Developmentally arrested *Austrofundulus limnaeus* embryos have changes in post-translational modifications of histone H3

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ABSTRACT

Although vertebrate embryogenesis is typically a continuous and dynamic process some embryos have evolved mechanisms to developmentally arrest. The embryos of *Austrofundulus limnaeus*, a killifish that resides in ephemeral ponds, enter diapause II (DII), a reversible developmental arrest promoted by endogenous cues rather than environmental stress. DII, which starts at 24-26 days post-fertilization and can persist for months, is characterized by a significant decline in heart rate and an arrest of development and differentiation. Thus, *A. limnaeus* is a unique model to study epigenetic features associated with embryonic arrest. To investigate chromosome structures associated with mitosis or gene expression we examined the post-translational modifications of histone H3 (phosphorylation of serine 10, mono-, di- and tri-methylation of lysine 4 or 27) in preDII, DII and postDII embryos. As seen by microscopy analysis, DII embryos have a significant decrease in the H3S10P marker for mitotic nuclei and an inner nuclear membrane localization of the H3K27me2 marker associated with silencing of gene expression. ELISA experiments reveal that the levels of methylation at H3K4 and H3K27 are significantly different between preDII, DII and postDII embryos indicating that there are molecular differences between embryos of different chronological age and stage of development. Furthermore, in DII embryos relative to PreDII embryos there are differences in the level of H3K27me3 and H3K4me3, which may reflect critical chromatin remodeling that occurs prior to arrest of embryogenesis. This work helps lay a foundation for chromatin analysis of vertebrate embryo diapause, an intriguing yet greatly understudied phenomenon.
SUMMARY STATEMENT

Killifish embryos enter into a developmental arrest called diapause II. We found that the chromatin state differs between embryos prior to, during and post diapause II.
INTRODUCTION

Animal development is often regarded as a dynamic and continuous process along a progressive trajectory. However, there are instances in which the active succession of development is arrested, likely as a means to increase individual or population survival. Metazoan developmental arrest is observed in a variety of species and in response to various environments. Examples of vertebrate developmental arrest include delayed implantation of mammalian embryos, anoxia-induced suspended animation, and diapause (Mead, 1993; Padilla and Roth, 2001; Podrabsky and Hand, 1999; Renfree and Shaw, 2000; Wourms, 1972). Delayed implantation is a reproductive strategy in which the young mammalian embryo does not implant into the uterus and is maintained in an arrested state of dormancy (Renfree and Calaby, 1981). In laboratory conditions severe O$_2$ deprivation (anoxia) can induce a reversible arrest termed suspended animation (Padilla and Roth, 2001). Diapause is a state of animal dormancy, often correlated with seasonal changes, in which growth and development are suspended (Mead, 1993). Diapause can occur in vertebrates and invertebrates and is typically accompanied by a decrease in metabolism (Podrabsky and Hand, 2015). Insects can diapause for extremely long periods of time and manage metabolic resources to meet the demands of survival during the long bouts of limited food (Hahn and Denlinger, 2011). Diapause among vertebrates is less frequent and not well understood.

Annual killifish maintain a permanent population in temporary bodies of water in Africa and South America that experience extreme dry and rainy seasons (Berois et al., 2012; Wourms, 1972). *Austrofundulus limnaeus* is an annual killifish from the Maracaibo basin of Venezuela (Podrabsky and Hand, 1999). The ponds that these fish inhabit dry out on a seasonal basis resulting in the dying off of adult and juvenile killifish. The survival of this killifish population is hinged upon the survival of embryos embedded in the pond sediment. The embedded killifish embryos are in a state of diapause and upon return of the rainy season
the embryos will hatch and continue development and growth. *A. limnaeus* can display three distinct stages of diapause (DI, DII and DIII). DI occurs early in development and is induced by environmental stress, DII is promoted by endogenous cues and DIII occurs in fully developed embryos that delay hatching in response to environmental cues. The phenomenon of DII is part of a normal developmental program in that embryos incubated in favorable conditions will still developmentally arrest. However, the number of embryos that enter DII can be influenced by maternal age and incubation temperature (Podrabsky et al., 2010; Pri-Tal et al., 2011). For example, the majority of *A. limnaeus* embryos reared at 20°C or 25°C will enter into DII whereas those raised at 30°C can escape DII and progress in development (Anderson and Podrabsky, 2014). DII occurs in embryos with 38-40 somite pairs and primordial organ development. The onset of DII, 24-26 days post-fertilization (dpf), is marked by the arrest of developing somites, lack of pigment and a significant decline in heart rate (Podrabsky and Hand, 1999). Although development is arrested, one should note that the DII embryos are not in a complete biologically arrested state in that, albeit significantly reduced, heartbeats and slight movement are observed in the embryo (Fergusson-Kolmes and Podrabsky, 2007). DII can last for over 100 days and is associated with stress resistance and a hypometabolic state (Duerr and Podrabsky, 2010; Machado and Podrabsky, 2007; Podrabsky et al., 2007). The mechanisms inducing and regulating DII and how various stages of DII differ at a molecular level are not well understood.

