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Id4 Functional Analysis During Neurogenesis using Zebrafish as a Model Organism

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ABSTRACT

Id4 is a member of Id proteins which belong to a transcription factor family called "basic helix-loop-helix (bHLH)" proteins. bHLH family members contain a DNA binding domain and a helix-loop-helix domain that enables dimerisation between bHLH proteins. Id proteins are not capable of DNA binding due to absence of basic (DNA binding) domain. Therefore Id-bHLH heterodimerisation results in inhibition of bHLH activity which drives cellular differentiation. Id4 plays an important role in proliferating cells maintaining neural stem cell pool and controlling the timing of differentiation as well as apoptosis in the mouse. TALEN-mediated knockout of Id4 in zebrafish resulted in a reduction of Sox2 expression a marker for neural stem cells suggesting a reduction of the stem cell pool. In addition, premature GFAP expression a marker of astrocytes suggests that reduction of the stem cells pool is in part due to premature glial differentiation. HuC expression marking neurons was not changed however at 24 hpf. The observed phenotype was similar to that exhibited in mutants of the notch signalling pathway suggesting that Id4 could be a downstream target of notch. Indeed, Id4 expression was elevated in mind bomb mutants and embryos treated with DAPM an inhibitor of the notch signalling pathway suggesting that notch negatively regulates Id4 expression.

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ABBREVIATIONS

Anti-DIG	Anti-Digoxygenin
bHLH	Basic Helix-Loop-Helix
bp	Base Pairs
CNS	Central Nervous System
DAPM	N-[N-3,5-Difluorophenacetyl]-L-Alanyl-S-Phenylglycine Methyl Ester
DIG	Digoxygenin
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dpc	Days Post Coitum (Days of Gestation)
EDTA	Ethylenediaminetetraacetic Acid
HLH	Helix-Loop-Helix
hpf	Hours Post Fertilisation
Id	Inhibitor of DNA Binding Protein
Id4	Inhibitor of DNA Binding Protein 4
MAB	Maleic Acid Buffer
MABT	Maleic Acid Buffer with Tween 20
Mib	Mind Bomb
mRNA	Messenger Ribonucleic Acid
MOs	Morpholino Oligos
MS-222	Ethyl 3-Aminobenzoate Methanesulfonate Salt
NECD	Notch Extracellular Domain
NICD	Notch Intracellular Domain
NSC	Neural Stem Cell
PBST	Phosphate Buffered Saline with Tween 20
PCR	Polymerase Chain Reaction

PFA	Paraformaldehyde
PTU	N-Phenylthiourea
SVZ	Subventricular Zone
SSC	Saline Sodium Citrate
TALEN	Transcription Activator-Like Effector Nuclease
TBE	Tris/Borate/EDTA
WT	Wild Type

1. INTRODUCTION

1.1 Id Proteins

Id proteins belong to a group of transcription factors which are also known as basic helix-loop-helix (bHLH) proteins. Members of this large bHLH protein family contain a basic domain which facilitates DNA-binding and a helix-loop-helix (HLH) domain which enables homo or heterodimerisation with other bHLH proteins (Figure 1.1) (Massari and Murre, 2000). Id proteins lack the basic domain and are thus unable to bind DNA (Figure 1.1). However, Id proteins contain a HLH domain and can dimerise with Class A (also known as E proteins; i.e. E47, E12,) and Class B bHLH proteins (i.e. MyoD) (Table 1.1). Id-bHLH heterodimers are unable to bind DNA due to the absence of the basic domain within the Id proteins causing the inhibition of bHLH protein function. This inhibition process gives Id proteins a dominant-negative regulatory function on bHLH proteins (Benezra *et al*., 1990; Norton *et al*., 1998). Since the Class A and Class B bHLH transcription factors are upregulated during cell fate determination and cell differentiation, causing the inhibition of these proteins make Id proteins not just inhibitors of DNA-binding but also inhibitors of cell differentiation (Massari and Murre, 2000).

1.1.1 Different Types of Id Proteins

4 Id proteins have been described in mammals which are called Id1, Id2, Id3 and Id4 (Benezra *et al*., 1990; Sun *et al*., 1991; Christy *et al*., 1991; Riechmann *et al*., 1994). Id1 was isolated first by Benezra *et al.* in 1990. All Id proteins share a highly conserved HLH domain which consists of two amphipathic α-helices with a loop in-between. They are also similar in size (13-20 kDa). All 4 members of the mammalian Id proteins are encoded by individual genes with similar genomic organisation in terms of exon-intron boundaries and their coding regions which is indicative of a common ancestral Id gene (Norton *et al*., 1998). The chromosomal localisation of the mammalian Id1-4 members in humans are; 20q11 (Mathew *et al*., 1995; Nehlin *et al*., 1997), 2p25 (Mathew *et al*., 1995), 1p36.1 (Ellmeier *et al*., 1992; Deed *et al*., 1994) and 6p21-22 (Pagliuca *et al*., 1995) respectively.

