mutant.

Fig. 26 Characterization of harpy/specter double mutant embryos. (A-D), morphological phenotype at 20 hours, side view; (A'-D') nuclear phenotype (DAPI) at 20 hours; (A"-D") cellular phenotype (antibody staining for phosphorylated form of histone H3) at 24 hours side view, of wild-type, spr, hrp, hrp/spr double mutant embryos, respectively. Bar = 20μm.

Ongoing mitosis in the double mutant embryos suggested that the cell cycle progression is changed in these embryos. Therefore, I stained embryos for the phosphorylated form of histone H3 to analyze the cellular phenotype. The hrp mutant embryos do not have cells dividing; therefore, I assumed that if double mutant embryos would have cells in mitosis, then it would be an effect caused by
lack of Cyclin B1, because these cells might be arrested at this stage. The
double mutant embryos had some cells in mitosis, however fewer than in the spr
mutant embryos. Suggesting that spr is epistatic to hrp in terms of pH3 staining.

These data helped me to analyze how the cell cycle might progress when
zygotic Emi1 and Cyclin B1 are absent in mutant embryos. Emi1 is necessary for
Cyclin B accumulation during S phase. In general, the hrp mutant phenotype can
be distinguished earlier than the spr mutant phenotype (Railey et al., 2010).
Therefore, I hypothesize if there is a lack of maternal Emi1 earlier than cyclin B1,
then cells would undergo S phase, activate the APC/C complex and continue
resembling a normal phenotype until maternal Emi1 is depleted. As soon as
maternal supplies of Emi1 are depleted, cells cannot regulate the APC/C
complex, which leads to re-replication, observed in typical hrp mutant embryos.
The cell cycle is not affected in spr mutant embryos as it is in hrp mutant
embryos. This suggests, that there might be enough Cyclin B1 to ensure the
onset of mitosis. APC/C complex ensures degradation of Cyclin B, therefore,
double mutants cannot show a pure spr phenotype: possible supplies of Cyclin B
get degraded earlier by an activated APC/C complex, due to the absence of
Emi1.

Morphological and Cellular Phenotype of the Specter/Zombie Double Mutant

The zombie mutant embryos arrest at the metaphase-anaphase transition,
due to the lack of Cdc20. The spr mutant embryos delay in the cell cycle
progression and cells might get arrested before entering mitosis. In the spr/zom
double mutant embryos I hypothesize the *spr* phenotype to be epistatic, because Cyclin B1 acts earlier in the cell cycle. To check my hypothesis, I performed the same morphological, nuclear and cellular analysis, as described for the *spr/hrp* double mutant embryos (Fig. 27).

I got the following numbers after crossing the double mutant fish: 72 wild type, 36 *spr*, 26 *zom*, and 11 *spr/zom*. The *spr* mutant phenotype seemed to be epistatic by morphological characteristics compared to *zom* mutant embryos. The double mutant embryo did not have such a dark head like the *zom* mutant embryos did not have such a dark head, had longer body axes, and distinguishable somites (Fig. 27, A-D). The nuclear phenotype showed a lot of abnormal nuclei, apoptotic nuclei in double mutant embryos with a small group of cells in mitosis; this resembles the nuclear phenotype of the *spr* mutant embryos. I saw very few cells in mitosis in double mutant embryos; this was confirmed with phospho-histone H3 staining (Fig. 27, A"-D").

Double mutant embryos had more cells in mitosis, but at lower rates than in both *zom* mutant embryos and in wild-type embryos. Interestingly, enveloping layer cells (EVL), were positive for the pH3 mitotic marker, which does not occur as much in the *spr* mutant embryos.

The *zom* mutant phenotype acts earlier than the *spr* mutant phenotype; nonetheless, in this case, APC/C complex is not being activated, therefore it cannot interfere with Cyclin B1 that acts earlier. On the other hand, considering the fact that there are fewer cells arrested in mitosis in the double mutant embryos, it suggests that the *spr* mutation and Cyclin B1 depletion happen
sometime right after depletion of Cdc20, but not at the same time. In this case, most of the cells will be locked in S/G2/early M phase, with an apoptotic pattern which is a characteristic of the *spr* mutant phenotype.

