Is the zebrafish zombie mutant caused by a mutation in CDC20?

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Is the zebrafish *zombie* mutant caused by a mutation in *cdc20*?

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# TABLE OF CONTENTS

Acknowledgements .................................................................................. 2  
List of Figures .......................................................................................... 4  
Abstract .................................................................................................. 5  
Introduction .............................................................................................. 6  
Materials and Methods .......................................................................... 19  
Results ..................................................................................................... 23  
Discussion ................................................................................................. 30  
Future Directions ..................................................................................... 35  
Conclusion ................................................................................................ 38  
References ................................................................................................. 39
LIST OF FIGURES

Figure 1. Constructed Representation of cell cycle phases………………………… 7
Figure 2. Constructed Representation of APC/C<sup>cdc20</sup> in the cell cycle………………… 12
Figure 3. Schematic Representation of the CRISPR/Cas9 system………………………….. 15
Figure 4. Morphological and cellular characterization of zombie mutant………………… 18
Figure 5. Zombie mutant cells arrest in mitosis post gastrulation…………………………… 25
Figure 6. CDC20 mRNA injection does not rescue zombie mutants……………………… 27
Figure 7. CRISPR/Cas9 mRNA injected embryos have a higher count of cells in metaphase plate and metaphase……………………………………………………………………… 29
Figure 8. Different amounts of injected CDC20 mRNA have no affect on wild-type embryos………………………………………………………………………………………….. 32
Figure 9. Constructed Representation of the Dual FUCCI…………………………………… 37
ABSTRACT

The zombie mutant was identified as an early arrest mutant, stopping in development around the 10-somite stage (14 hours of development). Further inspection revealed that this mutant was a cell cycle mutant and cells in the mutant arrest during metaphase as early as the 5-somite stage (11.6 hours of development). A similar phenotype is seen in the Drosophila melanogaster cell cycle gene fizzy, known to be to be a homolog of the Saccharomyces cerevisiae gene, cell division cycle 20 (cdc20). CDC20 is an activator protein of the anaphase promoting complex/cyclosome (APC/C), an ubiquitin E3 ligase that is responsible for cell cycle progression. In previous unpublished studies, zombie was mapped to Chromosome 2 and was fine mapped to the vicinity of cdc20, suggesting mutant cdc20 as a candidate gene. However, failure to rescue the zombie mutant phenotype using Xenopus cdc20mRNA was unsuccessful. Here, we investigate whether cdc20 is the gene mutated in zombie via two experimental assays: rescuing the zombie mutant phenotype with wild-type zebrafish cdc20mRNA and mutagenizing the wild-type cdc20 chromosome to produce the zombie mutant phenotype by using the CRISPR/Cas 9 system. Antibody staining for phosphorylated histone H3, showed an increase in mitotic cells in zombie mutants compared to wild-type siblings as early as the 5-somite stage. Unfortunately, CDC20 injected zombie embryos showed no phenotypic rescue of the mutant phenotype. In addition, mutagenesis of the wild-type cdc20 chromosome showed a phenocopy of the zombie mutant phenotype, convincing us that zombie is a mutation of CDC20.
INTRODUCTION

Eukaryotic Cell Cycle

The cell cycle consists of four phases, Gap 1 phase (G1), Synthesis Phase (S), Gap 2 phase (G2) and Mitosis (M). During the G1 phase, a cell is metabolically active. During the S phase, DNA replication occurs. During the G2 phase, the cell continues to grow but ensures DNA replication from the previous, S phase, was done properly, preparing for mitosis. During the M phase, cell division occurs, resulting in two daughter cells. Cells that no longer divide enter a dormant phase called G0.

Mitosis (M phase) consists of six phases. During prophase chromosomes condense into a globular shape. In addition, the mitotic spindle develops and the nuclear envelope breaks down. During prometaphase, microtubules from the mitotic spindle attach to the kinetochores of condensed chromosome. During metaphase, the chromosomes align along the metaphase plate in the center of the cell. During anaphase, sister chromatid are pulled apart and migrate to opposite poles on the mitotic spindle. During telophase, two daughter nuclei form in their perspective daughter cells and the chromosomes de-condense. Mitosis ends with cytokinesis, where the parental cell in split into two daughter cells (Lodish et al., 2000, Copper et al. 2000) (Figure 1).
Figure 1. Constructed representation of cell cycle phases.
The cell cycle contains four major phases: G\textsubscript{1} phase, S phase, G\textsubscript{2} phase and Mitosis. Cells that no longer divide go into the dormant G\textsubscript{0} phase. Within mitosis, there are five phases (Prophase, Metaphase, Anaphase, Telophase and Cytokinesis (not shown)) that are responsible for cellular division.
**Cell Cycle Checkpoints**