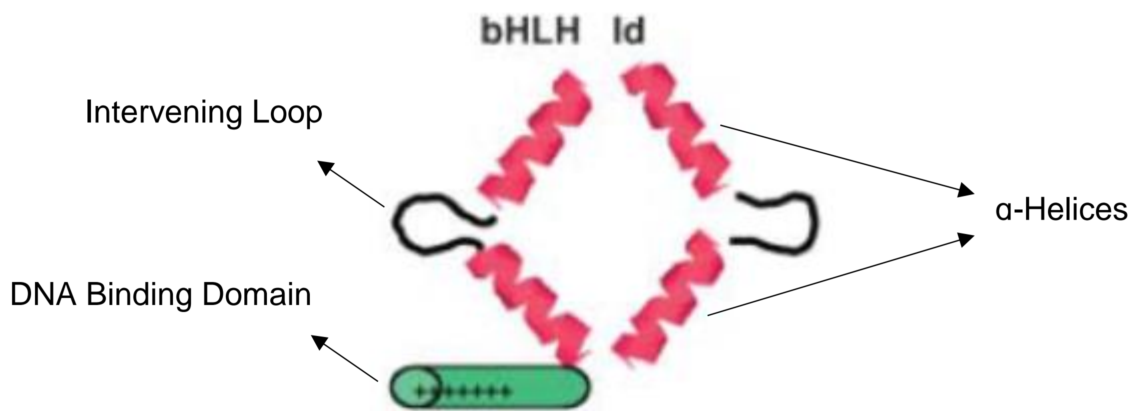

Figure 1.1: General bHLH and Id Protein Structure
Both bHLH and Id proteins contain two α-helices with an intervening loop (HLH domain) facilitating heterodimer formation. bHLH proteins also contain a basic domain (DNA binding domain) that is absent from Id proteins. Id-bHLH heterodimers are therefore unable to bind DNA. Only parts of the proteins are depicted (Adapted from Sikder *et al.,* 2003).

1.1.2 Heterodimerisation Between Id Proteins and bHLH Proteins

Class A and Class B bHLH proteins interact with each other to promote cell differentiation in a variety of cell lineages (Norton *et al*., 1998). The basic region of these proteins binds to DNA via a specific sequence which recognize the consensus “E-Box” [CANNTG] region (Ephrussi *et al.,* 1985; Murre *et al*., 1989). Because the Id proteins lack this basic region, heterodimer formation with bHLH proteins via the HLH domain prevents DNA binding. (Benezra *et al*., 1990; Norton *et al*., 1998). bHLH homo or heterodimers are positive regulators of differentiation in various cell lineages. For example, the homodimer between E12 and E47 proteins is B cell lineage-specific whereas heterodimerisation between MyoD and E12/E47 activates transcription in muscle cells (Figure 1.2) (Murre *et al.,* 1989; Murre *et al.,* 1991; Massari and Murre, 2000).

Protein Class	Protein Examples
Class A bHLH Proteins (E Proteins)	E12, E47, E2-5, E2-2, HEB
Class B bHLH Proteins	**Myogenic;** MyoD, Myf-5, MRF-4, Myogenin
	Neurogenic; Neurogenin, NeuroD1, NeuroD2 NeuroD3
	Neurogenic; Mash-1, Mash-2
	Neurogenic; NSCL-1, NSCL-2
	Haematopoietic; SCL, Lyl-1

Table 1.1: Examples of Mammalian Class A and Class B bHLH Proteins
The heterodimeric interactions usually take place between ubiquitously expressed Class A and tissue-specific Class B proteins (Adapted from Norton *et al.*, 1998).

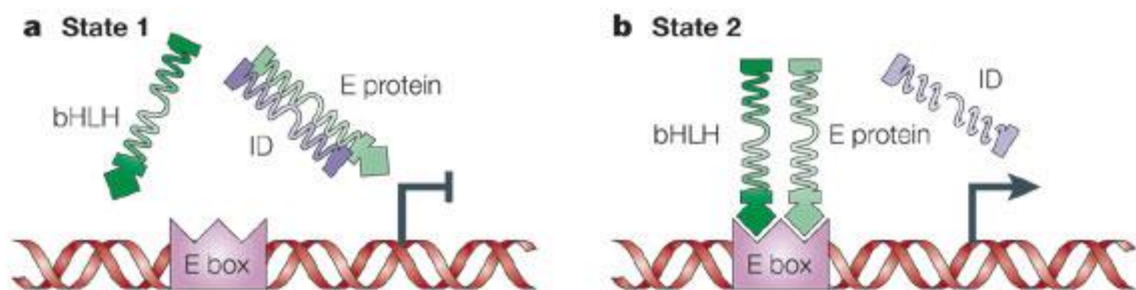

Figure 1.2: Cell Fate Determination by Id Proteins
(a) State 1: Id-E Protein interaction forms a heterodimer that cannot bind DNA. In addition, Id proteins restrain E proteins to bind other bHLH proteins.
(b) State 2: E Proteins can either form a homodimer with another E Protein (not shown in the figure) or a heterodimer with any other bHLH proteins. Formation of these homo or heterodimers are transcriptionally active complexes due to E-Box binding. Upregulation of Id proteins prevents cells to enter state 2 (Adapted from Perk *et al.*, 2005).