Fig. 27 Characterization of *zombie/specter* double mutant embryos. (A-D), morphological phenotype at 15 hours, side view; (A'-D') nuclear phenotype (DAPI) at 20 hours; (A"-D") cellular phenotype (antibody staining for phosphorylated form of histone H3) at 15 hours side view, of wild-type, *spr*, *zom*, *zom/spr* double mutant embryos, respectively. Bar = 20µm.

Summary

I showed that the *spr* mutant phenotype is epistatic to both, *hrp* and *zom* in morphological phenotype. The double mutant phenotype resembles characteristics of both mutations: for example, in the *spr/hrp* double mutant
embryos, cells enter mitosis and show signs of cells death. In the \textit{spr/zom} double mutant embryos, there are more cells in mitosis, but they are not arrested at the metaphase-anaphase transition. Observed phenotypes might be explained by when the Cyclin B1 or Emi1 or Cdc20 are required and when they are depleted in different cell lineages. For instance, this might explain EVL cells arrested in metaphase-anaphase in the \textit{spr/zom} double mutant embryos. EVL cells finish their cell cycle with division 11. If the \textit{spr/zom} double mutant embryos run out of Cdc20 by that time, than EVL cells cannot exit mitosis.

The \textit{spr/hrp} double mutant embryos probably get arrested before cells can enter mitosis, with come cells entering M phase. Due to the lack of Cyclin B1, the cells cycle in these mutant embryos is also delayed, which might cause nuclear instability and apoptosis like in the \textit{spr} mutant embryos. These results indicate that the mutations in Cyclin B1, Emi1 and Cdc20 are disruptive for the normal progression of the cell cycle.
Based on identification of the mutant sequence and reproduction of the mutant phenotype by complementation test with a newly generated CRISPR mutation, we have shown that the spr mutant is caused by the mutation in the cyclin B1 gene. Here we discuss changes in the spr mutant phenotype during early zebrafish development.

Introducing the premature stop-codon in exon 2 of the cyclin B1 gene resulted in a dysfunctional gene, which led to changes in the morphological phenotype, similar to the morphological phenotype of the crash-and-burn (crb, or b-myb) zebrafish mutant embryos: darkening of the head and elevated apoptosis (Stern et al., 2005). B-myb is a transcription factor that regulates progression through the G1 to S phases of the cell cycle. b-myb knockdown in human cells, Drosophila and zebrafish leads to a reduction of cyclin B1 and cdk1 expression, thus regulating G2 and M phase of the cell cycle (Okada et al., 2002; Shepard et al., 2005; Zhu et al., 2004). spr mutant embryos also have high rates of cell death, however the nature of this cell death remains unclear. In the case of b-myb mutant embryos, their abnormal monopolar spindles originated from a single or unseparated chromosomes, unorganized and multipolar spindles which cause catastrophic mitosis, mitotic arrest and cell death (Shepard et al., 2005; Stern et al., 2005). This genomic instability might not be caused directly by the mutation in b-myb, since Cyclin B1 is the key player in spindle formation (Nam and Deursen,
Therefore, reduced Cyclin B1 expression in b-myb embryos might be the reason for such cellular phenotype.