Cell cycle checkpoints are regulator controllers that ensure proper DNA replication and chromosome segregation occur for cell cycle progression. There are three checkpoints (G$_1$ checkpoint, G$_2$ checkpoint and Mitosis checkpoint) that regulate the cell cycle. The G$_1$/S checkpoint senses cell growth and DNA damage. The G$_2$ checkpoint senses unreplicated DNA. The Mitosis checkpoint regulates the alignment of chromosomes on the mitotic spindle (Lodish *et al.*, 2000, Foster *et al.* 2010) Damage DNA or abnormal chromosome segregation sensed by the cell cycle checkpoints could result in cellular apoptosis or cellular arrest.

**Zebrafish Development**

The zebrafish, Danio rerio is a model organism for embryonic development due to its rapid development, optical transparency, ability to lay around 200 eggs a day and accessibility to its genome (Bopp *et al.* 2006). In this thesis embryonic development terminology is from the Kimmel lab (Kimmel *et al.*, 1995). Zebrafish embryonic development is broken up into multiple periods, from the zygotic period (0-3/4h) to the hatching period (48-72h). The zygotic period begins after the fertilization of an egg. It lasts in this period for about 40 minutes before the first cleavage occurs. During the zygotic period, the egg is considered in a one-cell stage, in which the chorion swells as cytoplasmic movements are activated within 10 minutes.
During the cleavage period (3/4-2 ¼ h), blastomeres divide at 15 minute intervals. There are six cleavages that comprise this period, that occur at regular orientation. Blastomeres proceed through their cell cycles synchronously. The long axis of the ellipsoid predicts the orientation of the following cleavage.

During the blastula period (2 ¼ - 5 ¼ h), the embryo enters the midblastula transition, the yolk syncytial layer forms and epiboly begins. Epiboly continues through the gastrulation period. The midblastula transition begins at the 512-cell stage and occurs as each cell reaches a distinct nuclear-cytoplasmic ratio (Kane and Kimmel et al., 1993). This is the first time where some cells could be in the G1, S or G2 phase while others are in mitosis. Blastula period, near the end of epiboly begins the thinning and spreading of a percentage of the yolk cells that is covered by the blastoderm. The blastoderm consists of a flattened EVL monolayer and a deep cell multilayer. The blastula period ends with the 40% epiboly stage (Kimmel et al., 1995).

During the gastrula period (5 ¼ -10 h), epiboly continues as well as morphogenetic cell movements that produce primary germ layers and the embryonic axis (Kimmel et al., 1995). The gastrula period ends with the Bud Stage (10h), displaying the tail bud as the yolk of the embryo disappears, signaling the end of epiboly. Relevant to this study, the gastrula period is the time when many cell cycle genes begin to run out of maternal gene product (Kimmel et al., 1995).

During the segmentation period (10-24h), primary organs begin to form, somites appear, the tail bud becomes prominent, the embryo elongates, the first cells differentiate and the first body movements appear (Kimmel et al., 1995).
The final analyzed period was the pharyngula period (24-48h). During this period, the notochord develops, somites extend to the end of post-anal tail and the head straightens out (Kimmel et al., 1995). One significant observation of this period was muscular contractions. These contractions occur around the 17-somite stage, progressively becoming stronger, more coordinated and more frequent as they coordinate with the ingrowth of axons from primary motor neurons. By this period, cells have differentiated and some cells no longer go through their cell cycles (Hanneman and Westerfield et al., 1989, Kimmel et al., 1995)

**fizzy**: The *Drosophila melanogaster* homolog gene of *S. cerevisiae* cell cycle gene, Cell Division Cycle 20

Due to its ability to easily mutate and accuracy for genetic mapping, *Drosophila melanogaster*, the fruit fly has been used in laboratories to study development (Twyman et al. 2002).

In previous studies, the D. melanogaster cell cycle gene *fizzy* was found to be homologous to the *Saccharomyces cerevisiae* cell cycle gene, Cell Division Cycle 20 (*cdc20*), which is also homologous to mammalian *p55^cdc* gene. All three genes share similar WD-40 repeat domains in their carboxyl halves (Dawson et al., 1993). WD-domains are a series of approximately 40 amino acids that end in a Tryptophan-Aspartate or WD, using the single letter amino acid abbreviations (Manchado et al., 2010). The
fizzy mutation causes cellular arrest that shows an excess accumulation of tubulin in the spindle microtubules that prevents cells from exiting mitosis. Therefore, fizzy is required for cells to exit mitosis (Dawson et al., 1995).

