Understanding The Role of Protein Modifications in Development of Early Zebrafish Embryos

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ABBREVIATIONS

hpf	Hours post fertilisation
mpf	Minutes post fertilisation
mpa	Minutes post activation
RFP	Red fluorescent protein
GFP	Green fluorescent protein
AADC	Aromatic L-amino acid decarboxylase
(5-HT)	5-hydroxytryptamine
(5-HTP)	5-hydroxytryptophan
CNS	Central nervous system
PTM	Post translational modification
LB	Lysogeny broth
MQ	MiliQ water
TEMED	Tetramethylethylenediamine
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline and Triton-X 100
TBST	Tris-buffered saline and Tween-20
DAPI	4′,6-Diamidino-2-Phenylindole
RIPA	Radioimmunoprecipitation assay
PFA	Paraformaldehyde
TAE	Tris-acetate-EDTA
GABA	Gamma-aminobutyric acid
PBP	Periplasmic-binding proteins
cpGFP	circularly permuted Green Fluorescent Protein
GPCR	G-protein-coupled receptors
MDC	Monodansylcadaverine
TGM2	Transglutaminase 2

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ABSTRACT

Eukaryotic cells rely heavily on histones, a family of highly conserved proteins, to keep DNA in order and under tight control. These proteins have crucial roles in the organisation of chromosomes, which are made up of a complex of DNA and proteins called chromatin. Histones preserve genomic integrity by compacting and packing DNA, controlling gene expression, and regulating chromatin structure. Histones undergo what are known as post-translational modifications (PTMs) after they have been synthesised. The regulation of chromatin structure and gene expression relies heavily on these alterations. Histone post-translational modifications (PTMs) can regulate DNA accessibility to transcriptional machinery and attract specialised protein complexes. During embryonic development, different tissues and organs require careful regulation of gene expression. Histone modifications play an essential role in controlling gene expression, choosing cell fates, and establishing distinct cellular identities. Several histone modifications are well known and have been thoroughly studied, however a new histone modification - the serotonylation of histone H3 has been uncovered. Being fairly recent, there is much to be studied about this modification. Although serotonin is popularly known for its role as the "Happy Hormone", its functions extend far beyond, including protein modifications. Serotonin has been observed for years to be present at the pre-neural stages in several organisms, including in early zebrafish embryos, right from the one cell stage. In this body of work, we take the first steps to examine whether we can use a novel serotonin sensor as a tool to detect serotonin in early zebrafish embryos. Using western blotting and immunofluorescence, we also aim to lay the groundwork in studying this particular histone modification in early zebrafish embryos.

CHAPTER I

Detection of endogenous levels of serotonin in early zebrafish embryos

1.1 INTRODUCTION

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a neurotransmitter and hormone that plays a crucial role in the central nervous system (CNS) and various physiological processes in the human body. It is primarily synthesised in serotonergic neurons located in the raphe nuclei of the brainstem and is involved in regulating mood, appetite, sleep, memory, and cognition The biosynthesis of serotonin begins with the essential amino acid tryptophan, which is obtained from the diet. Tryptophan is converted into 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase. Following that, aromatic L-amino acid decarboxylase (AADC) decarboxylates 5-HTP to produce serotonin. This conversion process takes place in both the brain and peripheral tissues, including the gastrointestinal tract (Berger, Grey, & Roth, 2009; Young, 2007). Serotonin acts as a neurotransmitter by binding to specific receptors in the brain. It interacts with various receptor subtypes, including 5-HT1 and 5-HT7 receptors, each with distinct functions and distributions within the CNS (Hannon & Hoyer, 2008). These receptors are widely expressed in regions involved in mood regulation, such as the prefrontal cortex, amygdala, and hippocampus. The binding of serotonin to these receptors modulates neuronal activity, synaptic transmission, and the release of other neurotransmitters. Serotonin is a neurotransmitter known for its role in mood regulation, emotion regulation, appetite and satiety, sleep regulation, and cognitive functions. It is often referred to as the "feel-good" neurotransmitter due to its association with feelings of well-being and happiness. Serotonin inhibits brain circuits involved in anxiety and depression, which can lead to mood disorders like major depressive disorder and anxiety disorders. It also controls food intake and modulates hunger and fullness, acting on the hypothalamus. Disruptions in the serotonin system have been

linked to eating disorders like bulimia nervosa and binge eating disorders. Serotonin is also involved in cognitive functions such as learning and memory and modulates synaptic plasticity, which influences memory formation and consolidation processes. Alterations in serotonin levels or receptor functioning have been associated with cognitive impairments and neurodegenerative disorders, including Alzheimer's disease. (Hen, 2003)

Serotonin: more than a neurotransmitter

Although serotonin is primarily known for its role in the central nervous system, it has also been found to be present during early embryonic stages in vertebrates. This early presence of serotonin suggests that it plays a crucial role in early developmental processes. Several studies have provided evidence for serotonin's involvement in vertebrate embryogenesis. Research conducted on different vertebrate species has shown that serotonin is present during the early stages of embryonic development. In zebrafish embryos, serotonin and its synthesising enzymes have been detected as early as the one-cell stage. Serotonin appears to have important functions during embryogenesis. In zebrafish, serotonin signalling has been found to play a role in cell migration and tissue morphogenesis during gastrulation, which is a critical period of embryonic development. Furthermore, studies have shown that alterations in serotonin levels or disruption of serotonin signalling can lead to developmental defects. In zebrafish, interference with serotonin synthesis or signalling pathways results in abnormal development of the brain and craniofacial structures (Gutnick et al., 2011). Serotonin has also been found in the early stages of development of *Xenopus laevis* (African clawed frog) embryos, such as the gastrula and neurula stages (Lauder et al., 2000). Serotonin signalling has also been implicated in the regulation of left-right asymmetry during early development in vertebrates (Yasuo et al., 2009). Similarly, in Xenopus embryos, inhibition of serotonin signalling leads to abnormalities in neural development and axial patterning (Whitaker et al., 2003). Serotonin is also found to

be widely distributed in early mouse embryos across a range of tissues, including the developing nervous system, where it is involved in the regulation of axon guidance and synapse formation (Lebrand et al. 1996). Similarly, in Zebrafish embryos, the distribution of serotonin at different developmental stages and in a wide range of tissues has been observed, including in the brain, eyes, gut, and heart, as well as in non-neuronal cells such as blood and muscle cells (Hagenaars et al. 2015). The levels of various neurotransmitters at different embryonic stages in zebrafish were examined using Hydrophilic Interaction Liquid Chromatography (HILIC) coupled with tandem MS. Serotonin levels were stable, and only minor changes were visible during development from 0 dpf (days post-fertilization) to 6 dpf. The presence of serotonin during pre-neural stages suggests its involvement in fundamental developmental processes in vertebrates. Its likely functions as a signaling molecule that contributes to cell migration, tissue morphogenesis, and patterning during embryogenesis. However, further research is needed to fully understand the precise mechanisms and signaling pathways through which serotonin influences early vertebrate development. (Tufi et al., 2016)

Genetically encoded sensors for the detection of serotonin

Genetically encoded sensors are powerful tools that enable us to visualize the spatiotemporal dynamics of biomolecules within a biological system. These sensors are genetically engineered to code for a protein that consists of a native receptor for the recognition of the biomolecule in question and a luminescent / florescent protein that acts as the optical reporter. Once expressed, these sensors allow for the detection and measurement of various molecules or cellular processes in real-time, providing valuable insights into cellular dynamics and functions. One prominent class of genetically encoded sensors is the fluorescent protein-based sensor. These sensors utilise fluorescent proteins, such as green fluorescent protein (GFP) or its variants, which exhibit fluorescence upon excitation with specific wavelengths of light. By fusing these

fluorescent proteins with specific sensor domains, such as binding domains or cleavage sites, researchers can visualise and quantify the presence or activity of target molecules or cellular events. Genetically encoded sensors have emerged as powerful tools for the detection and monitoring of neurotransmitter levels in live cells and organisms. These sensors are designed by fusing specific neurotransmitter-binding domains with fluorescent proteins or other reporter molecules, enabling real-time visualisation and quantification of neurotransmitter dynamics. In recent years, genetically encoded sensors have become versatile instruments for detecting neural activity, particularly the release of several neurotransmitters such as glutamate, dopamine, norepinephrine, acetylcholine, and, more recently, serotonin. The majority of genetically encoded sensors use a unique G-protein coupled receptor (GPCR) as a sensing moiety linked to a circularly permuted fluorescent protein as the sensing moiety. Another sensor design is based on periplasmic binding proteins (PBP), which were created to assess neurotransmitters like glutamate, acetylcholine, and gamma-aminobutyric acid (GABA). A PBP-based 5-HT sensor (iSeroSnFR) was recently produced through controlled evolution of the acetylcholine sensor. Genetically encoded sensors such as iGluSnFR (improved Glutamate-Sensitive Fluorescent Reporter) have been engineered to detect changes in extracellular glutamate levels. iGluSnFR combines a glutamate-binding domain with a circularly permuted green fluorescent protein (cpGFP), allowing for sensitive and specific glutamate detection (Marvin et al., 2013). The GABA sensing protein GABABraD (GABA-Binding Receptor Activity Detector) utilizes a GABA-binding domain fused with a fluorescent protein. GABABraD enables real-time visualisation of GABA release and detection of GABA concentration changes in live cells (Wachowiak and Cohen,2001). Several genetically encoded dopamine sensors have been developed, including dLight1 and dLight2. dLight1 utilizes a circularly permuted GFP coupled with a dopamine-binding domain, allowing for high-affinity detection of dopamine release (Patriarchi et al., 2018). dLight2 employs a similar design and

enables fast and sensitive detection of dopamine dynamics (Sun et al., 2018). A genetically encoded serotonin sensor called GRAB-5HT (G-protein-coupled Receptor Activated by Light) has been engineered by replacing the intracellular domains of a serotonin receptor with light-sensitive components. This allows for optical control and monitoring of serotonin signalling (Oh et al., 2015).