However, there was no further and detailed histological or cellular description of the mutant lacking functional Cyclin B1 in the reviewed literature. We found that the lack of functional Cyclin B1 causes cells to acquire different cell cycle progression resulting in the majority of the cells appearing to be in S/G2 phase by 20 hours of development. According to our analysis of the mitotic cells, we hypothesize that cells seen in mitosis at the 7-somite stage are the cells that do not exit the cell cycle and might have a delayed and extended cell cycle progression if not get arrested, and no more cells enter mitosis after this stage. This would support the general importance of the Cyclin B1 for normal cell cycle progression (reviewed by Malumbres, 2001). Even low levels of nondegradable Cyclin B1 are sufficient to arrest cells at the metaphase-anaphase transition; therefore unless there is no Cyclin B1 in the cells during M phase, no cells can undergo mitosis (Chang et al., 2003). Enlarged nuclei in some cells of the spr mutant embryos, however, suggest that the spr mutant embryos undergo rounds of S phase without mitosis. However, with the obvious changes in morphology of the spr mutant embryos, there are no visible changes at the cellular level. One possible explanation for this, is that maternal Cyclin B1 can provide the embryo with enough levels of Cyclin B1. Once maternal Cyclin B1 is depleted, zygotic Cyclin B2 could compensate for Cyclin B1 and form a Cyclin B2/Cdk1 complex. It is possible that Cyclin B2 cannot fully replace Cyclin B1, and cells eventually undergo G2/M arrest of the cell cycle (Wu et al., 2010).
We found through *in situ* hybridization that there are fewer blood cells but they are bigger. This is a common characteristic observed in the cell cycle mutant embryos in the Kane lab. Blood precursors born in the gastrula undergo two more divisions during the segmentation stage in the wild-type embryos (Warga et al., 2009). Due to disruptions in the *spr* mutant embryos cell cycle, such as delay in cell cycle progression, possible arrest at G2/M phase and vast cell death, the last divisions of blood cell precursors (hemangioblast lineage) might not occur as fast as in wild type. In addition, endoreplication might explain why the blood cells are bigger in the mutant embryos.

Within the central nervous system of the *spr* mutant embryos, we found that there was fewer and bigger in size spinal interneurons, and sensory and motor neurons. Rohon-Beard (RB) cells are large sensory neurons that are specified at the neural plate stage, and are among the earliest specified neurons in the zebrafish (Korzh et al., 1993). We saw changes in the RB pattern possibly due to apoptosis, which normally occurs no earlier than at 3-4 days of development. Primary motor neurons undergo a final S phase between 9 and 16 hours. They have segmental organization that correlates with the pattern of adjacent axial muscles that they innervate (Eisen, 1992). However, the final cell cycle might be delayed in mutant embryos, giving rise to less motor neurons, a deficit that is compounded by the increase in apoptosis. The same pattern is seen for spinal interneurons. I have shown that the *spr* mutant embryos have a high number of apoptotic cells in the brain and along the body axes, as the embryo develops. These data suggest that cells born later might be affected by
both: first, cell cycle abnormalities, such as longer cell cycle progression and possible arrest at G2/M phase; second, by the increasing apoptosis that occurs predominantly in the nervous system, which explains the opaque head, and missing cells in the nervous system in the spr mutant embryos.

Histological analysis of muscle precursors showed delayed and weaker expression of MyoD. The segmentation clock coordinates the unsegmented presomitic mesoderm (PSM), the rhythm of somite formation (Venters et al., 2008). The Notch signaling pathway has long been known to be the first major player to regulate this signal (Kopan and Ilagan, 2009; Shin et al., 2007). Also, the hairy/E gene family of bHLH transcription regulators, such as her1 and her7 in zebrafish, and the Notch ligand Delta (Oates and Ho, 2002; Pasini et al., 2004) regulate somites formation. It was previously shown, that Notch signaling coordinates individual cells and synchronizes them (Zhou et al., 2010). We found that the somite borders in the spr mutant embryos are not disorganized. When Notch signaling fails, individual cells lose their synchrony, and changes in somite patterning occur (Lewis, 2003; Lewis et al., 2009). We showed that notch1b expression is delayed and weaker in the spr mutant embryos, which might explain delayed expression of MyoD. We did not identify whether there are any changes in her1 and her7 expression in the spr mutant embryos; however loss of the expression of these genes disrupts segmentation along the body axis (Kawamara et al. 2005; Oates and Ho, 2002).