Previous studies showed that stage 14 wild type embryos had fewer cells stained positively for either cyclin A or B, whereas, stage 14 fizzy mutants had more cells stained positively for cyclin A and B. The inability in the fizzy mutant to degrade cyclins A and B appears to cause cellular arrest (Dawson et al., 1995).

Cell Division Cycle 20 (CDC20): An activator protein of the anaphase promoting complex/cyclosome (APC/C)

CDC20 is an activator protein of the anaphase promoting complex/cyclosome (APC/C), a multi subunit ubiquitin-protein E3 ligase that targets proteins for degradation by the 26S proteasome. Activity of APC/C depends on cofactors CDC20 and CDH1 (Manchado et al., 2010). CDC20 regulates the metaphase- anaphase transition, a checkpoint during mitosis in which the cell monitors spindle fibers before inactivating the maturation promoting factor and initiating anaphase (Tavormina et al., 1998, Dawson et al., 1993). Although the binding of CDC20 to APC/C is unknown, APC/C\textsuperscript{CDC20} is a controlled target of the spindle checkpoint (Eytan et al., 2006). The spindle checkpoint doesn’t initiate the metaphase-anaphase transition until proper development of the mitotic spindle and bipolar attachment of each chromosome occurs (Wells et al., 1996) (Figure 2).
Cell Division Cycle 20 (CDC20) protein binds to the Anaphase Promoting Complex (APC), activating APC/C\textsuperscript{Cdc20}. Activated APC/C\textsuperscript{Cdc20} separates the securin-separase protein complex to initiate the onset of Anaphase.

**Figure 2. Constructed representation of APC/C\textsuperscript{Cdc20} in the cell cycle.**
Studies have shown that with the spindle checkpoint initiated in arrested cells, CDC20 might override spindle checkpoint and continue cell cycle progression (Geiser et al., 1997). Checkpoint complexes that contain BubR1 (Mad 3), Bub 3, Mad 2 and CDC20 directly bind to APC/C and inhibit its ligase activity (Tang et al., 2001, Yu et al., 2002). In prometaphase, the spindle checkpoint protects cyclin B1 and securin from the APC/C until all chromosomes reach bi-orientation. Release of the checkpoint at metaphase causes separase to cleave cohesion of sister chromatids which allow the sister chromatids to initiate segregation and anaphase (Hyun et al., 2013, Yu et al., 2002, Clijsters et al., 2013) (Figure 2).

Previous studies have shown that in addition to CDC20 being an activator protein of APC/C, it plays an important role in the development and progression of human cancers (Smolders et al. 2011).

**CRISPR/Cas 9 system: genome editing in zebrafish**

The CRISPR/Cas (clustered regularly interspaced palindromic repeats/ CRISPR-associated) system is a bacterial defense mechanism against invasion of foreign nucleic
acids derived from bacteriophages. Other organisms, such as zebrafish, utilize the CRISPR/Cas 9 system as a genome-editing tool to mutagenize wild-type alleles to mimic mutations (Hwang et al., 2013). This system is a two-component system that consists of a single guide RNA (sgRNA), that consists of a complimentary sequence to its target site, that guides the Cas9 nuclease to the target site. The Cas 9 nuclease then binds to the target site and cleaves the foreign DNA (target site), resulting in a mistake in the DNA sequence. Without template DNA to correct the Cas9 induced error, the DNA sequence encodes a mutation (Figure 3).
Figure 3. Schematic Representation of the CRISPR/Cas9 system.
The CRISPR/Cas9 system is a two-component system that consists of sgRNA and the Cas9 nuclease. gRNA, guides Cas9 to target site; GREEN in order to make nicks in the target site sequence that results in a mutation.
**zombie: an early arrest mutation in Danio rerio, the zebrafish**

The zombie mutant is a lethal recessive allele that was isolated from the Tübingen screen in which embryos arrest in the first 24 hours of zebrafish development (Kane et al., 1996). The zombie mutant phenotype becomes visible around the 5 somite stage (11 hours post fertilization), in which embryos are optically less transparent and large round cells appear in all tissues. The zombie mutant was distinguished during segmentation by the presence of dark regions in the embryo caused by the appearance of spherical cells, in which some appeared dead. Zombie mutant embryos developed small somites consisting of large cells (Kane et al., 1996) (Figure 4B-B’).

In zombie mutants, round cells contained visible plate-like structure, presumably mitotic nuclei that were not normally visible in normal dividing cells (Kane and Kimmel et al., 1993). The cell autonomy of zombie was assayed using cell transplantations. Within these experiments, mutant cells were transplanted into wild-type hosts, and in more than 90% of the transplantations, lysis of the mutant cells occurred, suggesting zombie was cell autonomous, and not dependent on external factors (Kane et al., 1996).