Developmental progression is well studied in a variety of animal models and involves the specification of cell types mediated through the expression or silencing of genes. How the chromatin landscape influences gene expression has been extensively studied in yeast and mammalian cells grown in culture but less is understood about chromatin modifications relative to the developmental stages of vertebrate embryos (Bannister and Kouzarides, 2011; Kishi, 2014; Martinez-Sales et al., 2014). The post-translational modifications of histones
have been mapped in mice embryos (Dahl et al., 2010; VerMilyea et al., 2009), zebrafish Danio rerio (Havis et al., 2006; Lindeman et al., 2010; Wardle et al., 2006) and Xenopus tropicalis embryos (van Heeringen et al., 2014). Together these studies indicate that histone modifications are associated with developmentally regulated genes. The post-translational modification of histones is highly conserved and occurs at the N-terminal tail of histones to either repress (silent heterochromatin) or activate (active euchromatin) gene expression (Ho et al., 2014). Furthermore, which histone amino acid is modified (e.g. lysine 4 or lysine 27 of histone H3) or the degree of modification (e.g. mono-, di- or tri- methylation) can have different effects on the chromatin structure or function (Bannister and Kouzarides, 2011). Histone modifications can also induce chromosome condensation (e.g. serine 10 phosphorylation of histone H3) and correlate with condensation of chromosome through mitosis (Shoemaker and Chalkley, 1978). Understanding the epigenetic features in blastomeres of developing organisms will lead to a greater understanding of the molecular mechanisms involved with regulating organismal and cellular development and differentiation.

The regulatory mechanism of endogenously controlled embryo arrest in vertebrates is not understood and A. limnaeus provides a unique opportunity to study this phenomenon. The endogenous cue to induce and maintain DII likely acts on all blastomeres as the arrest of growth and development occurs throughout the embryo. Here we examine and compare the chromatin modifications associated with preDII, DII and postDII embryos as a means to gain an understanding of the global chromatin structure associated with developmental arrest.
MATERIALS AND METHODS

Adult Fish Husbandry

The husbandry of adult *Austrofundulus limnaeus* was essentially as previously described (Podrabsky and Hand, 1999). The UNT Institutional Animal Care and Use Committee (IACUC) reviewed and approved research protocols involving *A. limnaeus*. Adults were maintained as spawning pairs in a recirculating rack. Water parameters were maintained to have a salinity of 1 ppt, pH of 6.5 and temperature of 26°C. The lighting cycle was on a programmed timer set to deliver 14 hours of light and 10 hours of darkness. Water was continuously changed at a rate of 4 liters per hour. Each mating pair was housed in a 6-liter tank that is divided into halves preventing males and females from constant direct contact. Animals were fed Hikari frozen bloodworms once a day normally and twice on days just prior to spawning. Embryo collections occurred twice weekly and were induced by the introduction of spawning medium in the form of approximately 300 ml of glass beads in a small plastic container. Each collection lasted between 2-3 hours and embryos were pooled into a single collection dish.

Embryo Collection and Husbandry

Embryos were collected twice weekly from 16-24 pairs of spawning of adults ranging from 6 months to 2 years of age. The embryos were immediately rinsed several times with approximately 500 ml of diH₂O; dead embryos were discarded. Living embryos were transferred at a density of 40 embryos per petri dish in diH₂O supplemented with salts that mimic their natural environment as described previously (Podrabsky and Hand, 1999). To reduce microbial contamination, the 4 dpf embryos were incubated in 0.03% hypochlorite for
5 minutes, rinsed with diH₂O, and stored at 25°C in diH₂O containing 10 mg gentamicin and HEPES. Petri dishes were checked daily and dead embryos were immediately removed. For experiments requiring postDII analysis, the DII embryos of 24-32 dpf were incubated in a 30°C incubator with an internal light source set to a cycle of L14:D10; within 2-3 days nearly all embryos were observed to have exited DII. Embryos were examined to ensure uniform developmental stage corresponding to 2-4 days postDII and outliers of extreme development or underdevelopment were removed. Live embryos at the designated stages were imaged using a Zeiss stereomicroscope and Axiocam mrc camera.