Emi1 mutant embryos zebrafish also have abnormalities in somite morphology: they are hyper-epithelialized, have no internal mesenchymne and
have elongated epithelial border cells that fuse and create segments of different sizes (Zhang et al., 2008). In the absence of the normal cell cycle in the emi1 mutant embryos, the internal mesenchymal cells exit the core of the somite after initial boundary formation. Zhang et al. (2008) showed that disruption of the cell cycle in the emi1 mutant embryos does not interfere with the segmentation period for the segmentation clock function, but is necessary for the normal segmentation arrangement of epithelial borders and internal mesenchymal cells (Zhang et al., 2008). Non-functional Cyclin B1 seems to be important for the segmentation clock function as well as segmentation morphology, perhaps due to disruptions in the Notch signaling pathway.

Histological analysis of notch1b and deltaa expression showed that expression is delayed and weaker for markers of the Notch signaling pathway, and much weaker for Delta. Predominantly, our results reveal that some population of notch cells do not enter the Delta pathway. Therefore, we observe fewer neurons forming. Some Notch positive cells seem to enter the Delta pathway and give rise to neurons.

Through the lack of complementation, we confirmed that the spr mutant phenotype is a result of a disruption in the cyclin B1 gene. Mutagenized clones created by the CRISPR/Cas9 system injection contain a splice site mutation that disrupts normal splicing of the cyclin B1 gene. It is possible that the translated product in these clones is not sufficient for the normal function and maintenance of the MPF complex. Morphological and cellular analysis of the transheterozygotes revealed characteristics of typical spr mutant embryos. This
suggests that the introduced mutation indeed gave rise to a non-functional Cyclin B1 product.

In summary, we have shown that the \textit{spr} mutation is a result of a mutation in the \textit{cyclin B1} gene that disrupts normal progression of the cell through the cell cycle causing morphological and cellular changes. By analyzing cell cycle mutant embryos we can reveal how cell cycle progression determines early development in not only zebrafish embryos, but in other organisms, since the cell cycle mechanism is conserved in many live systems.
CHAPTER VII

METHODS

Zebrafish Strains

The spr tu21 allele was isolated in a large-scale mutagenesis screen (Haffter et al., 1996). Embryos produced by natural spawning of heterozygotes were initially kept in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$ 2H$_2$O, 0.33 mM MgSO$_4$ 7H$_2$O) (Mullins et al., 1994). Manually dechorinated embryos were kept in Danieu’s media (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO$_4$ 7H$_2$O, 0.6 mM Ca(NO$_3$)$_2$, 5 mM HEPES) (Driever et al., 1998), incubated at 25°C to 33°C. For histological analysis embryos were fixed in 4% paraformaldehyde in 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$ 7H$_2$O, 1.4 mM KH$_2$PO$_4$).

To produce heterozygous fish for spr carrying the Dual FUCCI reporter (Bouldin et al., 2014), fish heterozygous for spr were outcrossed to the Dual FUCCI line, selecting individuals for the fluorescent reporter at 3 days of development. After fish were grown up, they were incrossed, selecting individuals heterozygous for spr and carrying Dual FUCCI reporter as viewed under a dissection microscope using the Texas Red filter.

spr/hrp and spr/zom double mutant embryos were produced by mating fish heterozygous for zom or hrp to fish heterozygous for the spr mutant allele. Embryos from this cross were grown and ¼ from each cross was identified as double heterozygous. Analysis of double mutant embryos was performed on G2.
Whole Mount In Situ Hybridization and Antibody Staining

Antibody staining was performed as described in Riley et al. (1999) and Phillips et al. (2006) using commercial antibodies against rabbit phospho-histone H3 (1:200) and rabbit phospho-caspase-3 (1:200). RNA in situ hybridization was carried out as described (Thisse et al., 1993) using riboprobes against hhbe, lplast, mpx, pax2a, islet 1, notch1b, deltaa, MyoD and krox20. For the in situ probe used the purple Nitro-Blue tetrazolium Chloride/Bromo Chloro Indolophosphate p-Toluidine chromogen product. Cleared in 70% glycerol and photographed with Sony F-707 Digital Camera.