DAPI staining of zombie mutant embryos at 18-22 hours, showed a high proportion of cells in metaphase or early anaphase (Kane et al., 1996) (Figure 4E). In previous studies, looking at cells in mitosis, it was found that already by the 3-somite stage, zombie mutants had more mitotic cells than wild-type embryos (E. Morgan Thesis). Zombie was mapped to Chromosome 2 and then fine mapped nearby cdc20 with micro-satellite zmarkers, showing the zombie mutant and cdc20 are close together (D.
Museav Honors Thesis and Kane, unpublished). Sequencing data showed a non-sense mutation in exon 7 of the zombie mutant cdc20 gene producing what is believed to be a non-functional CDC20 protein (T. Dams Honors Thesis).
Figure 4. Morphological and cellular characterization of *zombie* mutant.
(A) Side view of wild-type embryo at 15 hours and (A’) 24 hours. (B) Side view zombie mutant at 15 hours and (B’) 24 hours. (C) DIC view of zombie round cells; normal nuclei are indicated with an arrow while mitotic plate is indicated with an arrowhead. (D) DAPI staining shows wild-type cells have fewer nuclei in metaphase than (E) zombie cells. Scale bar is shown at 20μm for C and 50μm for D and E. Figure adapted from Kane et al. (1996).
METHODS AND MATERIALS

*Krox20 in situ* Hybridization

Stored dechondrinated 5 somite embryos were rehydrated in 200µl 3:1, 1:1 and 1:3 washes of Methanol in Phosphate Buffered Saline with Tween 20 (PBST; 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.76mM KH₂PO₄, 0.1% Tween 20) solutions for five minutes. Last rehydration wash was in 100% PBST for five minutes. Embryos were digested in 1000µl of 1:2000 Proteinase K(10 µg/ml) for 2 minutes and re-fixed in 4% paraformaldehyde (4% PFA; Sigma-Aldrich, St. Louis, MO) for 1 hour and 30 minutes. Embryos were washed 5 times in RNase free PBST for 5 minutes. Embryos were prehybridized in 200µl of hybridization Plus mix (60% Formamide, 5X SSC, 50µg/ml Heparin, 500µg/ml t(torula)RNA, 0.1% Tween 20, 1M citric acid to pH 6.0 and sterile dH₂O) in a 70°C water bath (VWR Scientific Inc., Cornelius, OR) for 2 hours and 30 minutes. Embryos were hybridized in 200µl of hybridization mix containing 200ng *Krox20* antisense RNA probe overnight in at 4°C. Embryos were washed quickly in 200µl of 100% hybridization minus mix (60% Formamide, 5X SSC, 0.1% Tween 20, 1M citric acid to pH 6.0 and sterile dH₂O) in 70°C. Embryos were washed three times in 200µl of RNase free 3:1, 1:1 and 1:3 Hybridization minus in 2X SSC at 70°C for 15 minutes each. Embryos were washed in RNase free 2X SSC at 70°C for 15 minutes before washing in RNase free 0.2X SSC twice for 30 minutes at 70°C. Embryos were washed in 200µl of 3:1, 1:1 and 1:3 RNase free 0.2X SSC in PBST at room temperature for 10 minutes each. Embryos were placed in 500µl of an Antibody Block Solution (1X PBST, 2% goat serum, 2mg/ml BSA) rocking at room temperature for two hours.
Antibody block solution was discarded and embryos were placed in 500µl 1:5000 anti-DIG dilution in 1X PBST, 2% goat serum and 2mg/ml BSA overnight, rocking at 4°C. Embryos were washed 3 times for an hour in PBST and 4 times for ten minutes each in staining buffer solution (100mM Tris HCL pH 9.5, 50mM MgCl₂, 100mM NaCl, and 0.1% Tween 20). Embryos were wrapped in aluminum foil while stained in staining solution (50mg/ml NBT, 50mg/ml BCIP and staining buffer) for 1 hour and 30 minutes at room temperature and monitored on a Zeiss Stemi 1000 microscope (Osterreich). Staining reaction was stopped by washing embryos in stop solution (1X PBS pH 5.5, 1mM EDTA) and PBST for 2 hours and 30 minutes.