**Fixation and Microscopy**

Embryos of the specified developmental stage (preDII or DII; 18-36 dpf) were collected, washed in 0.03% hypochlorite for 5 minutes and then rinsed twice in phosphate buffered saline (PBS). All media was removed with a pipette and embryos were submerged in 4.0% paraformaldehyde (Thermo Fisher, 28906). A 26-gauge needle was used to puncture the chorion and embryos were gently agitated with a pipette tip to allow perivitelline fluid to exchange with paraformaldehyde. Embryos were incubated in the fixative for 20 minutes at room temperature. The fixative was removed and embryos were washed three times with 20 ml of PBS and then placed in a PBS Block solution (PBS, 5% BSA and 0.25% Triton X-100). Using a stereomicroscope, the embryos were dechorionated while submerged in blocking solution and incubated in the PBS Block solution for 45 minutes at room temperature. The embryos were transferred to a microcentrifuge tube containing the specified primary antibody (anti-H3S10P, Millipore 06-570; anti-H3K27me2, Abcam ab24684; anti-H3K27me3, Abcam ab6002; diluted in PBS Block solution at a 1:200 concentration) and incubated overnight at 4°C. After antibody incubation, embryos were washed three times with PBS Block and the specified secondary antibody was added (anti-mouse Alexa Fluor 488, A-11034; anti-rabbit
Alexa Fluor 568, A-11011; diluted in PBS Block solution at a 1:1000 dilution) for a 35-minute incubation. Hoechst 33342 (Sigma Aldrich, 861405) was added to 2 µg/ml working solution and embryos were incubated for 10 minutes. The embryos were transferred into 3 ml of PBS Block, rinsed by gently swirling the media, placed into 1 ml of PBS and then transferred onto a Superfrost glass microscope slide. Vectashield mounting medium (VectorLabs, H-1400) or Prolong Gold (Life Technologies, P36930) was added directly to the tissue before covering the embryo with a glass cover slip. Embryos were imaged using an epifluorescent Zeiss Axioskop II and Axiocam camera. For fluorescent microscopy analysis the tail region of the embryo was imaged due to the decreased thickness of the sample and ability to distinguish nuclei. To determine the number of mitotic nuclei the number of nuclei recognized by anti-H3S10P was quantified in the whole animal. For each developmental stage six embryos at three independent times were quantified for the number of mitotic nuclei.

Acid Extraction of Histones

Analysis of the post-translational modifications of histones was conducted by combining two protocols (Nardelli et al., 2013; Shechter et al., 2007). Embryos of the specified developmental stage (preDII, DII, postDII; 18-36 dpf) were collected and rinsed in 0.03% hypochlorite for five minutes to remove external chorionic debris and microorganisms, and then rinsed once with diH2O. The embryos were placed in a 50 ml conical tube containing 10 ml of 2% (v/v) Tween-20 and vortexed for 5 seconds. The Tween-20 solution was removed using a pipette and embryos were rinsed four times with 50 mL diH2O. Embryos were transferred to a 1.5 ml microcentrifuge tube placed on ice and excess water was removed. To release the embryo from the chorion the embryos were homogenized on ice with Lysis buffer (0.25 M sucrose, 1 mM EDTA, 3 mM CaCl2, 0.05% saponin, 0.01 M
TrisHCl pH 7.4, Protease Inhibitor (Thermo Fisher, 88666), 1 mM PMSF, 1 mM DTT, 1 mM sodium fluoride, and 5 mM sodium butyrate). The supernatant was collected into a new 1.5 ml microcentrifuge tube. The homogenized sample was subjected to four repeats of a brief centrifugation at 2,000 X g for five seconds and supernatant collection to maximize the amount of lysate obtained. The collected supernatant was then centrifuged at approximately 4,000 X g for 10 minutes at 4ºC to pellet the nuclei. The supernatant was removed and the pellet was flash frozen in liquid nitrogen. The frozen pellet was resuspended in 0.4 N HCl and then allowed to rock at 4ºC overnight to fully solubilize histone proteins. Following overnight extraction the lysate was centrifuged at 16,000 X g for 10 minutes at 4ºC to remove debris. The supernatant was transferred to 8 ml of pre-chilled acetone and stored at -20ºC overnight to precipitate the histones. Following precipitation the histones were pelleted with centrifugation at 3,200 X g for 10 minutes at 4ºC. Acetone was removed and the pellet was allowed to dry completely at -20ºC overnight. The dry pelleted histones were resuspended in sterile resuspension buffer (ddH$_2$O with Trizma base and Tris HCl at pH 8.0) and stored at -80ºC until analyzed as described below.

**Extraction of Whole Embryo Lysate**

For analysis of proteins from whole embryonic lysate, embryos of the specified developmental stage (preDII, DII, postDII; 18-36 dpf) were rinsed in 0.03% hypochlorite for five minutes and rinsed four times with 50 ml of diH$_2$O. Approximately 100 embryos were transferred into a 1.5 ml centrifuge tube, excess water was pipetted out, and 200 µl of lysis buffer (Thermo Fisher Protease Inhibitor #88666, 1 mM PMSF, 100 mM Tris, pH 7.4, 2 mM Na$_3$VO$_4$, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 0.5% deoxycholate and 20 mM Na$_4$P$_2$O$_7$) was added. Embryo chorions were ruptured in lysis buffer with plastic pestle and the lysate was transferred to a 1.5 ml
microcentrifuge tube. Embryo lysate was placed in a beaker of ethanol saturated ice and exposed three times to a 5 second sonicator burst at 50% power (Branson Model 150 Sonic Dismembrator). The lysate was centrifuged for 10 minutes at 8,000 X g at 4°C to pellet remaining debris. The supernatant of this lysate was removed and stored at -80°C until analyzed as described below.