sDarken – A novel genetically encoded serotonin sensor

A novel genetically encoded sensor was developed that has superior spatio-temporal resolution, high sensitivity, and fast kinetics This sets it apart from other serotonin (5-HT) sensors, which either have fast kinetics or high sensitivity but not both. The initial design was based on the dopamine sensor dLight1 that has been described earlier and uses the native human 5-HT_{1A} receptor to detect serotonin due to its high affinity for 5-HT as compared to other receptors and circularly permuted GFP (cpGFP) as the reporter. It also has the unique property of being a darkening or quenching sensor, which means that there is a reduction in florescence upon the binding of serotonin to the receptor; hence, it was named sDarken (serotonin darkening sensor, 5-HT1A receptor-based sensor). Since detection of in vivo levels of serotonin is very dynamic, two other variants of the sDarken sensor were created. The low affinity variants, named L-sDarken, were created by introducing site-directed substitutions in the serotonin binding site. The high affinity variant was created by replacing the cpGFP with a superfolder GFP. These three variants together provide an excellent set of tolls for the detection of endogenous levels of serotonin in vivo. *(*Kubitschke, Martin et al, 2022)

In this chapter, we aim to take the first steps towards determining whether the sDarken sensors can be used to detect serotonin in early zebrafish embryos by first isolating the plasmid constructs that encode the sensors and then inserting the fragment that codes for all three sDarken variants into a vector backbone specifically for expression in zebrafish

1.2 MATERIALS AND METHODS

Transformation of *E.coli* DH5 alpha cells with plasmids

Competent cells and plasmid DNA were thawed on ice for about 20 minutes. 1-3 ul of plasmid DNA was added, mixed gently by tapping, and left on ice for 30 min. Cells were heat-shocked by keeping the tubes on a heat block for 30–40 seconds and then quenched on ice for 2–10 minutes. 400–500 ul of LB without antibiotics was added to the cells and kept in a shaking incubator at 37°C. After 1 hour, the cells were pelleted, and the supernatant was added to 25 ml of Luria broth (LB) containing the antibiotic kanamycin. The pellet was dissolved in 100 ul of LB and spread on an LB agar plate containing kanamycin. The liquid culture and culture plate were incubated overnight at 37°C.

Plasmid isolation

A single colony of the transformants was picked from the culture plate and inoculated in 2 ml LB broth with Kanamycin for 8 hrs. From this starter culture, 100 ul of culture was inoculated in 100 ml LB with Kanamycin and kept overnight in the shaker incubator at 37°C at 200 rpm. The cells were pelleted by centrifugation, and the supernatant was discarded. The cell pellet was resuspended in 8 ml of resuspension buffer. 8 ml of lysis buffer were added, mixed by inverting a couple times, and incubated at room temperature for 5 min. Next, the neutralisation buffer was added and mixed gently until the solution changes from blue to colourless. The precipitate was centrifuged at 5,000 x g for at least 10 min. Meanwhile, the NucleoBond® Xtra column with the filter was equilibrated with the equilibration buffer.

The lysate was centrifuged at 5000g for 10 min, and the supernatant was applied to the equilibrated NucleoBond® Xtra Column Filter. The column was allowed to empty by gravity

flow. The NucleoBond® Xtra Column Filter and Column were washed with Equilibration Buffer and allowed to empty by gravity flow. The NucleoBond® Xtra Column Filter was removed, and the column alone was washed with the wash buffer. The DNA was eluted into a fresh 15 ml flask using the elution buffer. Isopropanol was added to precipitate the DNA, which was centrifuged at 15,000 x g for 30 min at 4°C. The supernatant was discarded, and 70% ethanol was added to the pellet. After centrifuging at 15,000 x g for 5 min at room temperature, the supernatant was removed, and the pellet was allowed to dry at room temperature for 15–20 min. The pellet was then redissolved in 100 ul of autoclaved MilliQ by gentle pipetting and stored at -20°C for further use

Qualitative and quantitative estimation of isolated plasmid

Agarose gel electrophoresis was used to qualitatively assess the isolated plasmids. To make a small 1% gel, 30 ml of 1X TAE buffer was added to 0.3 g of agarose and melted in a microwave. A 3-ul SYBR-safe gel stain was added to the melted gel and poured into the gel casting tray with the comb in place. The gel was allowed to solidify for 30 minutes and placed in the gel-running apparatus containing 1X TAE buffer. 1 ul of 6x loading dye was added to 5 ul of the DNA sample and loaded into the wells of the gel along with a 1000 bp DNA ladder. The gel was run at 80 V for 1 hour and visualised using a UV transilluminator.

Using a nanodrop spectrophotometer, the concentration of the isolated plasmid was determined. The instrument was first calibrated using MilliQ, after which 2 ul of plasmid were loaded onto the instrument using a pipette and measured at 260 and 280 nm. The absorbance at 260 nm gives the concentration of DNA and ratio of absorbance at 260/280 indicates the purity of the sample, which should be 1.8. An absorbance ratio above 2 indicates RNA contamination, and below 1.8 indicates protein contamination.

Diagnostic digest of plasmids

Diagnostic digests were performed using Thermoscientific FastDigest restriction enzymes. 5 ul reactions were setup in a PCR tube according to the following reaction conditions

Mastermix						
	1 Rxn.	**2 Rxn.**	**3 Rxn.**	**4 Rxn.**	**5 Rxn.**	**6 Rxn.**
MQ	3.3	6.6	9.9	13.2	16.5	19.8
Buffer	0.5	1	1.5	2	2.5	3
HindIII	0.1	0.2	0.3	0.4	0.5	0.6
NotI	0.1	0.2	0.3	0.4	0.5	0.6
Plasmid DNA	1 ul					
Total volume	5 ul	10 ul	15 ul	20 ul	25 ul	30 ul

The reaction mixture was mixed gently and spun down after which it was incubated at 37°C in a water bath for 30 min. The restriction enzymes were inactivated by heating for 10 minutes at 80°C. The entire volume of the reaction mixture was loaded onto a 1% agarose gel along with a 1000 bp DNA ladder and visualised using a UV transilluminator.

Gel purification of isolated fragments

To extract DNA from an agarose gel, the gel was viewed using a transilluminator, and a fresh blade was used to remove the bands from the gel. The gel slice was transferred to a clean tube and weighed using a weighing balance. 200 ul of Buffer NTI was added for each 100 mg of agarose gel. The sample was incubated at 50°C for 5–10 minutes and vortexed every 2-3 minutes. Once fully melted, the sample was loaded into the Clean-up Column in a Collection Tube and centrifuged for 30 seconds at 11,000 x g. The flow-through was discarded. The silica membrane was then washed with 700 ul of Buffer NT3 and centrifuged for 30 seconds at

11,000 x g. The silica membrane was given a dry spin for 1 minute at 11,000 x g. The DNA was then eluted by adding 30 ul of Buffer NE. The eluted fragments were stored at -20°C for further use.

Cloning of sDarken fragments into pCS2P+ vector

The sDarken fragments were isolated from their original plasmid constructs using the restriction enzymes NotI and HindIII. The isolated fragments were run through a 1% agarose gel, and the specific bands were purified from the gel. Then, a suitable cloning vector, here pCS2P+, was prepared by digesting it with the same enzymes and was also gel purified.

The DNA fragment was then ligated into the vector using the T4 DNA ligase enzyme according to the following reaction conditions

Mastermix				
	1 Rxn.	**2 Rxn.**	**3 Rxn.**	**4 Rxn.**
5 x Reaction buffer	4 ul	8 ul	12 ul	16 ul
MQ	15.4 ul	31.6 ul	47.4 ul	63.2 ul
T4 DNA Ligase	0.2 ul	0.4 ul	0.6 ul	0.8 ul
Vector DNA	0.1 ul			
Insert	0.3 ul			
Total Vol.	20 ul	40 ul	60 ul	80 ul

The reaction mixture was mixed gently and spun down, after which it was incubated at room temperature for 5 minutes. This ligated plasmid was then transformed into competent *E.coli* DH5alpha cells, following which the recombinant plasmids were isolated

RESULTS

Recovery of sDarken constructs from plasmid stocks:

The three variants of the genetically encoded sensor sDarken were received as agar stabs and stored at 4 °C. These stock plasmids were given lab numbers for identification.

401: sDarken

402: L-sDarken

403: H-sDarken

The heat shock method was used to transform each of the three constructs into chemically competent *E.coli* DH5 alpha cells that were made in the lab. The transformed cells were then given an hour to recover at 37 in an orbital shaker suspended in LB broth without any antibiotic before being pelleted and then plated on selective media consisting of LB agar and the antibiotic kanamycin. The supernatant was added to 25 ml of LB broth with kanamycin. The following day, single colonies of the transformants were inoculated in 5 ml of LB Broth containing Kanamycin and allowed to grow overnight. The following day, the cultures were used to isolate the plasmids using a plasmid isolation kit, and the plasmids were qualitatively assessed by performing agarose gel electrophoresis (1% gel) as well as quantitatively assessed using nanodrop spectroscopy.

Two of the three constituents—402 and 403—were successfully isolated. Although 401 was successfully transformed, there were multiple issues with its isolation. The first issue was that the bacterial cells grown from the single colonies of transformants in liquid culture were resistant to lysis. In order to resolve this problem, several troubleshooting strategies were carried out, such as doubling the amount of lysis buffer and reducing the volume of culture used for plasmid isolation. After several rounds of reculturing the 401 colonies in order to weed

out any contaminants and optimize the amount of culture to be taken for efficient lysis of the cells, we obtained transformants that were successfully lysed. The second problem was that after the cells were lysed, the concentration of plasmid DNA being isolated using the Miniprep method, which uses 3-5 ml of culture, was far too low. So, to resolve this issue, 100 ml of culture containing 401 was grown, and the plasmid was isolated using the Midiprep method. This method finally produced a sufficient concentration of plasmid 401 to proceed to the next step. Finally, all three plasmid constructs were isolated using the Midiprep method and stored as stocks at -20°C for further experiments.

Diagnostic digest of the isolated plasmids:

In order to confirm that the correct constructs had been isolated before using them for further experiments, the plasmids were cut using HindIII and NotI restriction enzymes, which were on either side of the sDarken, L-sDarken, and H-sDarken inserts. This caused the inserts to be separated from the pN1 backbone, producing two bands of specific lengths. Of the three constructs, 402 and 403 produced clear, sharp bands of the required length, while 401 did not.

Since the diagnostic digest of 401 did not produce conclusive results, we suspected possible methylation of the 401 constructs, which was hindering the restriction digestion of the plasmid. In order to troubleshoot this problem, BglII, a methylation insensitive restriction enzyme, was used to cut all three constructs. 402 and 403 were successfully linearized and produced bands of the correct length; however, 401 did not.

Hence, we decided to move forward with 402 and 403 for cloning purposes while continuing to troubleshoot the issues faced with 401.