**pH3 Antibody Staining**

(Skip if you ran krox20 in situ hybridization) Dechorinated embryos were fixed in 4% PFA for two hours. 4% PFA was discarded and embryos were washed in PBST three times for ten minutes each. Embryos were permeabilized in dH₂O for 3-5 minutes and ice cold acetone for 3-5 minutes. Embryos were placed in 500µl of Antibody Block Solution (1X PBST, 2% goat serum, 1% DMSO) at room temperature for two hours. Antibody Block Solution was discarded and embryos were placed in a 48-well plate. Embryos were rocked in 200µl of anti-phospho histone H3 rabbit primary antibody (1:500 PBST Santa Cruz), rocking, for 5 hours at room temperature. Primary antibody was restored at 4°C and embryos were washed in PBST for 3 hours at room temperature. PBST was removed and 200µl goat anti rabbit peroxidase secondary antibody (1:1000 PBST; Vector Laboratories) was added to embryos in well plate, rocking, overnight at 4°C. Secondary antibody was discarded and embryos were washed in PBST for 3 hours at room
temperature. Embryos were then stained in a glass spot plate in aluminum foil with Vector SG Sk-4700 Peroxidase Substrate Kit (Vector Labs, Burlingame, CA) while rocking for 3 minutes, before analyzing on a Zeiss Stemi 1000 microscope. Staining was stopped with multiple PBST washes at room temperature and stored in 70% Glycerol (Mallinckrodt Baker, Inc., Philipsburg, NJ) from the light at 4°C. Positive nuclei staining was counted on a Nikon compound scope and photographed as whole mounts with a Cyber-shot 5.0 mega pixel Sony digital camera.

**Wild-type cdc20mRNA Injections**

*Zombie* carriers for CDC20 injections were placed in mating boxes and embryos were collected at fertilization in different clutches. Embryos were dechorinated in a Bacto™ agar (Becton, Dickinson and Company) coated petri dish. Embryos from *zombie* heterozygotes were injected between 1 and 4 cell stage with wild-type cdc20mRNA (50-260pg) while remaining embryos from the same clutch were not injected as the control. At the 5-somite stage, embryos were fixed in 4% PFA, rocking, for two hours at room temperature. 4% PFA was discarded and krox20 in situ hybridization staining and anti-phospho- histone- H3 antibody staining (pH3) was performed on all embryos as previously described.
**CRISPR/Cas 9 mRNA Injections**

Zombie carriers for CRISPR/Cas 9 mRNA injections were placed in mating boxes and embryos were collected at fertilization. Embryos were dechorinated in a Bacto™ agar (Becton, Dickinson and Company) coated petri dish. Embryos from zombie heterozygotes were injected between 2 and 4 cell stage with CRISPR/Cas 9 mRNA (60µM-100µM), while remaining embryos from the same clutch were not injected as the control. At the 10-somite stage, embryos were fixed in 4% PFA while rocking for two hours at room temperature. 4% PFA was discarded. pH3 staining was performed on all embryos as previously described.

**Statistical Analysis**

Independent T-tests with 0.05 as the limit for significance were used for analysis tests on embryo results.
RESULTS

To falsify the hypothesis that zombie is a mutation of CDC20, we conducted two experimental assays: 1) rescuing the zombie mutant phenotype using various of concentrations wild-type cdc20mRNA and 2) mutagenizing the wild-type cdc20 chromosome to produce zombie mutant cells using the CRISPR/Cas 9 system.

Zombie mutants have a higher mitotic cell count than wild type embryos

Previous studies had shown that as early as the 3-somite stage, zombie mutants had more mitotic cells than wild-type embryos (Morgan, unpublished). However, we repeated this experiment as a control for the zombie mutant phenotype at the 5-somite, 10-somite and 15-somite stages. In these experiments embryos were collected from a cross of zombie heterozygous carriers, therefore, 25% of the embryos are expected to have the zombie phenotype. krox20 in situ hybridizations were used to mark a reproducible area in the hindbrain region (rhombomeres 3 and 5) to conduct cell counts followed by staining with the phospho-histone-H3 antibody to visualize mitotic cells (Giudicelli et al. 2001). I found that at the 5-somite (Figure A-B), 10-somite (Figure C-D) and 15-somite
(Figure E-F) stages, there is a phenotypic difference in mitotic cells between wild-type and zombie embryos.

In Figure 5-G, there is a break (difference of 12 mitotic cells) between wild-type and zombie embryos. 28% of embryos had a high proportion of mitotic cells, presumably zombie embryos at the 5-somite stage. In Figure 5-H, there is a break (difference of 9 mitotic cells) between wild-type and zombie embryos. 15.15% of embryos had a high proportion of mitotic cells, presumably zombie embryos at the 10-somite stage. In Figure 5-I, there is a break (difference of 6 mitotic cells) between wild-type and zombie embryos. 15.6% of embryos had a high proportion of mitotic cells, presumably zombie embryos at the 15-somite stage. Figure 5-J shows that there was a significant difference (p=0.011) between the positive nuclei averages between wild-type and zombie mutants for the 5-somite, 10-somite and 15-somite stages. In summary, these results are in agreement with the earlier studies that zombie mutants have many more mitotic cells.
Figure 5. Zombie mutant cells arrest in mitosis post gastrulation.