**ELISA Assay**

The acid extracted histones and whole embryo lysate samples, isolated as described above, were diluted to 30X with 100 mM coating buffer (0.2M Na₂CO₃, 0.2M NaHCO₃, pH 9.6) and loaded into 96-well plates in triplicate with each sample row being loaded in duplicate. A row of wells was also loaded with coating buffer to assess background luminescence values. Plates were washed 4 times with wash solution (PBS supplemented with 0.05% Tween-20). Plates were then blocked with casein sodium salt for one hour at room temperature (Sigma Aldrich, C8654), washed 4 times with wash solution, and then incubated with the specified primary antibodies (1:1000 dilution or to equal 0.01 µg/mL) overnight at 4°C. Antibodies used for analysis include anti-H3K27me1, 2 or 3 (Active Motif, 39298, 39142, and 39156, respectively), anti-H3K4me1, 2 or 3 (Active Motif, 39378, 61435 and 39159, respectively), anti-histone H3 (Abcam, ab1791), anti-Hsp70 (Abcam, ab5442) and anti-RNA Pol II (Abcam, ab5408). Histone plates were loaded such that for every antibody of interest an equal number of wells with histone extract was probed with anti-histone H3 as a control. The whole embryo lysate ELISA plates were loaded at the same time as the Direct Detect protein quantification cards were processed (see below) and used as a loading control. Following primary antibody incubation the plates were washed 4 times with wash solution and incubated with anti-mouse or anti-rabbit secondary antibodies (Thermo Fisher Scientific, 32430 and 32460, respectively) for one hour at room temperature. Plates
were washed again four times with wash solution and immediately developed with Thermo Fisher Femto Super Signal (37075). Total luminescence (all wavelengths) was detected in a Biotek Synergy H4.

**Analysis of ELISA data**

The average of background signal, derived from antigen-negative wells, was subtracted from all sample wells to produce the background-subtracted values. The signal value of wells probed for modified histone was divided by the value of the anti-histone H3 signal to produce a normalized luminescence value. The ratio of post-translation modification (PTM) signal divided by the total histone H3 signal was used to calculate the relative PTM units (Dai et al., 2011; Dai et al., 2013; Mosesson et al., 2014). For quantification of RNA Pol II S5P and Hsp70 values are expressed as total luminescence relative to total protein for respective samples as detected on a Millipore Direct Detect system.

**Western Blot Analysis**

Acid extracted histones, low range protein ladder (Life Technologies, 26628) and a recombinant histone H3 protein (Active Motif, C110A) were loaded onto a 20% polyacrylamide gel and electrophoresed for 2.5 hours at 100V. Histones were transferred to a 0.2 µm nitrocellulose membranes (Bio-Rad, 162-0212) in CAPS buffer at 24V overnight. Membranes were blocked in casein (Sigma-Aldrich, C7594) for 1 hour at room temperature, probed with anti-histone H3 antibody for 1 hour at room temperature. Blots were washed three times with PBS-T wash buffer for 10 minutes at room temperature on a rocker. Blots were probed with secondary HRP-conjugated antibody (Thermo Fisher, 32430) for 1 hour at room temperature and washed 5 times for 5 minutes at room temperature prior to developing. To precipitate the HRP reaction 5 ml of 1-Step Ultra TMB (Thermo Fisher, 37574) was
poured directly onto membranes and bands were allowed to develop for approximately 3 minutes. The western blot was imaged using a Biospectrum 810 Imaging System (UVP).

Statistical Analysis

All data are expressed as mean ± standard error (s.e.m). Data sets were analyzed for Gaussian distribution using D’Agostino-Pearson omnibus normality test and Shapiro-Wilk normality test (alpha = 0.05, p>0.05). If the normality tests were not passed then the data set was arcsine square root transformed prior to analysis by one-way ANOVA. A Holm-Sidak’s multiple comparison post-test was used to compare between groups. For the ELISA assays the relative PTM unit (histone modification assays) or the total luminescent value relative to total protein (RNA Pol II, Hsp70) was the measurement variable and embryo stage (dpf) was the nominal variable. To analyze the number of mitotic nuclei present in animals relative to developmental stage one-way ANOVA followed by a Holm-Sidak’s multiple comparison test was conducted. P values reported are multiplicity-adjusted values. For all data analysis and graph generation Prism 6.0 was used. Statistical results for ELISA experiments are provided in Table S1, S2 and S3.

RESULTS

The Number of Mitotic Nuclei are Reduced in Diapause II Embryos

Arrested DII embryos, of different post-fertilization time points, are anatomically very similar to one another and to preDII (18 dpf) embryos (Figure 1). However, postDII embryos develop and differentiate, as seen by the presence of pigmentation, vascular tissue and other developmental changes (Figure 1). Here we investigate the changes in chromatin of preDII, arrested DII and postDII embryos. We elected to analyze the post-translational modifications
of histone H3 because there are commercially available antibodies that recognize specific histone H3 modifications, and have been used in many systems including yeast, humans, mice and fish (Baumgart et al., 2014; Wu et al., 2011). Furthermore, the predicted histone H3 protein in *A. limnaeus* shows 100% protein identity to the histone H3 protein in zebrafish and another annual killifish (*Nothobranchius furzeri*) and the fish protein has only two amino acid differences when compared to human histone H3 (Supplementary Figure 1A).