Isolating the vector backbone:

Since the constructs have to be used to produce RNA that will be microinjected into zebrafish, selecting a vector backbone that will easily facilitate that process was necessary. The lab had two empty vector backbone constructs that were specifically designed for expression in zebrafish, namely pCS2+ and pCS2P+. Both vectors were analysed, and pCS2P+ was chosen as the backbone to be used for cloning the L-sDarken and H-sDarken fragments. pCS2P+ had the restriction sites HindIII and NotI that would produce complementary sticky ends with the L-sDarken and H-sDarken inserts, allowing for ligation and cloning of the inserts into the pCS2P+ backbone. Chemically competent *E.coli* DH5 alpha cells were transformed using pre-existing stocks of pCS2P+, and the plasmid was isolated using the Midiprep method and stored for further use at -20°C. In order to confirm that the correct construct had been isolated, a diagnostic digest was conducted for pCS2P+ using NotI and HindIII.

Cloning of the inserts into the backbone:

Preparing the inserts and backbone for cloning

402, 403, and pCS2P+ were cut using the restriction enzymes HindIII and NotI in order to produce complimentary cohesive ends between the inserts and the backbone. The digested products were run through a 1% Agarose gel until the fragments were sufficiently resolved. All three fragments were then cut out of the gel and purified using a gel extraction kit (Macherey Nagel).

Ligation of inserts and backbone Using T4 DNA Ligase in a 1:5 ratio, the inserts were joined to the vector backbone, and then *E.coli* DH5 alpha cells were made from them. The transformed cells were plated onto a selective plate with ampicillin and incubated overnight

Plasmid	Conc. (ng/ul)	260/280 nm ratio
401	140.3	1.82
402	339.4	1.902
403	301.4	1.969

FIGURE 1: A: Transformants with sDarken (401), L-sDarken (402) and H-sDarken (403) on selective Kanamycin plates respectively **B:** Table depicting the concentration and 260/280 nm ratio of the isolated plasmids **C, D, E :** Plasmid maps of s-Darken, H-sDarken and L-sDarken respectively (created with created with SnapGene) **F:** Plasmid map of pCS2P+ (created with SnapGene)

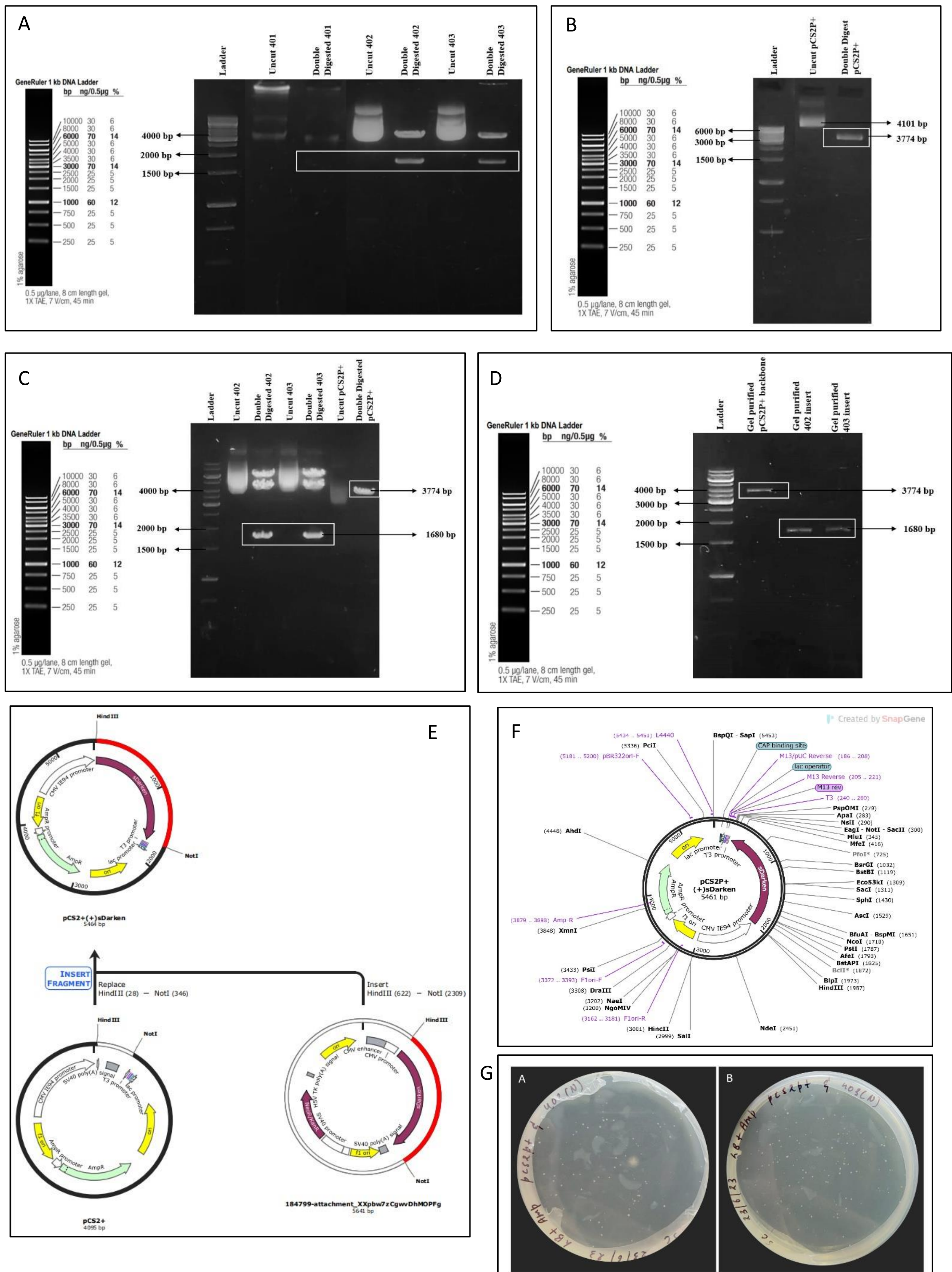


FIGURE 2: A: Gel depicting the diagnostic digest of sDarken (401), L-sDarken (402) and H-sDarken (403) using NotI and HindIII **B:** Gel depicting the diagnostic digest of pCS2P+ using NotI and HindIII **C:** Isolation of inserts L-sDarken (402), H-sDarken (403) and vector backbone pCS2P+ from gel **D:** L-sDarken (402), H-sDarken (403) and vector backbone pCS2P+ fragment after extraction from gel **E:** Schematic representing the ligation of inserts into vector backbone
F: Plasmid map of expected plasmid after cloning inserts into pCS2P+ **G:** Transformants of cloned L-sDarken (402) + pCS2P+ and H-sDarken (403) + pCS2P+ respectively

DISCUSSION AND CONCLUSIONS

The sDarken serotonin sensors have three variants: the normal sDarken sensor, the low affinity version of the Serotonin Sensor (L-sDarken), and the High affinity version of the Serotonin sensor (H-sDarken). These sensors were encoded within plasmids with pN1 as the vector backbone and are under the control of the CMV promoter. To increase the concentration of the original stock plasmids, they were transformed into chemically competent cells and recovered using plasmid isolation kits (Macherey Nagel). The plasmids were confirmed to be the right constructs by performing a diagnostic digest using two restriction enzymes on either end of the inserts, namely NotI and HindIII (Thermo Fast Digest Restriction Enzymes). Of the three variants, two, namely L-sDarken (402) and H-sDarken (403), were successfully isolated and confirmed, while the results of experiments with the third sDarken (401) have been inconclusive and are still being resolved. Next, an empty vector backbone known for expression in zebrafish, pCS2P+, was also transformed into chemically competent cells and isolated. A diagnostic digest using the same enzymes, NotI and HindIII, confirmed that the right construct had been isolated. Both the inserts and the backbone had restriction sites that would result in the formation of cohesive ends, ensuring that the inserts would be correctly oriented when subjected to ligation.

To clone the L-sDarken and H-sDarken inserts into the pCS2P+ backbone, all three constructs were cut using NotI and HindIII. The fragments were resolved using agarose gel electrophoresis (1%) and the appropriately sized bands were cut out of the gel and sequentially purified from the gel using a gel extraction kit (Macherey Nagel). Once the fragments were purified, the inserts were ligated to the pCS2P+ vector using T4 DNA Ligase. 2 ul of the ligated mixture was used to transform the chemically competent cells, and the transformants were plated on selective media containing ampicillin. Single colonies were obtained after overnight

incubation. Currently, these transformants are being screened in order to identify and isolate the correct constructs.

Once the correct cloned constructs are identified, the plasmid can be used to synthesize RNA in vitro. This RNA will then be injected into zebrafish oocytes at the 1-cell stage and tracked at various stages during its early embryonic development in order to deduce whether this genetically encoded sensor was expressed successfully in zebrafish and whether it can be used as an efficient tool to detect endogenous levels of serotonin early in its embryonic stages.

Chapter 2

Detection of the presence of serotonylated histones in early zebrafish embryos

Histones are a family of small, basic proteins that exhibit remarkable conservation across species. They are primarily responsible for packaging DNA and organising the genome within the confines of the cell nucleus. Through their interactions with DNA and other proteins, histones establish the foundation for chromatin structure and function. Histones are divided into five main types: H1, H2A, H2B, H3, and H4. These proteins form octameric complexes known as nucleosomes, which are the basic repeating units of chromatin. Nucleosomes consist of an octamer core composed of two copies each of H2A, H2B, H3, and H4, around which DNA is wrapped in a left-handed super helical turn. The assembly of DNA into nucleosomes creates a condensed chromatin structure that restricts access to the underlying genetic material. Chromatin remodelling complexes utilise ATP-dependent mechanisms to modulate nucleosome positioning, spacing, and occupancy, thereby regulating DNA accessibility and controlling gene expression. Histones undergo a variety of post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and more. These modifications occur on specific amino acid residues within the histone tails and modulate chromatin structure and function. Different combinations of modifications create a histone code that provides a dynamic and reversible mechanism for gene regulation. Histone modifications and the chromatin landscape can be stably transmitted through cell divisions and, in some cases, across generations. Epigenetic marks carried by histones can contribute to the establishment of cell identity, development, and responses to environmental cues. Embryonic development in vertebrates involves a series of intricate and precisely orchestrated events that give rise to a complex organism. Histone PTMs, by regulating chromatin structure and gene expression, play a pivotal role in modulating the spatiotemporal gene expression patterns that

drive embryogenesis. Histone modifications are involved in chromatin remodelling processes that occur during developmental transitions. Histone modifications can activate or repress the expression of developmental genes during embryogenesis. For example, histone acetylation, particularly at lysine residues, is associated with gene activation and an open chromatin state, allowing for the accessibility of transcriptional machinery to initiate gene expression. On the other hand, certain histone methylation marks, such as trimethylation of lysine 27 on histone H3 (H3K27me3), are associated with gene repression and a closed chromatin conformation. These modifications, along with ATP-dependent chromatin remodelling complexes, help modulate chromatin structure, nucleosome positioning, and accessibility to regulatory elements. Such remodelling is critical during key developmental events, such as gastrulation, neurulation, and organogenesis, allowing for the activation or repression of specific sets of genes required for each developmental stage.