DAPI Staining

Embryos were fixed and stained simultaneously in a solution of 4% PFA, Dimethyl sulfoxide and 0.01% DAPI. Embryos were dissociated by gently crushing between coverslips before photographing. Analyzed under using Zeiss Axiophot II equipped with a CDC-300-RC camera (DAGE MTI).

mRNA Injection

Embryos manually dechorinated in Petri dishes with 2% Bacto agar in 100% Danieau’s media (Driever et al., 1998) (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄ 7H₂O, 0.6 mM Ca(NO₃)₂, 5 mM HEPES) were injected at 1-8 cell stage with wild-type tailed cyclin B1 mRNA (Rachel Warga, unpublished). Control
embryos were injected with equivalent amounts of *kaede* mRNA. After the injection, Penicillin Streptomycin (1:100) was added into Danieau's media; embryos were incubated at 33°C.

CRISPR/Cas9 Mutagenesis

*Creating Cas9 mRNA and sgRNA*

Cas9 mRNA and single guide mRNA were created as described in Hwang et al. (2013). Briefly, Cas9 nuclease expression plasmid DNA encoding the Cas9 nuclease was amplified from the pMJ806 vector (Addgene Plasmid #39312) by PCR using the following primers:

OMM704: 5' ataagaatgcggccgctaatagactcactataggagagccgccaccATGGATAAGAAATACTCAATAGGCTTAG 3'

OMM705: 5' gtacataccggtcatcctgcagctccaccgctcgagactttcctcttcttgagaaccGT CACCTCCTAGCTGAC 3'.

The PCR product was digested with the NotI and AgeI restriction enzymes and inserted into plasmid pMLM651.

Single guide RNA (sgRNA) expression vector Vector pDR274RC harboring a T7 promoter positioned upstream of a partial guide RNA sequence was generated by commercial DNA synthesis (Integrated DNA Technologies). To create plasmids encoding sgRNAs bearing 20 nt targeting sequences, we digested with BsaI restriction enzyme, then cloned a pair of designed and annealed oligonucleotides into the vector backbone. The annealed
oligonucleotides have overhangs that are compatible with directional cloning into the BsaI-digested pDR274 vector.

To identify potential target sites for our sgRNA:Cas9 system, we used web-based ZiFit Targeter software.

To produce sgRNA and Cas9 mRNA, sgRNAs were transcribed using the DraI-digested gRNA expression vectors as templates and the MAXIscript T7 kit (Life Technologies). The Cas9 mRNA was transcribed using Pmel-digested Cas9 expression vector and the mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies). Following completion of transcription, the poly(A) tailing reaction and DNase I treatment were performed according to the manufacturer’s instructions for the Cas9-encoding mRNA. For sgRNA: to purify the plasmid we used QIAGEN plasmid midi kit, restriction digest using DRA-I, to purify DRA-I DNA we used QUAquick PCR purification Kit protocol. sgRNA was transcribed using MAXIscript T7 kit (Life Technologies).

Zebrafish Mutagenesis

Embryos derived from a wild-type parent and a heterozygote for spr were co-injected at 1-2 cell-stage with sgRNA and Cas9 mRNA. Further analysis was done using antibody staining against phospho-cas-3.

Embryos derived from zebrafish with wild-type background were co-injected at 1-2 cell-stage with sgRNA and Cas9 mRNA. These embryos were grown up and crossed heterozygotes for spr carrying Dual FUCCI transgene.
PCR Amplification

Target locus was amplified by PCR from pooled genomic DNA of 16 mutant and wild-type embryos derived from the CRISPR/Cas9 mutagenized fish crossed to fish heterozygous for spr mutant allele. Primers used:
F: 5’ acctaatggttaagccac 3’
R: 5‘ ccaccgtcatacagtaataac 3’
Reamplified PCR product was sent to University of Michigan Sequencing Core.