1.1.3 The Expression of Different Id Genes During Development

In general, Id gene expression is not restricted to a certain tissue, instead the transcripts of all four members of the mammalian Id genes can be found in a variety of adult tissues such as bone marrow, testis, brain, kidney and spleen (Riechmann and Sablitzky, 1995). In addition to their function to antagonise bHLH protein function, Id proteins have been shown to regulate cell proliferation. Highest expression of Id genes was observed within proliferating cells and lowest expression was within quiescent and/or terminally differentiated cells (Norton *et al.*, 1998).

Id1 expression was observed in undifferentiated neural precursors of ventricular zone and in the proliferative neuroepithelial layer of the CNS during mouse development. Id1 mRNA was first detected at 5.5 dpc, in the proximal side of the embryonic ectoderm and in the neural fold before closure of the neural tube at 8.5 dpc (Wang *et al.,* 1992; Jen *et al.,* 1997). At 12.5 dpc, Id1 expression was observed in the forebrain and also detectable in the ventricular zones of the midbrain, hindbrain and spinal cord. When the cells in the ventricular zone stop dividing and undergo migration and differentiation at 14.5 dpc, Id1 expression was unapparent. From 16.5 dpc, expression was seen in the walls of telencephalon, hippocampus and in some periventricular areas (Duncan *et al.*, 1992).

Id2 expression in the developing nervous system of the mouse is much higher than Id1 (Neuman *et al.*, 1993). Both Id1 and Id2 expression can be detected in cells of the roof plate but as the development of the CNS progresses, Id1 is expressed by dividing neuroblasts whereas Id2 expression is more prevalent in presumptive neurons that are undergoing maturation such as interneurons. Id2 expression can also be found in specific neurons in adulthood (Jen *et al.* 1997; Neuman *et al.*, 1993).

Id3 gene expression can be detected from 5.5 dpc during gastrulation along with Id1 within the tissues which derived from the inner cell mass in mouse development. At the very beginning of the spinal cord development, both floor and roof plate cells are expressing Id3 along with cartilage primordia and epithelial cells. Moreover, Id3 is the only Id gene expressed in the floor plate of the spinal cord. As the nervous system develops, Id3 and Id1 expression is localised within less-differentiated neuroblasts (Jen *et al.,* 1997; Riechmann *et al.,* 1995).

It was shown that Id4 is highly expressed in different tissue and organs such as testis, kidney and brain in adult mice whereas its expression is generally upregulated during embryonic development. A weak expression was observed in spleen, lung and skeletal muscle based on the *in situ* hybridisation analysis (van Crüchten *et al.,* 1998; Riechmann *et al.,* 1994). Between 9.5-17.5 dpc an obvious upregulation of Id4 expression was observed within neural cells of the developing brain, spinal cord, spinal roof and fifth cranial ganglions (Riechmann *et al.,* 1994; Riechmann *et al.,* 1995). No expression of Id4 was observed at 7 dpc (van Crüchten *et al.,* 1998). In humans, Id4 high expression was observed in a variety of tissues including osteoblasts, adipocytes, prostate epithelial cells, neurons, testicular Sertoli cells and during differentiation in glial cells. Low Id4 expression was seen in adult brain, thyroid, testis and pancreas. Moreover, Id4 seemed to be essential for normal mammary and prostate gland development (Patel *et al.,* 2015).

1.1.4 Neural Stem Cells

Stem cells are unique in that they can maintain themselves through self-renewal and can give rise to different cell lineages. Self-renewal refers to the ability of stem cells to divide symmetrically and expand therefore remain as stem cells or divide asymmetrically giving rise to one stem cell and one differentiated daughter cell such as brain or blood cells (Kennea and Mehmet, 2002).

Neural stem cells (NSCs) are multipotent stem cells that can self-renew and differentiate to neurons and glial cells (astrocytes and oligodendrocytes). NSCs can be found in several different places within the central nervous system (CNS) of an organism throughout its life. Adult NSCs are present mainly in 2 neurogenic regions in mammals which are the subgranular zone of the dentate gyrus in the hippocampus and subventricular zone of the lateral ventricles. NSCs also found in some non-neurogenic regions such as spinal cord (Figure 1.3; Temple, 2001; Butti *et al.,* 2014)