Zebrafish as a model to study development

Zebrafish (Danio rerio) is a widely used model organism for studying developmental biology due to its advantages such as external development, rapid development, large clutch size, genetic and molecular tools, regenerative abilities, evolutionary conservation, cost and ethical considerations, and relevance to human biology. Zebrafish embryos develop externally, allowing for easy observation and manipulation during various stages of development. They are transparent, allowing researchers to directly visualise developmental processes such as cell division, tissue formation, and organogenesis under a microscope without invasive techniques. Rapid development and large clutch sizes enable researchers to study multiple stages of development in a relatively short time. Zebrafish produce a large number of embryos in each clutch, facilitating large-scale experiments and statistical analysis. Genetic and molecular tools, such as gene knockdown, transgenesis, and tissue-specific gene expression control, enable

precise manipulation of gene function in zebrafish embryos. Regenerative abilities in the fins, heart, and central nervous system make zebrafish an excellent model for studying tissue regeneration and repair mechanisms, which have implications for regenerative medicine and understanding human developmental disorders. Evolutionary conservation in zebrafish embryos provides insights into fundamental developmental mechanisms relevant to human biology and disease. Lastly, the zebrafish's small size and minimal ethical concerns make them an ethically favourable choice for research. Overall, the zebrafish's versatility, genetic tractability, and relevance to human biology make it an invaluable tool for advancing our understanding of developmental processes and human health.

Serotonylation of proteins

Serotonin is primarily known for its role as a neurotransmitter in the central nervous system, where it plays a crucial role in mood regulation, appetite, and sleep. However, it has been found that serotonin can also covalently modify proteins outside the nervous system, suggesting that it may have broader biological functions. Serotonylation of proteins, also known as serotonylation, is a post-translational modification (PTM) process where the neurotransmitter serotonin (5-hydroxytryptamine, or 5-HT) is covalently attached to proteins. This modification is discovered relatively recently and is still an area of active research. The process of serotonylation involves the enzymatic transfer of serotonin to specific amino acid residues on target proteins. The exact mechanisms and enzymes responsible for this modification are not yet fully understood, but some evidence suggests that transglutaminase enzymes may be involved. Transglutaminases are a family of enzymes that can transfer various small molecules, including serotonin, to proteins. Serotonylation has been found to occur on a variety of proteins in different tissues and cell types. One of the well-studied targets of serotonylation is actin, a

cytoskeletal protein involved in cell structure and movement. Serotonylation of actin has been implicated in the regulation of cytoskeletal dynamics and cell migration.

Serotonin and transglutaminase are localized in the nucleus

Nuclear localization of serotonin has been studied in a range of cell types, including human lymphocytes (super helical et al. 2000), primary neurons (Côté et al. 2003), adrenal gland cells, and liver cells (Csaba and Kovacs 2006). Nuclear localization has been shown to regulate the expression of genes involved in neurotransmitter synthesis and metabolism in the rat brain (Konradi et al. 1994). The nuclear localization of serotonin in the early embryonic stages in both vertebrates and invertebrates is investigated in *Xenopus laevis* oocytes and early embryos, particularly during the stages of rapid cell division (Fillion and Trudeau 2002), as well as the expression and localization of serotonin receptors in the nucleus of blastomeres at the eight-cell stage of development in mouse embryos (Bonnin et al. 2001) Transglutaminase 2 (TGM2) is a multifunctional enzyme belonging to the transglutaminase family. TGM2 catalyzes the covalent crosslinking of proteins by forming epsilon-(gamma-glutamyl) lysine bonds between glutamine and lysine residues (Lorand and Graham, R. M. 2003). Using immunohistochemistry, western blot analysis, and transglutaminase inhibition experiments, nuclear localization of serotonin is observed in sea urchins (blastula stage) and zebrafish (32-128 cell stages), not only at various early embryonic stages but also at different phases of the cell cycle (Ivashkin E et al. 2019). TGM2 has been shown to play an important role in the nuclear localization of serotonin in early embryos of invertebrates and vertebrates, wherein blocking the activity of TGM2 using inhibitors such as cystamine or monodansylcadaverine (MDC) leads to a decrease in the anti-5-HT signal in the nucleus.

Histone serotonylation

Several proteins that have been reported to undergo serotonylation include tubulin, histones, and various signalling molecules. Serotonylation of histones is a relatively newly discovered post-translational modification, and the exact mechanisms and enzymes responsible for histone serotonylation are not yet fully understood. However, some studies suggest that transglutaminase enzymes, such as transglutaminase 2 (TGase2), may be involved in this modification. Transglutaminases are enzymes capable of transferring small molecules, including serotonin, onto proteins. The functional consequences of histone serotonylation are still being investigated. It is proposed that this modification may influence chromatin structure, gene expression, and cellular processes regulated by histone modifications. Histone modifications, including serotonylation, can affect the accessibility of DNA to transcriptional machinery, thereby influencing gene transcription.

Histone modifications are covalent post-translational modifications that occur on amino acid residues of histone proteins. These modifications play a critical role in regulating gene expression by modulating the accessibility of DNA to transcriptional machinery. Serotonylation of histone H3 at the fifth glutamine position is the first monoaminyl modification identified. (Farrelly et al 2019). It is definitively proven that H3 is a substrate for the serotonylation catalysed by TGM2, while H2A, H2B, and H4 were not, and TGM2 is in fact critical for the serotonylation of H3, resulting in a H3Q5ser modification.

Histone H3 proteins are known to be highly conserved across all eukaryotes and are subject to many post-translational modifications (Waterborg, J. H., 2012). Histone H3.1 is a nucleosome core component that is exclusive to mammals. It is expressed during S phase, and then its expression significantly decreases as cell division pauses during the differentiation process.

Expression of histone H3.1 is shown to be replication-dependent. It functions as the standard histone that is incorporated during DNA replication. (Zhang et al., 2012). Serotonin-producing organisms can serotonylate glutamine at position 5 (Q5ser) on histone 3.1. Both the single modification (H3Q5ser) and the dual modification (H3K4me3Q5ser) were detected in cultures of serotonergic cells, but only the dual modification H3K4me3Q5ser is identified in vivo in the brain of mice and across a wide range of 5-HT-producing organisms from drosophila to humans. H3K4me3Q5ser is found to have a ubiquitous pattern of expression, enriched in organs producing 5-HT (such as brain and colon) and showing more-limited signal in some non-serotonergic organs. Robust signals were also observed in the heart, circulating blood, and testes. Despite the fact that methylation at lysine 4 and serotonylation at glutamine 5 are in proximity to each other, no cross-talk between the two modifications is observed. The functional role of H3K4meQ5ser is examined in human serotonergic neurons derived from hPSC's, developing mouse brains, and cultured serotonergic RN46A-B14 cell culture model using CHIP-seq analysis. Consistent results were obtained across all three models, revealing enrichment of the H3K4meQ5ser marker in differentiated cells. 60% of the genes displayed alterations in H3K4me3Q5ser signal post-differentiation. Most of these alterations were found to be an increase in the H3K4me3Q5ser signal within the gene promoter regions.

H3K4me3 is a common histone modification that plays an important role in regulating gene expression. This refers to the trimethylation of the lysine 4 residue on histone H3, one of the core histone proteins that make up the nucleosome. H3K4me3 is generally associated with active transcription of genes and is thought to be involved in the recruitment of transcription factors and other chromatin-associated proteins to gene regulatory regions (Shilatifard, A. 2012).

TFIID is a multi-subunit protein complex that plays a critical role in initiating transcription by binding to the promoter regions of genes which has been shown to interact with modified

histones, specifically H3K4me3, to facilitate transcriptional activation (Lauberth, Shannon M et al. 2013).

The presence of the Q5ser mark, along with the K4me3 mark on H3, potentiated the binding of TFIID to H3K4me3. Of all potentiated K4me3 readers examined, 15 were TFIID complexes. The association of TFIID with the dual modification H3K4me3Q5ser is enhanced in the post-differentiation phase and peaked at Transcription Start Sites (TSS).

Western blotting

SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) and Western blotting are commonly used techniques in molecular biology and protein analysis. SDS-PAGE is used to separate proteins based on their molecular weight in a denatured state.

A polyacrylamide gel is prepared with varying concentrations to achieve different resolving ranges. The gel is cast between glass plates, and a comb is inserted to create sample wells. Proteins are denatured by boiling in the presence of SDS, a detergent that disrupts protein structure and imparts a negative charge. A reducing agent (e.g., mercaptoethanol or DTT) is often added to break disulfide bonds. The denatured protein samples are loaded into the sample wells, and an electric current is applied. The negatively charged proteins migrate through the gel matrix, with smaller proteins moving faster than larger ones. After electrophoresis, the proteins within the gel are visualised by staining with a protein-specific dye (e.g., Coomassie Brilliant Blue). Western Blotting, also known as immunoblotting, is a technique that deals with protein detection using specific antibodies. It allows the identification of a target protein within a complex protein mixture. Following gel electrophoresis, the proteins are transferred from the gel onto a solid membrane (commonly nitrocellulose or PVDF) using electroblotting. This process transfers the proteins into their separated states, maintaining their relative

positions. The membrane is blocked with a protein-based blocking agent (e.g., BSA or milk) to prevent the non-specific binding of antibodies. The membrane is incubated with a primary antibody specific to the target protein of interest. The primary antibody binds to the target protein if it is present on the membrane. The membrane is then incubated with a secondary antibody conjugated with an enzyme or a fluorescent tag. The secondary antibody recognises and binds to the primary antibody, enabling the detection of the target protein. The presence of the target protein is detected by visualizing the enzyme-conjugated secondary antibody using a substrate that produces a signal (e.g., chemiluminescence) or by directly visualizing the fluorescent tag. By combining SDS-PAGE separation and antibody-based detection, Western blotting is used to identify specific proteins within a complex mixture, determine protein size and investigate protein modifications.