Previously, others used flow cytometry analysis to show that most blastomeres of DII animals are in the G1/G0 stage of the cell cycle (Podrabsky and Culpepper, 2012). To examine cell division relative to the onset of DII, embryos at various stages of preDII and DII were examined for the presence of mitotic nuclei using an antibody that detects the phosphorylation of serine 10 on histone H3. The phosphorylation of serine 10 of histone H3 is a marker for mitotic nuclei in a wide variety of eukaryotic organisms from yeast to human cells (Prigent and Dimitrov, 2003; Shoemaker and Chalkley, 1978). The number of mitotic blastomeres was highest in young embryos (preDII, 18 dpf) and subsequently decreased as the preDII embryos approached DII (20, 22 dpf) (Figure 2A, B). The embryos at the onset of DII (24, 26 dpf) contained mitotic nuclei but at a reduced number relative to the preDII embryos. The number of mitotic nuclei was significantly reduced to near zero in older DII embryos (30-36 dpf) (Figure 2B). These data indicate that cell division gradually decreases as the embryos approach DII and that the number of mitotic nuclei remains significantly reduced in older DII embryos in comparison to embryos that entered into DII.

**Histone Methylation**

The molecular mechanism(s) that signals the killifish embryo to arrest development and enter DII is unknown yet hypothesized to be an endogenous cue. Furthermore, it is likely that all blastomeres within the embryo receive the DII arrest signal since a coordinated arrest
of development and differentiation is observed throughout the embryo. In addition to developmental arrest, other cellular processes, such as chromatin modifications and gene expression, are likely to be modified in DII embryos relative to developing embryos. Here we used site-specific antibodies to discriminate between different post-translational modifications of histone H3 in preDII, DII and postDII embryos. The methylation of histone H3 at lysine 27 (H3K27) is well studied and critical for the repression of developmental genes (Bannister and Kouzarides, 2011; Ho et al., 2014; Kouzarides, 2007; Strahl and Allis, 2000). When H3K27 is trimethylated (H3K27me3) it is often associated with the inactivation of gene promoters and is an epigenetic marker for inactive genes. The H3K27me2 modification is less studied but is thought to have a similar silencing effect on genes as H3K27me3. In contrast H3K27me1 is thought to positively effect gene expression (Ferrari et al., 2014). Here we stained preDII and DII embryos with antibodies that recognize either H3K27me3 or H3K27me2. The nuclei, in preDII and DII embryos, were detected by anti-H3K27me3 and the distribution of the chromatin appeared throughout the nucleus in both preDII and DII embryos (Figure 3). The distribution of chromatin detected by anti-H3K27me2 differed between preDII and DII animals (Figure 4). In preDII embryos, H3K27me2 was distributed throughout the nuclei. However, in DII embryos the localization pattern of the chromatin detected by anti-H3K27me2 displayed an inner nuclear membrane localization pattern (Figure 4). The inner nuclear membrane localization of H3K27me2 occurred in 44.3% percent of the DII embryos yet was never observed in the preDII embryos (counts analyzed with Fisher's exact test, p=0.0035). These data indicate that there are significant differences in chromatin modifications between preDII and DII embryos, that these chromatin differences may affect localization within the nucleus and that the localization pattern of the chromatin may vary between the animals that are in DII.
To better quantify the histone modifications initially observed by microscopy we purified histones (Supplemental Figure 1b) and used ELISA to measure the relative post-translational modification (PTM) of H3K27 (me1, me2, me3) and H3K4 (me1, me2, me3), relative to histone H3, in preDII (8 dpf, 16 dpf), DII (24 dpf, 32 dpf) and postDII embryos (Figure 5). The relative PTM of H3K27me1 did not differ between the two stages of preDII embryos. There was a significant decrease in relative PTM in 8 dpf preDII embryos compared to 24 dpf DII and postDII embryos (Figure 5A). The relative PTM of H3K27me1 was lowest for 24 dpf DII embryos and postDII embryos (Figure 5A). The relative PTM of H3K27me2 did not differ between the embryo stages (Figure 5B). In regards to the relative PTM of H3K27me3, it was significantly higher in 16 dpf preDII embryos relative to 24 dpf DII embryos (Figure 5C). Together, these data indicate that 1) 24 dpf DII embryos have a significant difference in H3K27me1 and H3K27me3 levels compared to preDII embryos, 2) H3K27me2 is not significantly different in preDII, DII and postDII embryos and 3) Post DII embryos have a significant decrease in H3K27me1 in comparison to 36 dpf DII embryos. Table S1 contains statistical results for the ELISA assays using antibodies to detect methylation of H3K27.