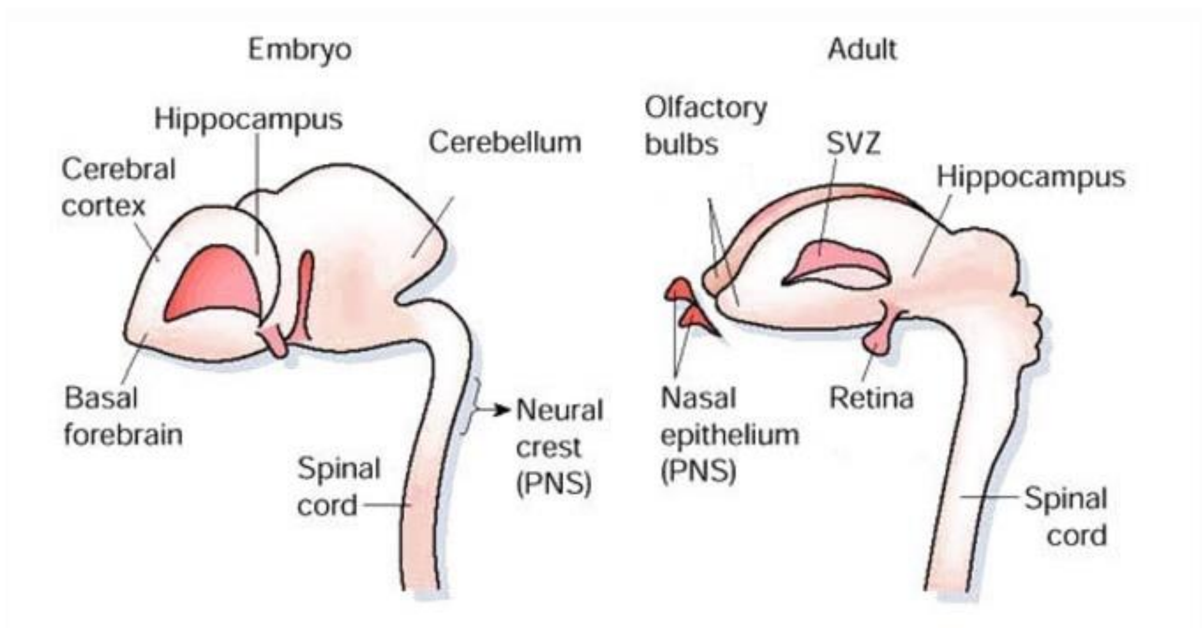

Figure 1.3: The Location of Neural Stem Cells in Embryonic and Adult Mammalian Nervous System
Regions where neural stem cells have been isolated during embryogenesis (left) and adult brain (right) as indicated (Adapted from Temple, 2001).

The development of the CNS starts early during embryogenesis when NSCs emerge in neural plate within neuroectoderm. NSC proliferation and neural differentiation is tightly regulated by external signals and intrinsic regulatory networks of gene expression. One of the example of these signals is bone morphogenetic proteins (BMPs). BMPs induce ectodermal cells to become epidermal and suppress neural differentiation. Signal molecules secreted from cells in the organiser such as noggin, follistatin and chordin act as BMP antagonists, therefore preventing BMP function, resulting in neural differentiation of neuroectodermal cells. Those molecules provide a signalling network in order to promote proper development of the neural plate. (Kennea *et al.,* 2002; Briscoe *et al.*, 2001).

As neurogenesis proceeds, cells of the neural plate proliferate to form the brain and neural tube. NSCs in the ventricular zone expand and differentiate into neurons and glia cells. Extrinsic signals such as BMPs and Wnt that are produced from cells in the roof plate as well as sonic hedgehog (Shh) that is produced from cells in the notochord and floor plate of the neural tube are key in the cell specification in the spinal cord. These signal molecules form a gradient along the dorsal ventral axis and depending on their concentration trigger downstream target gene activation that determine the fate of the developing neurons and later glial cells (Figure 1.4) (Jessell, 2000; Lee *et al.,* 2001).

During the development of the CNS neurogenesis happens first followed by gliogenesis. The transition from neurogenesis to gliogenesis is determined by proneural genes which are intrinsic regulators. Prior to neurogenesis, extrinsic neurogenic signals induce proneural gene expression. One of the well-known induction example in this context is Mash1 (a proneural gene) upregulation by BMP2 (extrinsic signal) (Lo *et al.,* 1997). Accumulation of sufficient amount of proneural proteins drives neuronal differentiation and inhibits glial differentiation. Proneural gene activity is regulated by Notch signalling which is a pathway that inhibits proneural gene expression in the neighbouring cells therefore preventing neuronal differentiation via a process called lateral inhibition. Subsequently, gliogenesis is induced by several extrinsic signals like FGF2 (Fibroblast Growth Factor 2) and BMPs (Artavanis-Tsakonas *et al.,* 1999; Johe *et al.,* 1996). While these signals drive glial differentiation, neurogenesis is simultaneously inhibited via various mechanisms including Id and Hes (Hairy and Enhancer of Split) protein families (Figure 1.5) (Nakashima *et al.,* 2001).