Immunofluorescence of whole zebrafish embryos

Immunofluorescence is a technique used to visualize the presence and distribution of specific molecules, such as proteins or other antigens within cells or tissues. Using antibodies that are selectively designed to recognize and bind to specific protein modifications this technique provides valuable insights into the localization, abundance, and spatial distribution of various post-translational modification in proteins. Two-step immunofluorescence employs primary antibodies that specifically recognize the target antigen of interest. These primary antibodies are not directly conjugated to fluorophores but are instead detected using secondary antibodies that are conjugated to fluorophores. The secondary antibodies recognize and bind to the primary antibodies, enabling their detection my fluorescent microscopy. Zebrafish embryos are collected at the desired developmental stage and fixed using an appropriate fixative which preserves the morphology and integrity of the embryos. Fixed embryos are then permeabilized to allow the antibodies to penetrate the tissues. To reduce nonspecific binding of antibodies,

the embryos are treated with a blocking solution containing proteins, such as bovine serum albumin (BSA) after which the embryos are incubated with primary antibodies specific to the antigen of interest. Primary antibodies can often show cross-species reactivity which means that antibodies raised in one species can recognize and bind to its target antigen in another species, given that there is homology between the amino acid sequences. The embryos are then incubated with secondary antibodies that are conjugated with fluorochromes. The secondary antibodies should be chosen based on the species from which the primary antibodies were raised. Excess secondary antibodies are washed away to minimize background staining. If nuclear proteins are being targeted, the embryos are also stained with DAPI which marks DNA. The embryos are then mounted on slides in a mounting medium that preserves the fluorescence signal and imaged using a fluorescence microscope equipped with the appropriate filters for the fluorophores used in the staining process.

In this chapter, we aim to use the techniques of western blotting and immunofluorescence to detect the presence of the serotonylated histones in early zebrafish embryos.

MATERIALS AND METHODS

WESTERN BLOTTING:

Dechorionation of embryos

In-vitro fertilized eggs or water-activated oocytes were collected in a glass dish using a plastic Pasteur pipette. A pen was placed under one edge of the glass dish to maintain the dish tilt. To prevent lysis, the extra E3 embryo medium was

removed, while ensuring that the embryos were not exposed to air. 100 embryos were treated with 100 µL of pronase (20 mg/ml dissolved in autoclaved MilliQ water), ensuring that every embryo was submerged. To achieve uniform treatment, embryos were incubated in pronase solution for 5 min, followed by mild agitation. The embryos were examined under a microscope to check for chorionic membranes. To facilitate dechorionation, E3 medium was gradually introduced along the glass dish walls, causing the embryos to move in a swirling manner. This swirling process was performed numerous times until all the embryos were dechorionated. To avoid lysis, the dechorionated embryos were gardened using a glass pipette and kept in an additional glass plate containing E3 medium between cycles of E3 medium washes. By switching out the E3 medium, pronase was eliminated from the dechorionated embryos.

Deyolking embryos

The embryos were dechorionated using pronase, counted, and then transferred to a 1.5 ml microcentrifuge tube. Most of the embryo media was removed, and 200 ul of deyolking buffer per 100 embryos was added. The embryos were gently pipetted to disrupt the yolk sac. The sample was then placed in a Thermomixer for 5 minutes at 1100 °C. The cells were pelleted at 300 g for 30 seconds, and the supernatant containing the yolk was removed and stored at -20°C

for later use. 1000 ul per 100 embryos of washing buffer was added to the cell pellet and placed in the thermomixer again for 2 minutes at 1100 rpm at 28 °C. The sample was centrifuged at 300 g for 30 seconds, and the supernatant was discarded. The cell pellet was stored at -20 °C until used for protein extraction.

Protein extraction from zebrafish embryos:

For protein extraction, embryos were drained of embryo media and transferred to a 1.5 ml Eppendorf microcentrifuge tube on cold. The extraction of proteins was performed using ice-cold RIPA lysis buffer. At 70 ul/ 100 embryos of lysate buffer was used for extraction. After the addition of lysis buffer, the sample was homogenized with a micro pestle. After homogenization, the samples were centrifuged at 4°C for 20 minutes at 17,970 g. The particle was discarded, while the supernatant was transferred to a new tube. The supernatant was labelled and stored at -20 degrees C for future use.

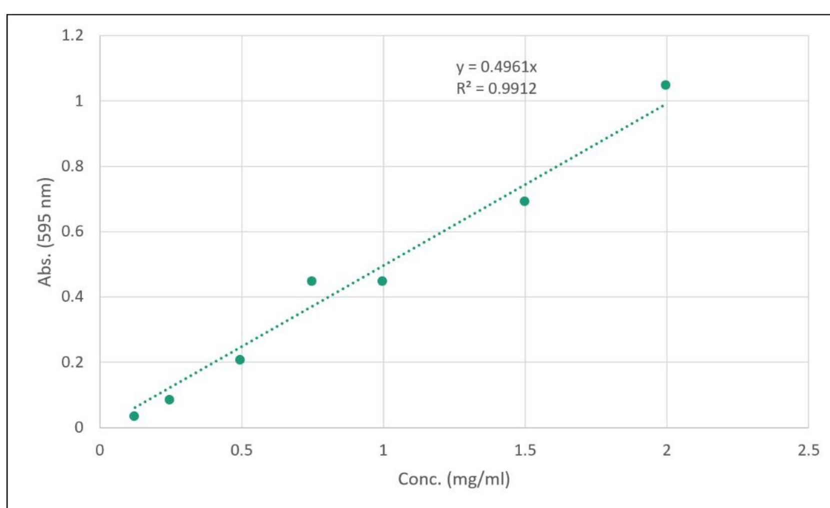
Serial dilutions of BSA used to create the standard curve

Tube no.	Concentration of BSA (mg/ml)	Volume of BSA (µl)	Volume of PBS (µl)
1.	2.0 mg/ml	70 µl	-
2.	1.5 mg/ml	30 µl from Tube 1	10 µl
3.	1.0 mg/ml	20 µl from Tube 1	20 µl
4.	0.75 mg/ml	20 µl from Tube 2	20 µl
5.	0.50 mg/ml	20 µl from Tube 3	20 µl
6.	0.25 mg/ml	20 µl from Tube 5	20 µl
7.	0.125 mg/ml	20 µl from Tube 6	20 µl
8.	Blank (2 Blank Tubes)	-	20 µl

Estimation of protein concentration

The Bradford method was used to calculate the protein concentration. BSA (bovine serum albumin) was used in varying concentrations as the protein standard. The protein test sample consisted of 10 ul of protein lysate diluted with 10 ul of PBS. On a spectrophotometer, the absorbance was measured at 595 nm. The absorbance values of standard protein samples yield a linear graph and equation necessary to calculate the concentration of unknown protein samples.

Conc.(mg/ml)	Abs. (595 nm)
0.125	0.034
0.25	0.084
0.5	0.205
0.75	0.446
1	0.448
1.5	0.691
2	1.046



Calculation for protein concentration of sample:

Concentration (x) = absorbance (y) / m (here, m = 0.4961)

Sample	Abs. (595 nm)	Conc. of samples (mg/ml)
Deyolked	0.51	1.028
Non-deyolked	1.42	2.862

Representative table and graph of standard curve for estimation of protein concentration in samples

SDS-PAGE (Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis)

The volume of the components of the resolving gel was decided according to the percentage of the gel required which depends on the size of protein to be isolated. The gel was loaded onto a 1 mm glass plates and overlaid with isopropanol. The gel to allowed to polymerize for 30 minutes. A stacking gel was made using the same recipe which contains 1M Tris of pH 6.8. TEMED was added to the gels just before loading. The Mini Protean Tetra cell tank was

filled with Running Buffer up to the mark mentioned on the tank and the comb was removed only after adding Running Buffer. Protein sample was prepared by mixing 50 µg - 70 µg of protein sample with Laemmli Dye and RIPA Buffer with protease inhibitor. The sample mixture was vortexed for 10 seconds, subjected to a short spin for 20 seconds and heat the protein sample mixture at 95°C for 5 minutes. The mixture was loaded into the wells and the gel was run until dye front reaches the base of the glass plate. The separated proteins were then transferred to a PVDF (polyvinylidene fluoride) membrane using transfer apparatus and buffer, overnight for 16 hours at 55 mA. The PVDF membrane was charged with methanol and the transfer cassette was set up with the gel towards the negative side and the PVDF membrane towards the positive side, cushioned between a sandwich of sponges and cotton pads. Once the transfer was complete, the PVDF membrane can be stained with ponceau stain to confirm the efficient transfer of the bands.

STACKING GEL

Component	2 ml
Water	1.4
Acrylamide mixture	0.33
1 M Tris pH 6.8	0.25
10% SDS	0.02
10% APS	0.02
TEMED	0.002

15% RESOLVING GEL

Component	5 ml
Water	1.2
Acrylamide mixture	2.5
1.5M Tris pH 8.8	1.3
10% SDS	0.05
10% APS	0.05
TEMED	0.004

Calculations for protein sample preparation:

Since samples were diluted 1:1 in 1X PBS:
Final protein concentration = 2* conc. of samples

To load 50 ug of protein:
Volume of sample to be taken = 50 / final protein conc.

For a sample mixture of 50 ul:

Volume of Laemmli dye = Size of the well (here 30 ul) / Laemmli stock concentration (here 6x)
= 5 ul

Volume is made up to 50 ul using RIPA buffer

Sample	Conc. of samples (mg/ml) *2	Amount of sample to be taken (ul)	Laemmli Dye (ul)	RIPA (ul)
Deyolked	2.056	24.318	5	20.681
Non-deyolked	5.724	8.734	5	36.265

Representative table for protein sample preparation to run a SDS – PAGE gel

Primary and secondary antibody incubation

To remove Ponceau stain the PVDF membranes were washed with MilliQ water and stored at 4°C in TBST before blocking them with 5% skimmed milk. The membranes were rinsed with TBST to remove excess blocking solution. The primary antibody solutions were prepared in 5% BSA and stored at 4°C. A small container was chosen for incubation and a minimum of 500 ul of the antibody was added. The blot was incubated overnight at 4°C on a rocker after which the entire Antibody content was recovered and stored back at 4°C. The blot was washed in TBST four times for five minutes each. A secondary antibody dilution was made in 5% skimmed milk and the blot was incubated at room temperature for an hour. The blot in then washed in TBST four times for five minutes each and can be stored in TBST before imaging. A Luminol/Enhancer solution and Stable Peroxide Solution mixture (1:1) was made in an Eppendorf covered in aluminum foil. For a blot with three lanes, 500 µl mixture

was sufficient. For chemiluminescent Western blots, the Image Quant LAS 500 system was used.