Each methylation state (mono-, di-, or tri-) of histone H3 at lysine 4 is thought to have a different impact on gene expression. In the yeast *Saccharomyces cerevisiae* the trimethylation of histone H3 at lysine 4 (H3K4me3) is associated exclusively with the promoters of active genes and is associated with initiated forms of RNA pol II (RNA Pol II S5P) whereas the di-methylation of K4 (H3K4me2) occurs at both inactive and active euchromatic genes (Lauberth et al., 2013; Santos-Rosa et al., 2004). The impact of H3K4me1 is more ambiguous but has been associated with enhancers (Barski et al., 2007; Heintzman et al., 2007). Here we find that the relative PTM of H3K4me1 was significantly less for postDII animals relative to preDII and DII animals (Figures 5D). The relative PTM of H3K4me2 did
not differ between the embryo stages (Figure 5E). The relative PTM of H3K4me3 was significantly higher in 8 dpf preDII embryos relative to 24 dpf DII (Figure 5F). Together, this indicates that 1) H3K4me1 is at a similar level in the preDII and DII animals and 2) that H3K4me3 levels are higher in preDII animals relative to DII embryos. Table S2 contains statistical results for the ELISA assays using antibodies to detect methylation of H3K4.

We also graphed the relative PTM for the modifications assayed relative to developmental state to determine which histone H3 modification we examined was more prevalent. For each developmental stage, the H3K27me3 or H3K4me3 was most abundant in comparison to the H3K27me1/me2 or H3K4me1/2 states, respectively (Supplementary Figure 2). The H3K27me1 and H3K27me2 levels were significantly different from one another in 32 dpf and PDII embryos but there was no difference in these two modifications in embryos of other developmental stages. Overall, the trimethylated state of H3K27 or H3K4 is a more prevalent chromatin state for the embryos we examined.

**Hsp70 Levels and Active RNA Pol II Varies in DII Embryos of Different Time Points**

A characteristic of DII embryos is that they are drought tolerant and resistant to a variety of stresses including anoxia and hypersalinity (Podrabsky et al., 2008; Podrabsky et al., 2007; Podrabsky et al., 2012). In DII embryos, there is a depressed rate of protein synthesis (Podrabsky and Hand, 2000) yet western blot analysis indicates a constitutive level of expression of Hsp70 in preDII and DII embryos (Podrabsky and Somero, 2007). We found that Hsp70 was detectible in all embryos, yet the level of Hsp70 was significantly higher in postDII embryos, relative to preDII and DII embryos. These data indicates that the level of Hsp70 is highest in the postDII embryos. Note that we used an antibody whose epitope is located between amino acid 437-479 of human Hsp70 and thus can potentially detect the family of Hsp70 proteins (Hsp70/Hsc70).
Transcription of mRNA by RNA Pol II is positively influenced by the phosphorylation status of the C-terminal domain (CTD) of the RNA Pol II largest subunit. Here we use anti-RNA polymerase II CTD repeat YSPTSPS phospho S5 antibody (anti-RNA Pol II S5P) to examine the activated form of RNA Pol II in preDII, DII and post DII embryos (Ahn et al., 2004). The preDII, DII and postDII embryos did not differ significantly in the relative amount of RNA Pol II S5P (Figure 6B). These data suggest that RNA Pol II is potentially poised to transcribe even in the DII embryos. Table S3 contains statistical results for the ELISA assays using antibodies to detect HSP70 and RNA Pol II S5P.
DISCUSSION

*A. limnaeus* are one of a few vertebrates whose embryos developmentally arrest in environments that are conducive for growth and development. To assess chromatin changes associated with DII we examined highly conserved histone H3 modifications associated with mitotic nuclei (H3S10P) and regulation of gene expression (methylation of H3K27, H3K4). A decrease in mitotic nuclei was observed as animals progressed through preDII suggesting that the DII program is initiated prior to substantial slowing of the heart rate and arrest of somite development. The gradual decrease in mitotic nuclei in the embryos approaching DII suggests that blastomeres do not uniformly arrest at the same time. However, nearly all blastomeres do exit mitosis by the time the DII embryo reaches the 30 dpf (Figure 2). These data show that there are molecular differences between animals in the first stages of DII in comparison to those that have been in DII for a longer period of time and that DII is not a “static” state at the molecular level.