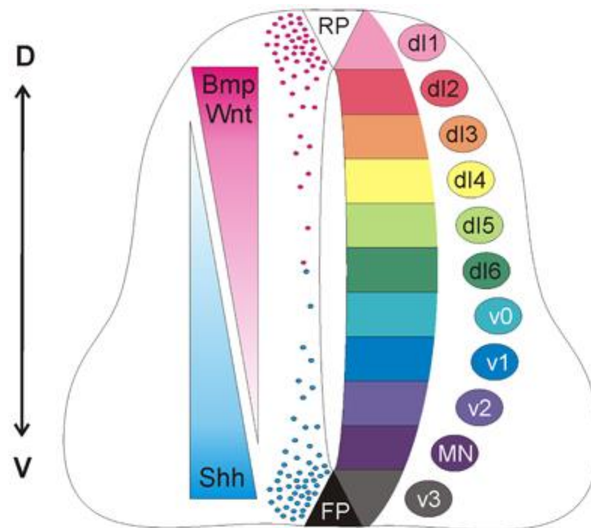

Figure 1.4: Sonic Hedgehog (Shh) and Wnt/BMP Concentration Gradient in the Developing Spinal Cord

Shh secreted from the floor plate cells and BMP/Wnt secreted from the roof plate cells form a gradient that leads to a concentration-dependent specification of precursor cells along the dorso-ventral (D-V) axis. RP: Roof Plate, FP: Floor Plate (Adapted from Aviles *et al.,* 2013).

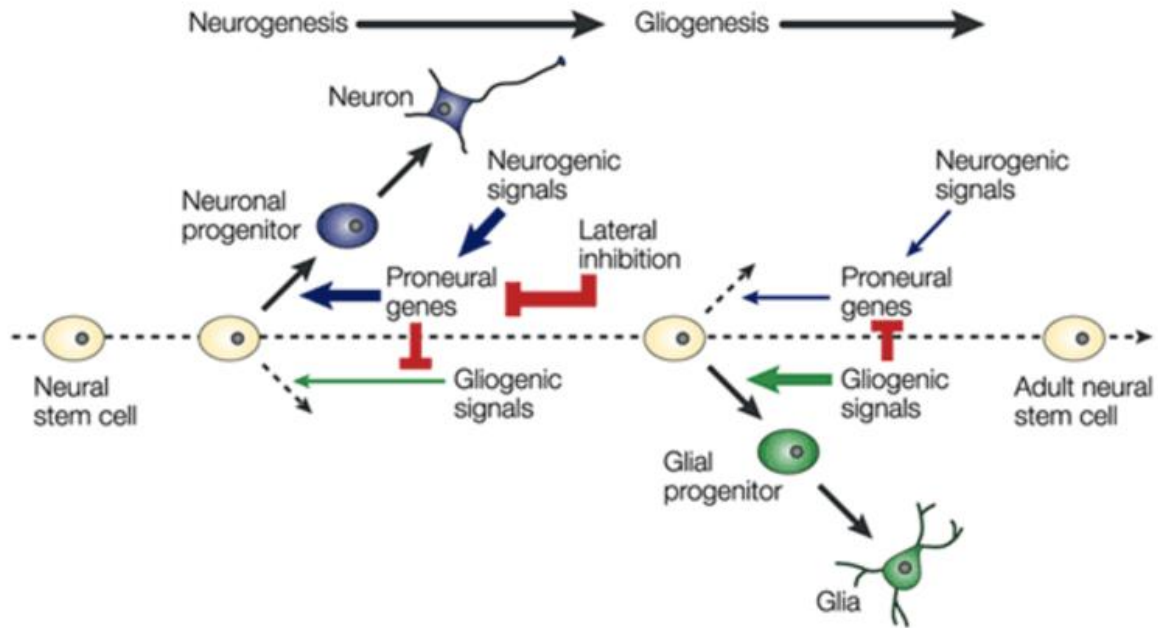

Figure 1.5: Vertebrate Neuronal and Glial Differentiation Model
Neural stem cells are capable of giving rise to all neural cell types which are neurons, astrocytes and oligodendrocytes. NSCs differentiate first into neurons and then into glial cells. This process is regulated through external signals and intrinsic regulatory elements. Proneural genes drive neuronal differentiation therefore lead the formation of neurons. When neuronal differentiation is inhibited via lateral inhibition, gliogenic signals induce the formation of glial cell types (Taken from Bertrand *et al.,* 2002).

1.1.5 Id4 as a Member of Id Proteins

Id4 was discovered, as a novel dominant negative HLH protein in 1994 by Veit Riechmann *et al.* Initially, it was shown that Id4 prevents DNA-binding of E47 homodimers as well as E47/MyoD heterodimers therefore establishing that Id4 acts as an inhibitor of bHLH proteins similar to the other Id proteins (Id1-3) (Riechmann *et al.,* 1994). The Id4 gene is localised on chromosome 13 of the mouse, 6p21-22 of humans (van Crüchten *et al.,* 1998; Pagliuca *et al.*, 1995) and chromosome 16 of zebrafish (ZFIN.org).