BUFFERS :

1X Deyolking Buffer (50 ml)

Reagents	Required Concentration	Stock	Volume taken from stock
NaCl	55 mM	1M	2750 µl
KCl	1.8 mM	250 mM	360 µl
$NaHCO_3$	1.25 mM	1M	62.5 µl

Dissolve in 50 ml MQ H_2O

1X Washing Buffer (50 ml)

Reagents	Required Concentration	Stock	Volume taken from stock
NaCl	110 mM	1M	5500 µl
KCl	3.5 mM	250 mM	700 µl
$CaCl_2$	2.7 mM	250 mM	540 µl
Tris HCl pH 8.5	10 mM	250 mM	2000 µl

Dissolve in 50 ml MQ H_2O

RIPA lysis buffer (10 ml)

Reagents	Required Concentration	Stock	Volume taken from stock
NaCl	150mM	1M	1500 µl
Triton-X 100	1%	100%	100 µl
SDS	0.1%	10%	100 µl
Tris pH 7.4	50mM	250mM	2000 µl

Running Buffer 10X:

Dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H_2O. The pH of the buffer should be 8.3 and no pH adjustment was required. Store the running buffer at room temperature and dilute to 1X before use.

Transfer Buffer 10X:

Dissolve 29.0 g of Tris base, 145.0 g of glycine in 1000 ml of H_2O. The pH of the buffer should be around 9.2 and no pH adjustment was required. Store the transfer buffer at 4 °C and dilute to 1X before use.

To make 1X Transfer Buffer from 10X: Mix 100 ml of 10X Transfer Buffer, 200 ml of methanol and 700 ml of double distilled water. (Methanol was to be added only to 1X working solution before use)

IMMUNOLABELLING OF EARLY ZEBRAFISH EMBRYOS

Animal husbandry

Zebrafish (Danio rerio) were maintained in an aquatic facility under 14 h light and 10 h dark cycles at 27°C. Fish were paired in mating cages the night before embryos were harvested the next morning.

Extrusion of Eggs from Gravid Female Zebrafish

The night before the tests, two adult male and female zebrafish were placed in the mesh-bottomed aquariums. The females were observed for egg ejection the next morning. Egg-laying pairs were separated, and the females were maintained in a separate fish tank to disrupt their mating behavior to ensure that females retained eggs for manual egg extrusion. Then, in 250 ml glass beakers, 20 ml of a 0.4% MESAB (common anesthetic for fish) stock solution was diluted in 150 ml of fish facility water to anesthetize the primed females. After being submerged in the anesthetic until their gill movements began to slow down, they were removed from the beaker and given a quick rinse with facility water. To avoid egg activation by water, eggs were stored on a tissue bed and gently wiped dry before extrusion. The anal fin and cloacal region of the fish were then exposed by placing them horizontally on a dry petri dish. The urogenital entrance was wiped dry to remove any water remaining after the anal fin was extended. To expel mature eggs, the abdomen was gently squeezed downward towards the cloacal opening using a flat spatula. To decrease stress on females, the spatula was always kept parallel to the path of action. The extruded eggs were carefully designed to avoid contact with water. Afterward, the females were returned to the fish tanks so they could recover from the

extrusion procedure.

Preparing the solution for the spermatozoa

A male fish was anesthetized in 20 ml of 0.4% MESAB diluted with 90 ml of facility water for 10 min. Fish were retrieved from the glass beaker using a plastic spoon until all gill movements ceased (typically after 4-5 minutes). It was then gently rinsed with facility water and patted dry prior to dissection. The fish were decapitated and placed on a Petri plate for dissection under an incident light microscope. An incision was made from the abdomen to the ventral side of the pelvic fin. After carefully removing the gut with forceps, the swim bladder testicles on either side were examined. The testicles were then separated and stored in 1 mL Hank's buffer. To release sperm into the fluid, the testes were macerated with a pipette tip and pipetted up and down once. The 1.5 ml tube was filled with tissue, which was allowed to sink to the bottom. The supernatant was then examined under an incident light microscope to identify motile sperms. The procedure was repeated if no visible motile sperm was found.

Water activation of Oocytes

Gravid female zebrafish were used to extrude eggs, which were then spread in a Petri dish. MilliQ water was added to the eggs to water activate them after which the plate was flooded with E3 media.

In-vitro fertilization

Gravid female zebrafish were used to extrude eggs, which were then spread out in a monolayer on a Petri dish. 400-500 eggs were extruded from a healthy female, and 200ul of spermatozoa solution was placed on top, ensuring that the sperm solution was uniformly distributed. To start

egg activation and subsequent fertilization, 2-3 drops of E3 medium were introduced to the egg monolayer ten seconds after addition. To distribute the sperm fluid and fertilize the eggs equally, the eggs were spun. E3 medium was introduced and allowed to completely fill the petri plate completely after two minutes.

Fixation of embryos

The embryos/ oocytes were dechorionated using pronase. To study different time points, fixations were performed using batches of synchronously fertilized or water-activated eggs. Water-activated eggs were fixed at 10, 20, and 30 mpa (minutes post activation), whereas fertilized eggs were fixed at 10, 15, and 20 mpf (minutes post fertilization). Embryos were fixed using 4% paraformaldehyde (PFA) after in vitro fertilization, water activation, and dechorionation. The embryos were incubated in fixative for 6 h at room temperature, after which they were stored at 4°C overnight.

Immunolabeling of fixed embryos

After overnight fixation, the fixative was discarded, and the embryos were gently rinsed with 1 ml PBS for 5 min. They were then placed into a 1 ml tube with 100% methanol and maintained at -20°C for permeabilization the following day. Embryos can be maintained in 100% methanol for up to 4 weeks.

After permeabilization overnight, the embryos were rehydrated using MeOH: PBS serial washes of 75%, 50%, and 25% MeOH in 1X PBS for 5 min each, without rocking.

Then, without rocking, they were washed twice with PBS for five minutes each. The tubes were inverted two or three times for five minutes each and the embryos were washed three times in PBST.

The embryos were carefully deyolked using forceps under a microscope after being transferred to a glass dish containing PBST. The embryos were deyolked, placed in tubes containing PBST, and washed twice for five minutes each on a rocker.

The embryos were then incubated for 2 h at room temperature on a horizontal rocker in 1 ml of blocking solution (1% BSA in PBS). After discarding the block, embryos were treated with 300–500ul of primary antibodies at the appropriate dilution and incubated overnight at 4°C on a rocker. The primary antibody was recovered and stored for future use.

Primary Antibodies	**Dilution**
Rabbit Anti-H3Q5ser Antibody (Merckmillipore ABE1791)	1:1000
Mouse Anti-Gamma Tubulin Antibody (T3559 Invitrogen)	1:2000

The embryos were rinsed with PBST on a horizontal rocker for 30 min at room temperature. This procedure was performed six to eight times. The PBST was subsequently discarded. Embryos were incubated in 1 ml of blocking solution on a horizontal rocker for 2 h at room temperature. The block was discarded and the embryos were incubated overnight at 4°C on a horizontal rocker in the dark with 100 ul/tube of secondary antibodies at the appropriate dilution. To avoid photobleaching, the secondary antibodies were kept away from light.

Secondary Antibodies	**Dilution**
Goat Anti-Rabbit Alexa Fluor 488 (A21206 Invitrogen)	1:100
Goat Anti-Rabbit Alexa Fluor 555 (A-31572 Invitrogen)	1:100
Donkey Anti-Mouse Alexa Fluor 555 (A31570 Invitrogen)	1:100

The secondary antibodies were either removed the following day or stored for use the following day. The embryos were washed 6–8 times with PBST for 30 min on a horizontal rocker.

The embryos were then stained for DNA for 10 minutes with 100ul of 0.5 ug/ml DAPI solution (in PBS). Subsequently, they underwent three PBST washes lasting five minutes each. The embryos were then kept at 4°C until imaging with 50% glycerol (in PBS).

Mounting of embryos and imaging

The tagged embryos were flat-mounted on glass slides and covered with reinforcing slips before being submerged in 6 ul mounting medium. Clear nail paint was used to seal the coverslips.

Composition of mounting media

70% glycerol	1 ml
1M Tris-HCl pH 8	20 ul
Pinch of DABCO	A pinch

All samples were imaged using a Zeiss LSM 900 confocal laser-scanning microscope. The 63x objective was used to take images of the nuclei within the blastodisc of the embryos (at 1X magnification or as necessary). Two or three data channels were recorded as required red for gamma-tubulin, green for the serotonylated histone marker (H3Q5ser), and blue for DAPI in the first set of replicates. In the second set of replicates red for serotonylated histone marker (H3Q5ser) and blue for DAPI

BUFFERS AND SOLUTIONS:

PBS 10X:

Dissolve 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2PO_4 and 2.4 g of K_2PO_4 in 1000 ml of H_2O. The pH of the buffer should be 7.4 and no pH adjustment was required. Store at room temperature and dilute to 1X before use.

PBST :

Mix 500 ml of 1X PBS with 500 ul Triton-X 100 to make 500 ml of PBST.

Blocking Solution:

To make 10 ml of blocking solution, mix 9 ml PBST AND 1 ml 10% BSA.

RESULTS

Protein extraction and estimation:

The initial testing of the antibody against the histone serotonylation mark was carried out on water activated oocytes at 10-, 20-, and 30-minutes post activation. Freshly extruded eggs from gravid zebrafish females were water activated in batches of 100–300 at a time such that they could be dechorionated using pronase and deyolked in order to arrest the oocytes within the 10-, 20-, or 30-minute time frame. Varying amounts of water activated oocytes were taken in different batches in order to standardize the number of oocytes that were required to obtain the optimum protein concentration.

Batches of 200 to 500 oocytes were taken for protein extraction, and the concentration of protein in mg/ml was quantified using the Bradford Assay. Proteins were also extracted and quantified from 24 hpf embryos to be used as controls. While the concentration produced using 400 or more water activated oocytes was comparable to the concentration obtained using 150 embryos at the 24 hpf stage, we tried to use as few embryos as possible to get crisp bands on the SDS-PAGE gel.