(A-F) Embryos shown in dorsal view oriented with Krox20 in situ hybridization. Mitotic cells expressed by pH3 antibody staining. (A-B) Representative wild-type (n=23) and mutant (n=9) sibling at 5-somite stage. (C-D) Representative wild-type (n=27) and mutant (n=5) sibling at 10-somite stage. (E-F) Representative wild-type (n=28) and mutant (n=5) sibling at 15-somite stage. (G-I) Positive nuclei count between Krox20 stripes at (G) 5-somite, (H) 10-somite, (I) 15-somite stage. Boxes indicate presumed zombie mutants based on positive pH3 staining. (J) Comparative positive nuclei averages between wild-type and mutants for 5-somite, 10-somite and 15-somite stage (p=0.011)
**CDC20 Injected Embryos Showed No Phenotypic Rescue**

Embryos were collected from a cross of zombie heterozygous carriers, making 25% of embryos zombie mutants. Wild-type embryos injected with CDC20 mRNA were not affected by injection (Figure 6A-B). Zombie embryos injected with Kaede, a green fluorescent protein, as a control displayed normal zombie phenotype (Figure 6C).

Prior to injecting embryos with a fixed amount, Dr. Rachel Warga injected different amounts of CDC20 mRNA in wild-type embryos to see if it affected the wild-type embryos. Dr. Rachel Warga injected 26pg, 50pg, 52pg and 260pg of CDC20 mRNA in wild-type embryos. The different amounts of injected CDC20 mRNA did not have an affect on the wild-type embryos, so 260pg CDC20 mRNA was injected in zombie embryos (Figure 8A-E). Zombie embryos injected with 260 pg CDC20 mRNA showed no rescue (Figure 6D). There was no statistical difference in pH3 positive nuclei was seen between Kaede and CDC20 injected embryos (Kaede control n=41; CDC20 n=25) (p=0.81) at 5-somite stage (Figure 6E-F). There was no significant difference in pH3 positive nuclei seen between Non-Kaede un-injected control and CDC20 injected embryos at the 10-somite stage (Non-Kaede control n=32; CDC20 n=11) (Figure 6G-H). In summary, injected CDC20 mRNA does not rescue the zombie mutant phenotype.
Figure 6. CDC20 mRNA injection does not rescue zombie mutants.
(B) Wild-type embryos injected with CDC20 mRNA were not affected by injection.
(D) Zombie embryos injected with CDC20 showed no rescue. (E-F) No statistical difference in pH3 positive nuclei was seen between Kaeda control and CDC20 injected embryos. Kaeda control n=41; CDC20 n=25 (p=0.81) at 5-somite stage. (G-H) No significant difference in pH3 positive nuclei was seen between Non-Kaeda control and CDC20 injected embryos. (C) Non-Kaeda control n=32; CDC20 n=11 at 10-somite stage.
Wild-type *cdc20* allele can be mutagenized by CRISPR/Cas 9 mRNA

Embryos were collected from a cross of zombie heterozygous carriers and wild-type homozygous. One half of embryos from this cross must carry two wild-type alleles, and the second half should be heterozygous and carry one wild-type allele. These embryos were injected with 60-100μM of constructed CRISPR/Cas 9 (Kane, unpublished) mRNA that recognized 18 nucleotides in the wild-type *cdc20* allele at 2-4 cell stages. We expect to see a mosaic *zombie* phenotype in 50% of the embryos that were heterozygous for the *zombie* mutation (Figure 7A).

Control embryos have a higher average and percentage of positive nuclei cells in prophase (22.22%) and a lower average percentage of cells in metaphase (34%) than CRISPR/Cas 9 mRNA injected embryos (Injected1 8.9% in prophase, 45% in metaphase; Injected2 9.43% in prophase, 50.22% in metaphase; Injected3 9.3% in prophase, 44.42% in metaphase (Figure 7B). In summary, the wild-type *cdc20* allele was mutagenized by the CRISPR/Cas9 system to mimic the *zombie* mutation.
Figure 7. CRISPR mRNA injected embryos have a higher count of cells in metaphase plate and metaphase. (A) Constructed CRISPR sequence. sgRNA; BLUE in CRISPR recognized 18 nucleotides in the wild-type CDC20 allele; GREEN to guide the Cas9 nuclease to target site that results in a mutation. PAM site; RED is required for Cas9 nuclease function. Target site was proximal to original mutation site; YELLOW. (B) Histogram representation of Control embryos and CRISPR/Cas 9 injected embryos. Control embryos (n=30) have a higher average percentage of cells in prophase and a lower average percentage of cells in metaphase than CRISPR injected embryos (Injected#1 n=35; Injected#2 n=23; Injected#3 n=31)
DISCUSSION