The intron exon configuration of Id4 is conserved in these species: Id4 has 3 exons, 2 of them (exon I and exon II) contain coding regions. Exon I encodes the HLH domain whereas exon II encodes the C-terminal 14 amino acids. Transcription of the mouse Id4 gene results in 4 different RNAs due to differential use of polyadenylation sites within the 3'-untranslated region (Figure 1.6). However, all four murine Id4 transcripts encode a single Id4 protein containing 161 amino acids (16.6 kDa) (van Crüchten *et al.,* 1998). In zebrafish, the Id4 gene has one transcript which encodes 126 amino acids giving rise to a protein with a size of 14.44 kDa (ZFIN.org).

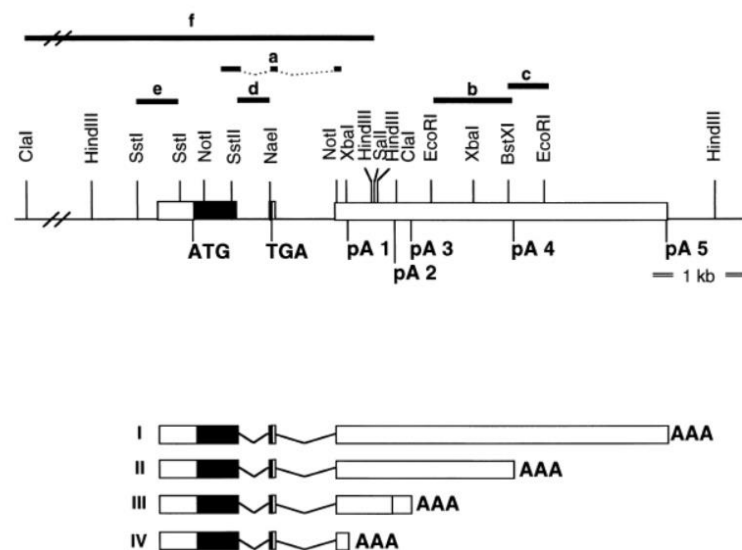

Figure 1.6: Genomic Structure of the Murine Id4 Gene
Upper figure shows the restriction map of the murine Id4 gene. Exons are indicated as boxes and pA1-5 are the putative polyadenylation sites. ATG is the translational start codon and TGA is the stop codon. Lower figure shows the transcripts of Id4. Transcripts indicated as open and filled boxes, they differ in their alternative polyadenylation sites in their 3'-end (Adapted from van Crüchten *et al.,* 1998)

1.2 Id4 Function in the Developing Nervous System of the Mouse

In general, highest expression of Id genes was observed in undifferentiated and proliferating cells and their expression decrease as cells differentiate (Norton *et al.,* 1998; Coppé *et al.,* 2003). The critical role of Id4 in the proliferating cells is maintaining neural stem cell pool and controlling the time of transition from proliferation to differentiation was shown in knockout mouse models (Yun *et al.,* 2004; Bedford *et al.,* 2005). Id4 expression during mouse embryogenesis is restricted to the developing nervous system. No Id4 expression could be detected during early gastrulation and the earliest stage that Id4 expression was observed is at 6.5 dpc (Riechmann and Sablitzky, 1995; Jen *et al.,* 1997). Id4 is mainly expressed in the ventricular zone were NSC and progenitor cells proliferate and Id4 expression is particularly high in the developing telencephalon. There are two mouse models which were developed by Fred Sablitzky's group and Mark Israel's group. Sablitzky's group replaced a region within Id4 gene, including HLH domain coding sequence, with a lacZ/neomycin cassette resulting in the expression of the N-terminal end of Id4 fused to β-galactosidase (Bedford *et al.,* 2005). Israel's group replaced the first two exons of Id4 with GFP and the neomycin-resistance gene resulting in a complete lack of Id4 (Yun *et al.,* 2004). Both models revealed that Id4 is essential for the normal development of the brain. Lack of Id4 caused not only reduced brain sizes which was apparent from E11.5 onwards, but also smaller ventricular surface of the telencephalon, especially the dorsal and medial pallial regions, primordia for the neocortex and the hippocampus. Compared to wild type, Id4 knockout mice exhibited an approximately 30% reduction of proliferating NSC and progenitor cells. A reduction was also observed within lateral ventricles and ventricular surface at E15.5 and E18.5 (Yun *et al.,* 2004). These observations indicated that absence of Id4 caused loss of neural precursor cells suggesting a role for Id4 in neural precursor cell proliferation (Bedford *et al.,* 2005).

Indeed, it was shown that NSC and progenitor cells had a prolonged G1-S transition which resulted in a 15% thicker ventricular zone at E12.5. Further analysis with markers for cell cycle phases revealed that the attribution of the thicker region corresponds to the G-phases of the cell cycle. It seems therefore that lack of Id4 keeps neural stem and progenitor cells in the G1 phase for longer causing a disruption of the cell cycle (Yun *et al.,* 2004).