Histone H3 post-translational modifications are extensively studied due to their impact on chromatin structure and function and their central role in epigenetic mechanisms. However, our understanding of chromatin modification in developing vertebrate embryos is limited and has only been examined in few species including mice (Dahl et al., 2010; VerMilyea et al., 2009), zebrafish (Havis et al., 2006; Lindeman et al., 2010; Wardle et al., 2006), *N. furzeri* (Baumgart et al., 2014) and *Xenopus tropicalis* embryos (van Heeringen et al., 2014). The chromatin state in an arrested vertebrate embryo has not been fully examined. We hypothesized that markers for chromatin silencing would be high in DII embryos, relative to preDII and postDII embryos, since hypometabolic states require energy conservation. The H3K27me3 modification is a repressive mark commonly found on silenced gene promoters in different organisms and is involved in the regulation of several genes (Oct3/4 and Sox2) key
to pluripotency and differentiation of embryonic stem cells (Ho et al., 2014). The demethylation of H3K27me3 has been shown to alter the repressive mark resulting in gene activation (Agger et al., 2009; Hong et al., 2007). The upregulation of H3K27me3 signal is also associated with the aging brain (posterior optic tectum) in the short-lived fish *N. furzeri* (Baumgart et al., 2014). Here we find that H3K27me3 is highest in preDII embryos, relative to DII and postDII embryos, and that there is no difference in the levels of H3K27me3 between DII and postDII animals (Figure 5C). It makes sense that some markers for repression will decrease in postDII embryos, as these embryos are rapidly growing and differentiating, which likely requires expression of specific genes. The reduction of the H3K27me3 repressor marker for gene expression in DII embryos, relative to preDII could be interpreted in several ways. First, it is possible that the early embryo relies on maternal stores to support aspects of embryogenesis to the DII stage. Second, it is possible that the DII embryo expresses genes vital for maintenance of the DII embryo and contains chromatin poised for expression of growth and development genes upon exit from DII. This is an intriguing possibility and is conceptually in line with some work involving the nematode *Caenorhabditis elegans*. Baugh et al. showed that active transcription by RNA Pol II of starvation-response genes and RNA pol II accumulates on the promoters of growth and development genes were observed during starvation-induced L1 larvae arrest in *C. elegans*. Upon release from the arrest by feeding the accumulation of RNA Pol II at promoters decreased and mRNA levels increased (Baugh et al., 2009). Our studies do show that the level of activated RNA Pol II is high in the 32 dpf DII embryos relative to 24 dpf DII embryos. Additional studies would be needed to determine if specific genes required for entry, maintenance and exit from DII are expressed and if the expression machinery is poised to initiate gene expression upon the signals that regulate DII. It is possible that genes that are known regulators of cell cycle and epigenetically regulated may also regulated in DII.
embryos. For example, the methylation of the INK4A-ARF locus, which encode for the tumor suppressor proteins p16\textsuperscript{INK4A} and P14\textsuperscript{ARF}, are known to be epigenetically silenced by H3K27me3 and that modulation of the JMJD3 demethylase contributes to the activation of p16\textsuperscript{INK4A} and cell senescence (G1 arrest) thus establishing that chromatin modifications can have impact on cell cycle progression (Agger et al., 2009).

H3K27me2 is broadly distributed across the genome, is thought to fill regions of the chromatin to prevent uncontrolled deposition of acetylation on H3K27 and is associated with gene silencing in embryonic stem cells (Ferrari et al., 2014). The level of H3K27me2 did not differ between the embryo stages (Figure 5B). However, microscopy analysis indicates that unlike preDII embryos, 44.3% of the DII embryos contained H3K27me2 chromatin localized to the inner nuclear membrane (Figure 4). We did not observe the inner nuclear localization pattern in preDII or postDII embryos. These data indicates that the chromatin localization within the nucleus may vary in DII embryos relative to preDII embryos. Note that chromatin associated with the inner nuclear membrane is often but not always associated with gene silencing. We suggest that not only the chromatin mark but also the chromatin nuclear location might be a means to regulate gene expression within DII embryos.

In other systems H3K27me1, H3K4me1 and H3K4me3 are positively associated with gene expression or associated with promoters of active genes (Ferrari et al., 2014; Robertson et al., 2008). However, there is also some evidence that H3K4me3 can negatively regulate gene expression (Kim and Buratowski, 2009). We find that the markers for active gene expression (H3K4me1, H3K27me1) were decreased in postDII embryos (Figure 5A, D). The fact that the markers associated with gene expression are lowest in the postDII embryo is puzzling. It could be that other chromatin markers for active gene expression, that were beyond the scope of this paper, are associated with postDII embryos. Alternatively, it is possible that mRNA molecules that serve as templates for translation are present in DII
embryos that transition to postDII and that protein synthesis supports the rapid growth and development observed in postDII embryos. The dramatic increase in HSP70 protein (Figure 6A) in postDII embryos is consistent with this idea.

The chromatin of all embryos we examined had a higher level of H3K4me3 and H3K27me3 relative to the mono and dimethylated states (Supplementary Figure 2) indicating that trimethylation is the more common chromatin state in these killifish embryos. Thus, from an experimental pragmatic point of view H3K4me3 and H3K27me3 can be easily detected. The 24 dpf DII embryos, but not 32 dpf DII embryos, had a decrease in level of H3K4me3 and H3K27me3 relative to preDII embryos (Figure 5C, F) suggesting that there are chromatin differences between the DII embryos.