Separation of proteins using SDS-PAGE:

A 15% polyacrylamide gel was used to separate proteins from the whole cell lysate because the histone H.3 against which the histone serotonylation antibody (H3Q5ser) was to be tested was around 16 kDa and the sizes of proteins separated by a 15% gel range from 12 to 45 kDa. Usually 50–70 ug of protein was sufficient to get crisp bands on the gel. The volume required to be taken for 50 ug of protein was calculated, and the protein was mixed with Lamella dye. The volume was made up to 50 ul using RIPA lysis buffer and then heated for 10 minutes to denature the proteins. Each well of a 1mm thick gel can contain up to 25 ul of the protein

sample mixture. However, when a smaller number of waters activated oocytes (around 300) were used to extract proteins and the concentration of the proteins obtained was low, a higher volume of the protein sample had to be taken in order to load 50 ug of protein in the gel. The volume of the protein sample far exceeded 50 ul; hence, the volume of the mixture was increased to 150 ul. But since the sample was too diluted, the protein sample was left to heat for a longer stretch of time so that the excess liquid evaporated and the volume came down to 50 ul. Each protein sample was split into two (25 ul each), creating two sets of protein samples. After the gel run was complete, one set was cut from the gel and stained with Coomassie dye, while the other set was further processed for western blotting.

Western blotting:

The proteins were transferred from the gel onto the PVDF membrane overnight. The following day, the PVDF was stained with Ponceau stain to confirm that the transfer was successful. The membrane was blocked in 5% skimmed milk in TBST and incubated overnight with 500 ul of the primary antibody against the histone serotonylation mark, H3Q5ser. The following day, the membrane was washed with TBST and incubated with the HRP linked secondary antibody for an hour. The membrane was washed with TBST and stored at 4 °C until imaging. The membrane was imaged using the Image Quant LAS system 500 after the addition of a mixture of hydrogen peroxide, which reacts with the HRP linked secondary antibody to produce chemiluminescence.

In the SDS-PAGE of proteins extracted from 24 hpf embryos and 20 and 30 mpa oocytes, proteins of molecular weight 20 kDa and above produced sharp bands; proteins of lower molecular weight (below 20 kDa) were seen only as a smear and no clear bands were observed. However, the proteins were transferred to the PVDF membrane on the off chance that the proteins we wanted to detect were present but in too low a concentration to give a

sharp band. However, after transferring the proteins to the PVDF membrane and incubation with the antibodies, no bands were observed on imaging.

Immunolabelling:

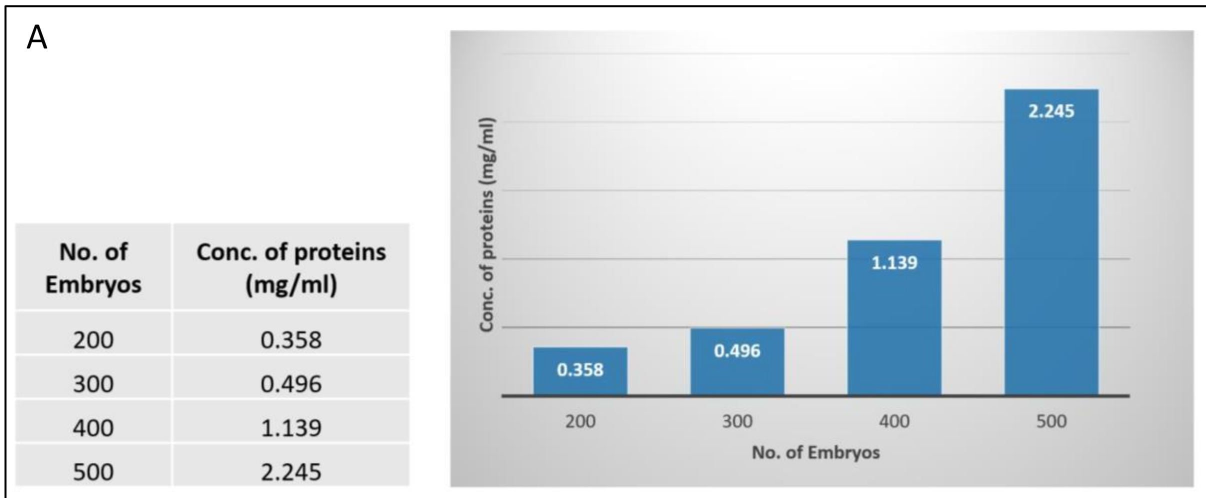
Water activated oocytes at 10-, 20-, and 30-minutes post activation (mpa), as well as embryos at 10-, 15-, and 20-minutes post fertilization (mpf), were fixed. The anti-histone H3Q5ser antibody was a highly specific rabbit polyclonal antibody that targets Histone H3.1 serotonylated at the glutamine 5 position.

The water activated oocytes were stained with the anti-H3Q5ser antibody alone, while the fertilized embryos were stained with both the anti-H3Q5ser antibody and the anti-gamma tubulin antibody. The embryos were imaged using confocal microscopy. This was because the water activated oocytes contain only the female nucleus due to the lack of fertilization. Meanwhile, the fertilized embryos have both male and female pro- nuclei. the male pronuclei and surrounded by sperm-aster microtubules that facilitate the union of the male and female pronuclei after fertilization. The anti-gamma tubulin antibody stains the gamma tubulin present around the male pronuclei, hence distinguishing it from the female pronuclei.

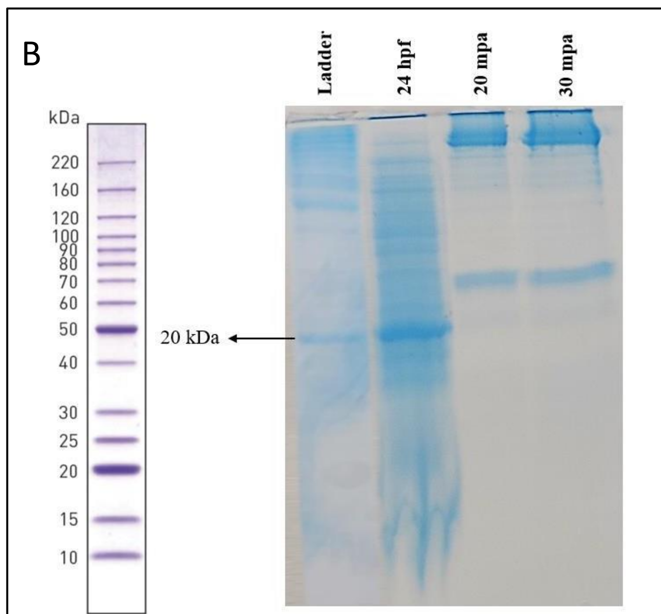
Two different secondary antibodies were used against the anti-H3Q5ser antibody. First, an anti-rabbit antibody with a GFP fluorophore was used; however, it appears to have cross-reacted with the anti-gamma tubulin antibody, as the RFP signal of the secondary antibody against the anti-gamma tubulin antibody and the GFP signal appear to overlap. No GFP signal was observed within the DAPI stained nuclei, however.

In order to avoid cross reactivity, both the water activated oocytes and the fertilized embryos were stained with only the anti-H3Q5ser antibody, and a different anti-rabbit secondary

antibody with an RFP fluorophore was used to detect the primary antibody. No RFP signal has been observed yet within the nuclei at these particular developmental stages.



No. of Embryos	Conc. of proteins (mg/ml)
200	0.358
300	0.496
400	1.139
500	2.245



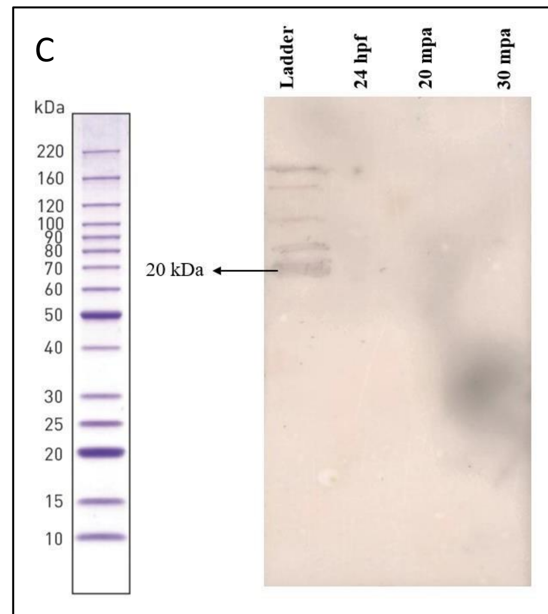


FIGURE 3: A: Table and graph depicting the number of embryos at the 1-cell stage used for protein extraction vs the concentration of protein extracted **B:** SDS-PAGE Gel depicting protein bands at 24 hpf , 20 mpa and 30 mpa **C:** Western blot of proteins extracted at 24 hpf , 20 mpa and 30 mpa with the anti-H3Q5ser antibody