Furthermore, the upregulation of early neuronal markers revealed premature neuronal differentiation in the absence of Id4 in neocortex at E11.5 (Yun *et al.,* 2004) and also at E12.5 and E14.5 (Bedford *et al.,* 2005). These results indicate, in the absence of Id4, a disrupted cell cycle of neural progenitors alter the timing of neuronal differentiation resulting in premature differentiation and eventually causing a reduction in brain size.

In addition, absence of Id4 resulted in a 3-fold increase of cell death through apoptosis in the neocortex (Bedford *et al.,* 2005) and increased apoptosis was also observed within oligodendrocyte progenitor cells lacking Id4 (Marin-Husstege *et al.,* 2006).

Taken together, Id4 is required for the maintenance of NSC and progenitor cells in the mouse ensuring balanced cell proliferation, death and differentiation.

1.3 Loss-of-Id4-Function in Zebrafish

1.3.1 Morpholino-Mediated Gene Knockdown

In order to understand the function of specific genes by inhibiting their function in the developing embryos, morpholino oligos (MOs) were introduced in 2000 as a reverse genetic tool. MOs are chemically modified antisense oligonucleotides which creates a temporary gene knockdown by inhibiting either the protein synthesis or splicing of the pre-mRNA via RNA binding. To execute the inhibition of the protein synthesis, MOs bind to the start site of the mRNA, therefore they are called “start-site morpholinos.” On the other hand, MOs binding to splice sites of pre-mRNA block splicing result in defective transcripts. These MOs are called “splice-site morpholinos” (Figure 1.7) (Nasevicius and Ekker, 2000; Timme-Laragy *et al.,* 2012).

Although antisense MOs appeared to be powerful tools for loss-of-function studies in zebrafish, it also brings some limitations. Knockdown of gene expression is variable, often not complete and lasts only for a restricted period of time during the first few days of the development. Off-target effects might occur and injection of MOs sometimes triggers upregulation of p53 (Nasevicius and Ekker, 2000; Heasman, 2002; Robu *et al.*, 2007).

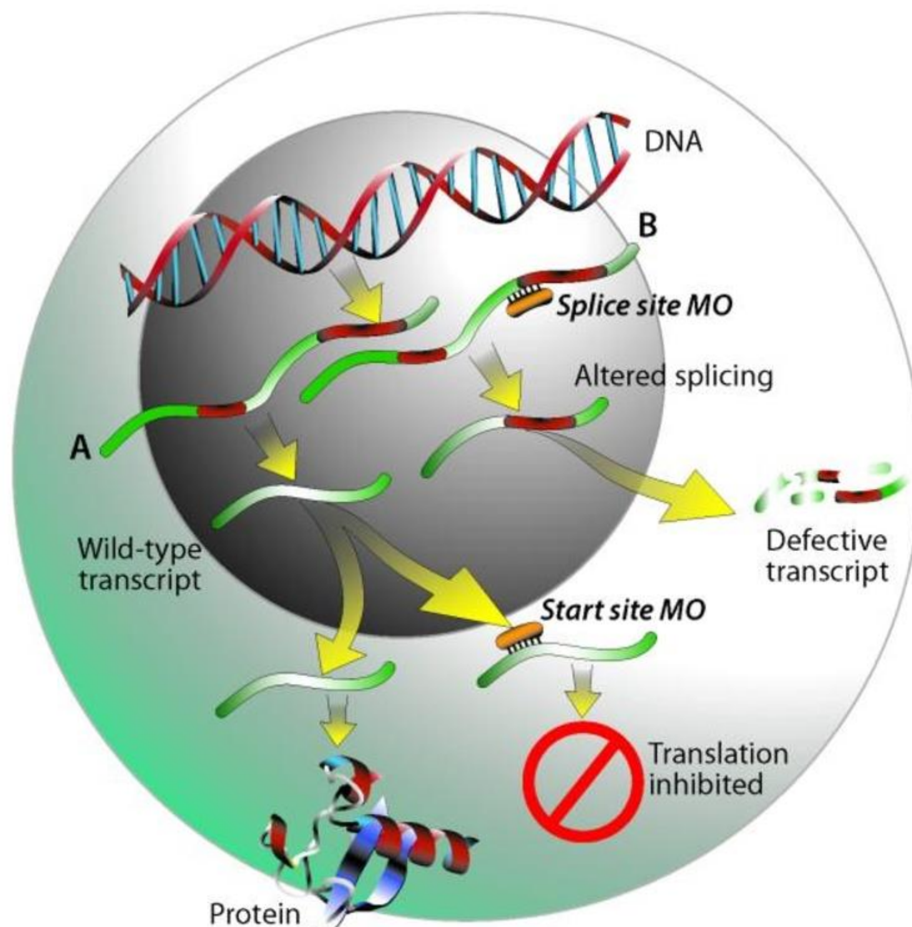

Figure 1.7: The Way MOs Work
(A) Start-Site MOs: MOs bind to the ATG start site or to a sequence close to the ATG of the mRNA transcript. This blocks initiation of translation resulting in reduction or loss of the protein.
(B) Splice-Site MOs: They binds t intron/exon junctions within pre-mRNA and prevent splicing at this junction. When the altered mRNA transcripts transport to the cytoplasm they will either be degraded, or give rise to a defective transcript (Adapted from Timme-Laragy *et al.,* 2012).