*zombie* mutants have a higher proportion of mitotic cells than wild-type embryos

The anti-phospho-histone H3 antibody binds to show cells in mitosis. *Krox20 in situ* hybridization was used to limit a small region of the embryos to make counting of mitotic cells easier. Experiments showed that *zombie* could be seen as early as the 5-somite stage (3-somite stage in previous studies). Positive nuclei representing mitotic cells coincided with previous *zombie* data, that *zombie* embryos have a higher proportion of mitotic cells than wild-type siblings at the 5-somite, 10-somite and 15-somite stages.

The higher proportion of mitotic cells at older stages in the *zombie* mutant embryos used in this experiment, supports the idea that *zombie* embryos enter a mitotic cellular arrest, resulting in an accumulation of cells in mitosis. In the 10-somite and 15-somite embryo category, the number of embryos that show the *zombie* mutant phenotype was less than a quarter. Although both 10 and 15-somite are an older stage, it is plausible that *zombie* mutant embryos were loss during their experiments. If all original *zombie* mutants were present during mitotic cell count, it is possible they lacked antibody staining, lowering the amount of mitotic cells seen between the krox20 stripes, resulting in a lower amount of *zombie* mutant embryos at the 10-somite and 15-somite stage. This experiment consists with the idea that *zombie* is a mutation in *CDC20*. 
Rescue of the *zombie* mutant phenotype with 260pg wild-type *cdc20*mRNA

To test lethality of wild type *cdc20*mRNA embryos, we injected various amounts into wild-type embryos. We found that embryos survived with amounts ranging between 50pg-260pg (Figure 8). It appears that injecting 50pg wild-type *cdc20*mRNA is not enough to rescue the *zombie* phenotype. We see the *zombie* phenotype due to the absence of *cdc20*, resulting in mitotic arrest during the metaphase-anaphase transition. Higher injections of wild-type *cdc20*mRNA are needed to resume normal cell cycle progression in *zombie* mutants (Kane *et al.*, 1996). It appears that injecting embryos with 260pg wild-type *cdc20*mRNA didn’t rescue the *zombie* mutant phenotype.

Failure to rescue the *zombie* mutant phenotype could due to CDC20 mRNA degradation in earlier stages of embryonic development before the *zombie* mutant phenotype can be seen, resulting in a mitotic arrest phenotype seen in the data. A previous experiment showed CDC20mRNA expression at the 64-cell stage in *zombie* mutants after being injected with CDC20mRNA at 2-cell stage (P. Johnston Honors Thesis). Another previous study showed *emil* injections were able to rescue another zebrafish mutant with a phenotype seen around 50% epiboly, but was unable to rescue the mutant by the tailbud stage (Zhang *et al.* 2008).
Figure 8. Different amounts of injected CDC20 mRNA have no affect on wild-type embryos. (A) Wild-type embryos injected with Kaede as a control displayed normal wild-type phenotype. (B) Wild-type embryos injected with 26pg CDC20 mRNA displayed normal wild-type phenotype. (C) Wild-type embryos injected with 50pg CDC20 mRNA displayed normal wild-type phenotype. (D) Wild-type embryos injected with 52pg CDC20 mRNA displayed normal wild-type phenotype. (E) Wild-type embryos injected with 260pg CDC20 mRNA displayed normal wild-type phenotype.
Another explanation of the failure to rescue the *zombie* phenotype is the stability of the *zombie* embryos. Due to a weakened condition, it is possible that *zombie* embryos can’t withstand a high dosage of injected CDC20 mRNA. Thus, the 260pg of CDC20 mRNA used during the experiment was degraded earlier than it could have if a higher dosage was injected.

**CRISPR/Cas 9 mRNA possibly can mutagenize the wild-type *cdc20* allele and produce *zombie* mutant cells**

Within CRISPR mRNA, target sequence binds complementary to cdc20mRNA. Nuclease Cas 9 cleaves double strands, creating mutations in cdc20mRNA (Auer et al., 2014). Embryos injected with constructed CRISPR mRNA that recognized 18 nucleotides in Exon 7 of the wild-type CDC20 allele by Dr. Donald Kane, displayed a visible *zombie* mutant phenotype with a higher amount of mitotic cells in metaphase than in prophase compared to un-injected embryos that had a higher amount of mitotic cells in prophase than in metaphase coinciding with the evidence that the usage of the CRISPR/Cas 9 system in embryos, mutagenizing the wild-type cdc20 allele mimics the *zombie* mutant.