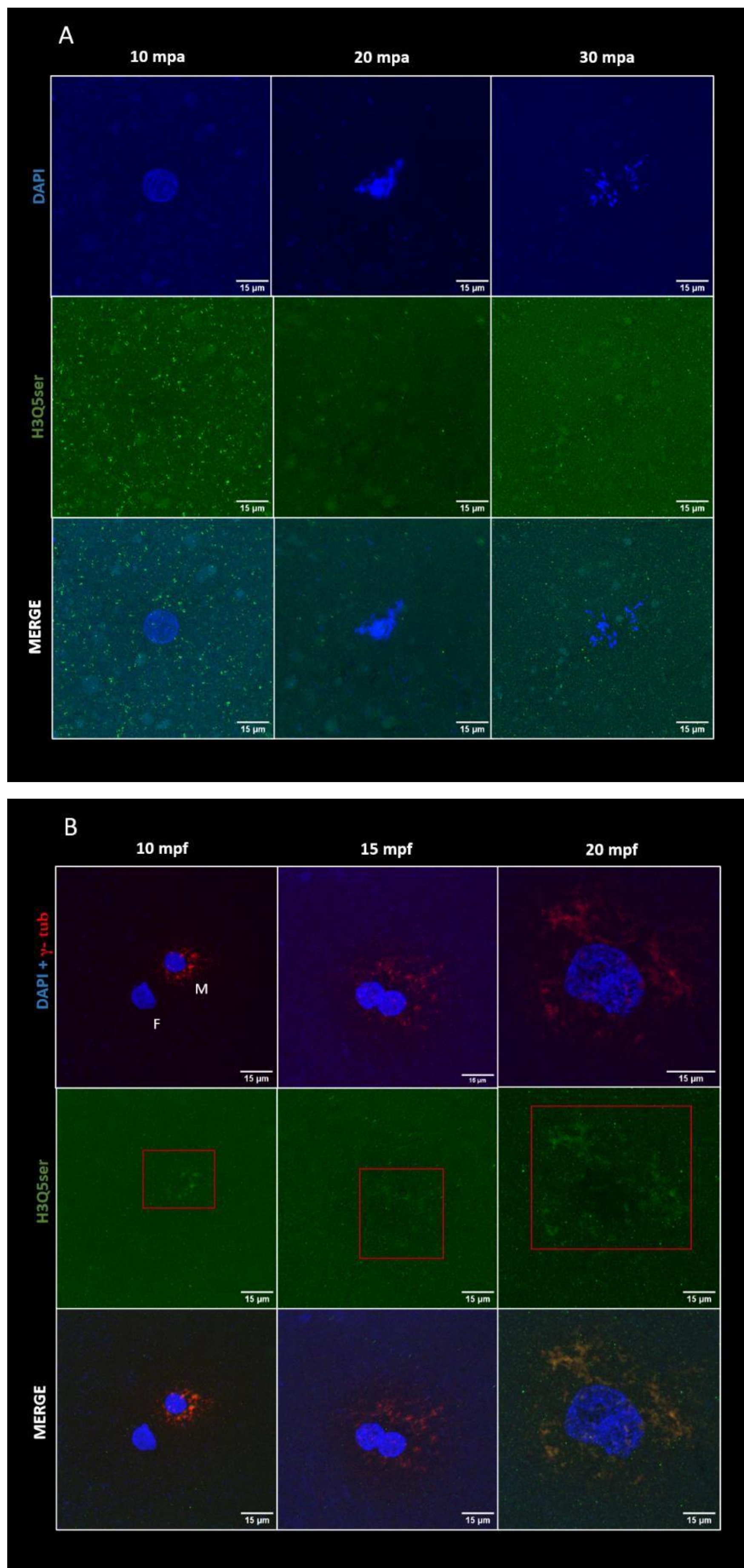


FIGURE 4: A: Panels showing 10, 20 and 30 mpa oocytes stained with DAPI (blue channel) and anti-H3Q5ser antibody (green channel) **B:** Panels showing 10, 15 and 20 mpf embryos stained with DAPI (blue channel), anti-γ tubulin (red channel) and anti-H3Q5ser (green channel) antibodies
Scale bar – 15 um Magnification – 63x n = 30 (for each time point)

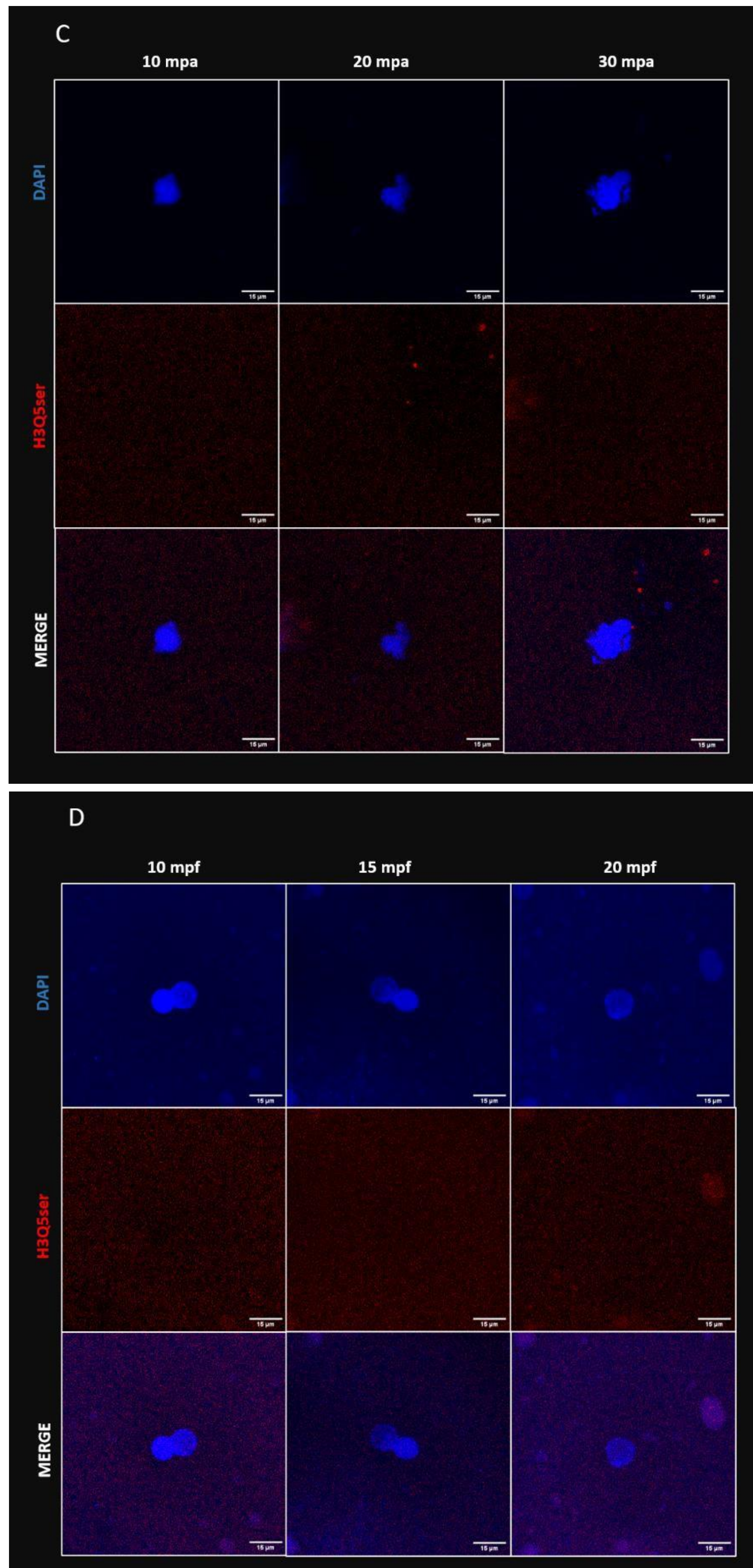


FIGURE 4: C: Panels showing 10, 20 and 30 mpa oocytes stained with DAPI and anti-H3Q5ser antibody (red channel) **D:** Panels showing 10, 15 and 20 mpf embryos stained with DAPI (blue channel) and anti-H3Q5ser antibody (red channel)
Scale bar – 15 um Magnification – 63x n = 30 (for each time point)

DISCUSSION AND CONCLUSIONS

In order to determine whether the anti-H3Q5ser antibody, which had previously been tested on mice and humans, could also be used on zebrafish we turned to the techniques of western blotting and whole embryos immunofluorescence. Since we are interested in the developmental role this marker may play in the early embryonic stages of zebrafish, we decided to test the antibody on both water-activated oocytes and fertilized embryos at various time points during the one-cell stage.

Western blotting was used to examine the cross-species reactivity of the anti-H3Q5ser antibody in water activated oocytes. Several rounds of protein extraction were undertaken with varying amounts of oocytes in order to optimize the number of oocytes that would be required to obtain a sufficient concentration of proteins so that crisp bands were obtained during SDS-PAGE. However, since oocyte extrusion was a strenuous process that also causes stress to the fish, being judicious with the number of oocytes required for protein extraction was crucial. So, with a lower protein concentration, it became necessary to make small changes to the protein sample preparation before loading it on the gel. Protein sample preparation was a crucial step that, if done incorrectly, produces smeared bands. Since a larger volume of the extracted proteins would be required in the sample mixture to load 50 or 70 ug of protein in the gel, the sample was heated at 95 °C to concentrate the sample by evacuating the excess liquid. The sample was then spun down at 11,000 g for 1 min to pellet down any artifacts, which can also hamper the smooth running of the gel.

The samples run in the gel produced crisp bands for proteins whose weight was 20 kDa or above; however, proteins of molecular mass lower than 20 kDa did not produce bands, and only a smear was seen. This could be due to the degradation of proteins of lower molecular

weight. It was possible that in order to efficiently isolate histone proteins, a protein extraction protocol specifically tailored to histone proteins, such as the acid-based extraction method, can be followed. Another possible solution could be to use a tris-tricine gel, which has been shown to give better resolution for proteins smaller than 30 kDa.

Simultaneously, the antibody was also used to immunolabel water activated oocytes and fertilized zebrafish embryos. Water activated oocytes were chosen in order to examine whether the histone serotonylation mark was maternally inherited. Across the various time points in water activated oocytes, we can see the female pronuclei which was intact at 10 mpa, gradually decondense at 20 and 30 mpa. While the water activated oocytes were only stained with the anti-H3Q5ser antibody the fertilized embryos were stained with a combination of H3Q5ser and gamma tubulin antibodies. In the fertilized embryos, over the course of time we see the male and female pronuclei being pulled towards each other and eventually fusing. Male pronuclei are identified by the presence of sperm-aster microtubules around it. In the fertilized embryos, it was observed that there was some potential cross reactivity between the secondary antibody. The anti-mouse RFP tagged secondary antibody which was complimentary to the anti-mouse gamma tubulin primary antibody hence marking the gamma tubulin in red. The anti-rabbit RFP tagged antibody which was complimentary to the anti-rabbit H3Q5ser primary antibody was found to produce a green signal that overlapped with the red signal of the gamma tubulin, indicating possible cross-reactivity.

In order to resolve this problem, fertilized embryos were stained with the H3Q5ser antibody alone and a new RFP tagged anti-rabbit secondary antibody was used.

So far, the search for the H3Q5ser mark in both the water activated oocytes and fertilized embryos has been inconclusive and further experiments are being conducted to confirm the presence or absence of the histone serotonylation mark in early zebrafish embryos. Serotonin

and the enzyme transglutaminase which was responsible for the serotonylation of proteins as well as the nuclear localization of serotonin has been shown to be present at the 128-cell stage in zebrafish embryos (Ivashkin E. et al. 2019), so it was possible that the H3Q5ser mark appears later in development. It has also been shown that although the H3Q5ser single modification was present in cultured cells, only the dual modification H3K4me3Q5ser was present in-vivo. hence, it was possible that in the zebrafish system as well, the dual modification alone may be was present. Experiments to test this hypothesis are also currently underway.

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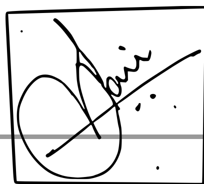

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