Time-Lapse and Data Analysis

For in vivo observations of embryos carrying Dual FUCCI transgene, embryos were mounted and recorded in multi-plane as previously described (Warga and Kane, 2003) using Nikon C2 confocal microscope. Embryos were mounted during gastrulation in 0.2% agarose between coverslips and sealed with Vaseline. Recorded using 60x water and oil emersion lens at 10 to 15 planes using 5 to 10 µm z-spacing for periods of 2 to 6 hours. In a single experiment, groups of 6 to 8 individuals were recorded using a computer controlled stage, each location was recorded every 5 to 10 minutes. Temperature in the room was maintained around 30°C. By the end of recording, embryos were carefully removed from between the coverslips, placed intro Danieu’s media with Penicillin Streptomycin (1:100), incubated at 33°C, and their phenotypes were determined the next day. Older embryos with distinct phenotypes were sorted by the
phenotype before mounting. Afterward, the recordings were analyzed using NIS Elements Viewer 4.20 Software and Adobe Photoshop.

Statistical Analysis

To determine statistical significance of number of apoptotic cells and cells in mitosis standard deviation and standard errors were used.
BIBLIOGRAPHY


APPENDICES

A. Individual IACUC Approval

Date: March 12, 2015
To: Don Kane, Principal Investigator
    Rachel Warga, Co-Principal Investigator
From: Lisa Baker, Chair
Re: IACUC Protocol Number 13-03-02

This letter will serve as confirmation that the change to your research project “Zebrafish Breeding Colony” requested in your memo received February 27, 2015 (to add student investigator Tanya Petrachikova and Peyton Johnston) has been approved by the Institutional Animal Care and Use Committee.

Approval Termination: March 11, 2016
B. Approval Letter from IACUC

WESTERN MICHIGAN UNIVERSITY
Institutional Animal Care and Use Committee
ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

PROJECT OR COURSE TITLE: Zebrasfish Breeding Colony
IACUC Protocol Number: IACUC Protocol 13-03-02
Date of Review Request: 25 February 2015 Date of Last Approval: 11 March 2014
Purpose of project (select one): Teaching Research Other (specify): Breeding Colony

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: Don Kane Title: Associate Professor
Department: BIOS Electronic Mail Address: don.kane@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Rachel Warga Title: Adjunct Faculty
Department: BIOS Electronic Mail Address: rachel.warga@wmich.edu

1. The research, as approved by the IACUC, is completed:
   Yes (Continue with items 4-5 below.)  x No (Continue with items 2-3 below.)
   If the answer to any of the following questions (items 2-4) is “Yes,” please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? Yes  x No

3. Have there been any new findings or publications relative to this research that require you to alter your study? Yes  x No
   Describe the sources used to determine the availability of new findings or publications:
   No search conducted (Please provide a justification on an attached sheet.)
   Animal Welfare Information Center (AWIC)
   Search of literature databases (select all applicable)
   AGRICOLA
   Current Research Information Service (CRIS)
   Biological Abstracts
   x Medline
   Other (please specify):
   Date of search: 1 January 2015 Years covered by the search: 1965-2015
   Key words: key words: gastulation, vertebrate, aquaculture, mutant, genetics
   Additional search strategy narrative:

4. Are there any adverse events, in terms of animal well-being, or mortalities to report as a result of this research? Yes  x No
   Cumulative number of mortalities:

5. Animal usage: Number of animals used during this quarter (3 months): 250
   Current cumulative number of animals used to date: 6000

Principal Investigator/Faculty Advisor Signature: 26 Feb 15
Co-Principal or Student Investigator Signature: 26 Mar 15

IACUC REVIEW AND APPROVAL
Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature.

IACUC Chair Signature: 31 Dec 15

Revised 01/2013 WMU IACUC ALL OTHER COPIES OBSOLETE.