CONCLUSIONS

Our analysis of the chromatin modifications of histone H3 provides insight into the molecular changes associated with DII. We conclude that there are molecular differences between A. limnaeus embryos of different developmental time points. Also, in the DII embryo there could be some active transcription and/or the chromatin is in a state that facilitates RNA Pol II to be poised for gene expression upon exit of DII (postDII). The fact that the chromatin marks for active gene expression were significantly reduced in the postDII (4 days after DII) suggest that it is perhaps translation rather than a high increase in mRNA expression that will support the rapid growth and development that occurs in postDII embryos. This work helps lay a foundation for chromatin analysis of vertebrate embryo diapause, an intriguing yet greatly understudied phenomenon.
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There are no competing interests the authors need to declare.

LIST OF SYMBOLS AND ABBREVIATIONS

DII      Diapause II  
dpf      days post fertilization  
H3K27    Histone H3 lysine 27  
H3K27me1 Histone H3 lysine 27 monomethylation  
H3K27me2 Histone H3 lysine 27 dimethylation  
H3K27me3 Histone H3 lysine 27 trimethylation  
H3K4     Histone H3 lysine 4  
H3K4me1  Histone H3 lysine 4 monomethylation  
H3K4me2  Histone H3 lysine 4 dimethylation  
H3K4me3  Histone H3 lysine 4 trimethylation  
H3S10P   Histone H3 serine 10 phosphate
<table>
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<tr>
<td>preDII</td>
<td>Prediapause II</td>
</tr>
<tr>
<td>postDII</td>
<td>Postdiapause II</td>
</tr>
<tr>
<td>PTM</td>
<td>Post translational modification</td>
</tr>
<tr>
<td>RNA Pol II S5P</td>
<td>RNA Polymerase II serine 5 phosphate</td>
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**Figures**

**Figure 1. Representative images of preDII, DII and postDII embryos.** White arrow points to the anterior region of the preDII embryo; yolk, and a large lipid droplet is as noted. Given that the embryo rotates within the chorion forceps can be used to hold the embryo steady without damage to the embryo. White arrow on preDII (18 dpf) denotes anterior region of developing embryo. Black arrow on 9 day postDII embryo points to developing vascular tissue. Scale bar = 500 µm.
Figure 2. The number of mitotic blastomeres decreases as embryos progress towards diapause II. Embryos were fixed and stained with anti-histone H3 serine 10 phosphate (H3S10P) to detect mitotic nuclei and Hoechst 33342 to stain DNA. (A) The anterior tail region is shown for all images. 18 dpf embryo represents a pre DII embryo, 24 dpf embryo represents an embryo in early DII, and 32 dpf is a DII embryo. Scale bar = 50 µm. Representative embryos are shown (at least 18 embryos were analyzed; ≥6 embryos per trial (n=3) for each embryo stage). (B) The number of positive H3S10P cells was quantified in the tail region of embryos at various ages of preDII and DII. The data represents at least 18 embryos (analysis of ≥6 embryos per trial (n=3) for each embryo stage). The values are mean ± s.e.m. Each identical letter indicates embryo groups with no significant difference; different letters indicate a p value < 0.05. The p values were determined using one-way ANOVA followed by a Holm-Sidak’s multiple comparison test.
Figure 3. The preDII and DII embryos display similar distribution of H3K27me3. Embryos were stained with anti-H3K27me3 and Hoechst 33342. The anterior tail region is shown for all images. Two different magnifications are shown for each embryo. Scale bar = 50 µm. The data represents at least 18 embryos (analysis of ≥6 embryos per trial (n=3) for each embryo stage.
**Figure 4.** DII embryos stained with anti-H3K27me2 shows an inner nuclear membrane localization pattern. Embryos were stained with anti-H3K27me2 and Hoechst 33342. The anterior tail region is shown for all images. Two different magnifications are shown for each embryo. Scale bar = 50 µm. The data represents at least 18 embryos (analysis of ≥6 embryos per trial (n=3) for each embryo stage.
Figure 5. The post-translational methylation of H3K27 or H3K4 relative to histone H3, was determined in preDII (8 dpf, 16 dpf), DII (24 dpf, 32 dpf) and postDII embryos. ELISA was used to quantify (A) H3K27me1, (B) H3K27me2, (C) H3K27me3, (D) H3K4me1, (D) H3K4me2 and (F) H3K4me3. Each identical letter indicates embryo groups with no significant difference; different letters indicate a p value < 0.05. The p values were determined using one-way ANOVA followed by a Holm-Sidak’s multiple comparison test. The values are mean ± s.e.m. The data is obtained from 80-100 embryos per trial (n=3) for each embryo stage.
Figure 6. ELISA was used to quantify the levels of (A) HSP70 and (B) RNA polymerase II CTD repeat YSPTSPS phospho S5 (RNA Pol II S5P) from whole embryo lysate, relative to total protein, in preDII (8 dpf, 16 dpf), DII (24 dpf, 32 dpf) and postDII embryos. Each identical letter indicates embryo groups with no significant difference; different letters indicate a p value < 0.05. The p values were determined using one-way ANOVA followed by a Holm-Sidak’s multiple comparison test. The values are mean ± s.e.m. The data is obtained from 80-100 embryos per trial (n=3) for each embryo stage.
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