However, mitotic cell count for CRISPR/Cas 9 injected and non-injected embryos were inconsistent as the orientation of the embryos changed periodically as the view of the embryos deepened. Thus, non-consistent mitotic cell count of embryos doesn’t analyze the data properly. In previous studies, DAPI staining of CRISPR/Cas 9 mRNA
injected embryos showed a difference in the presence of mitotic cells in CRISPR/Cas9 mRNA injected embryos compared to wild type embryos.

In wild type cells, there is a higher count of cells in prophase with a lower count of cells in metaphase and anaphase cells, suggesting cells were properly regulated during cell cycle division. In CRISPR/Cas 9 injected embryos, there was a clone of cells in metaphase something that isn’t visible in normal dividing cells of wild-type embryos, indicating that mutagenizing the wild-type cdc20 chromosome works (Kane et al, 1993).
FUTURE DIRECTIONS

Inability to rescue the zombie phenotype with CDC20mRNA injections, poses the question as to when specifically during the cell cycle, does the zombie mutation occur. One way to eliminate the idea that the zombie mutation occurs during S-phase is through the use of Bromodeoxyuridine (BrdU) labeling along with pH3 antibody staining. Bromodeoxyuridine is a thymidine analog and incorporates into DNA only if cells are at S-phase (Ergul et al. 2013). Since prior data has shown the zombie mutant to experience cellular arrest during the metaphase-anaphase transition. We hypothesize to not see positive BrdU labeled cells, but see positive nuclei staining further suggesting zombie is a mutation of CDC20.

In addition, another way to determine when the zombie mutation occurs during the cell cycle is to use zombie dual Fucci transgenic fish. The dual Fucci transgene monitors cell division. It uses a ubiquitin promoter to express a Cerulean-tagged-zGeminin degron, that is visible in cells during S/G2/M due to the degradation of chromatin licensing and DNA replication factor 1 (Cdt1), and accumulation of its inhibitor, geminin and a mCherry-tagged-zCdt1 degron, that is visible in cells during G1 due to the degradation of geminin and accumulation of Cdt1 (Bouldin et al., 2014) (Figure 9).
Previous studies have shown that zombie mutants have a higher proportion of cells in metaphase (E. Morgan Thesis) than their wild-type siblings, suggesting cellular arrest during mitosis. Based on the constructed dual Fucci transgene, we can expect to see colorless cells in zombie mutants, since nuclear envelope breakdown occurs by metaphase with a possible degradation of geminin (Sakaue-Sawano et al., 2008). However, if CDC20, an activator protein of APC is inactive, inactivating the binding of cdh1, a second substrate of APC during G1 phase, geminin possibly could be expressed, resulting in blue cells of the zombie mutant.

If both substrates of APC are activated earlier in the cell cycle, geminin is inactive, allowing the expression of cdt1 to occur, resulting in red cells of the zombie mutant. These potential experiments could further verify whether zombie is a mutation of CDC20.

We were successful in mutagenizing the wild-type CDC20 allele with CRISPR mRNA. We should raise CRISPR fish by first, crossing two wild-type fish together. We inject the progeny with constructed CRISPR mRNA that recognizes 18 nucleotides in the wild-type CDC20 allele, at the 2-cell stage to knockdown the wild-type allele. We could then cross a CRISPR mRNA injected fish with a zombie heterozygous fish. If their progeny fail to complement during complementation testing, we expect to see the zombie phenotype. This experiment could further verify whether zombie is a mutation of CDC20.
Figure 9. Constructed representation of the Dual FUCCI (fluorescent ubiquitination-based cell cycle indicator) system. (A) Schematic representation of the Dual Fucci transgene that uses an ubiquitin promoter to regulate the expression of Cerulean-tagged-ZGeminin S/G2/M indicator and mCherry-tagged- ZCdt1 G1 indicator. Figure adapted from Bouldin et al. (2014) (B) Constructed representation of expressed Dual FUCCI indicators based on cell cycle phases. (C) Hypothesized Dual FUCCI indicator expression for a *zombie* mutant.
CONCLUSION

Although, we were unable to rescue the zombie mutant phenotype, we used other experimental assays that convince us that zombie is a mutation of CDC20. This study was able to demonstrate that the zombie mutation can be mimicked by the use of the CRISPR/Cas 9 system designed to mutagenized wild-type cdc20 mRNA. We further showed that zombie mutants have a higher mitotic cell count than its wild-type siblings via krox20 in situ hybridization and the degradation of CDC20 mRNA is developmental stage dependent, giving us hope that zombie is the mutation of CDC20.
REFERENCES