1.3.2 TALEN-Mediated Gene Knockout

TALEN (Transcription Activator-Like Effector Nucleases) appeared to be one of the most effective genome editing tools in 2011. TALEs (Transcription Activator-Like Effectors) are effector proteins secreted by the bacteria belong to the *Xanthomonas* genus which are plant pathogens. This group of bacteria synthesises TALEs in order to sensitise the plant to their infection. TALEs are capable of activating their target genes via DNA binding and consist of 3 main domains: a central DNA binding domain which is also responsible for sequence recognition, a C-terminal target gene transcription activating domain and a N-terminal nuclear localisation domain (Schornack *et al.,* 2006). Each monomer that makes up the DNA binding domain consists of 34 amino acid residues. Amino acids at positions 12 and 13 determine the specificity of nucleotide recognition. These amino acids are also called “repeat variable diresidue (RDV)”. The surrounding amino acids are highly conserved tandem repeats. Each RDV is composed of an amino acid pair which is specific to a nucleotide. The last residue at the 3’-end of the DNA-binding domain contains 20 amino acid residues therefore it is called a half-repeat. To construct TALENs, FokI restriction endonuclease is fused with the TALE domain which consists of 15,5-19,5 single repeats (monomers). TALENs work as pairs and a second TALEN has to be designed and placed on the opposite DNA strand with a 12-25 bp spacer sequence in between the two. When both TALENs bind to their specific DNA sequence, FokI dimerisation takes places and results in a double-strand break which leads to indel mutations by DNA repair mechanism (Figure 1.8) (Miller *et al.* 2011; Nemudryi *et al.* 2014).

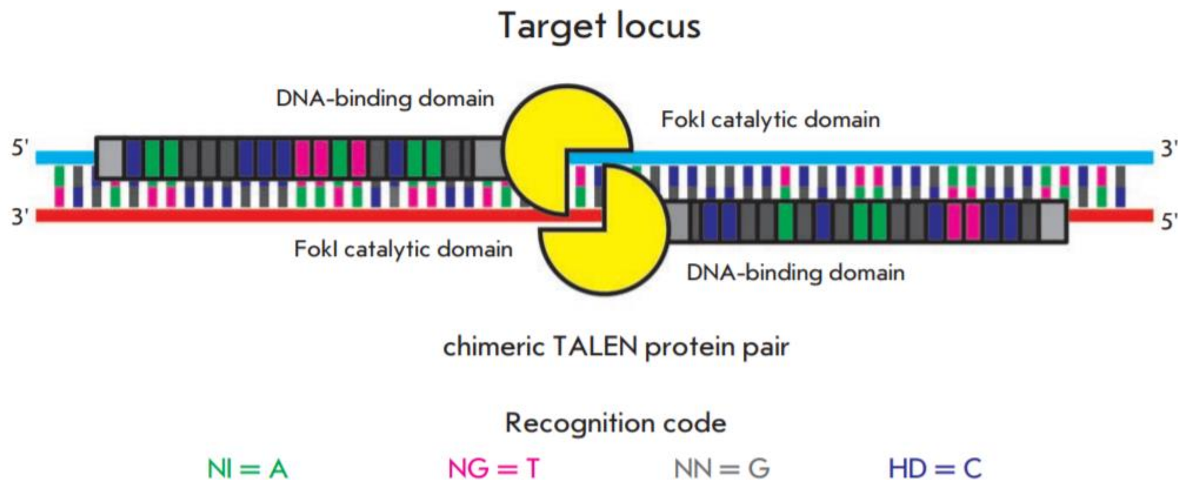

Figure 1.8: TALEN Mediated Genome Editing Mechanism
Each RDV monomer is color-coded based on the nucleotide-specific amino acids at positions 12 and 13. NI (Asn-Ile) binds A, NG (Asn-Gly) binds T, NN (Asn-Asn) binds G and HD (His-Asp) binds C. Dimerisation of FokI results in a double-strand break in the spacer region (Adapted from Nemudryi *et al.,* 2014).

1.4 Id4 Function in the Developing Nervous System of Zebrafish

In developing zebrafish embryos, Id4 expression is not restricted to the central nervous system (Thisse *et al.,* 2001; Dhanaseelan, 2011) and the earliest expression was detected at 6 hpf (shield stage) by RT-PCR (Bashir, 2010) as well as *in situ* hybridisation (Dhanaseelan, 2016) (Figure 1.9). At 70% epiboly, Id4 expression is extensive includes the precordal plate. Around tail bud stage (10 hpf), when somite formation begins and proceeds, Id4 expression becomes more restricted to the developing nervous system and is highly expressed in midbrain, hindbrain, telencephalon, diencephalon, retina and tegmentum (Figure 1.9) (Dhanaseelan, 2016).

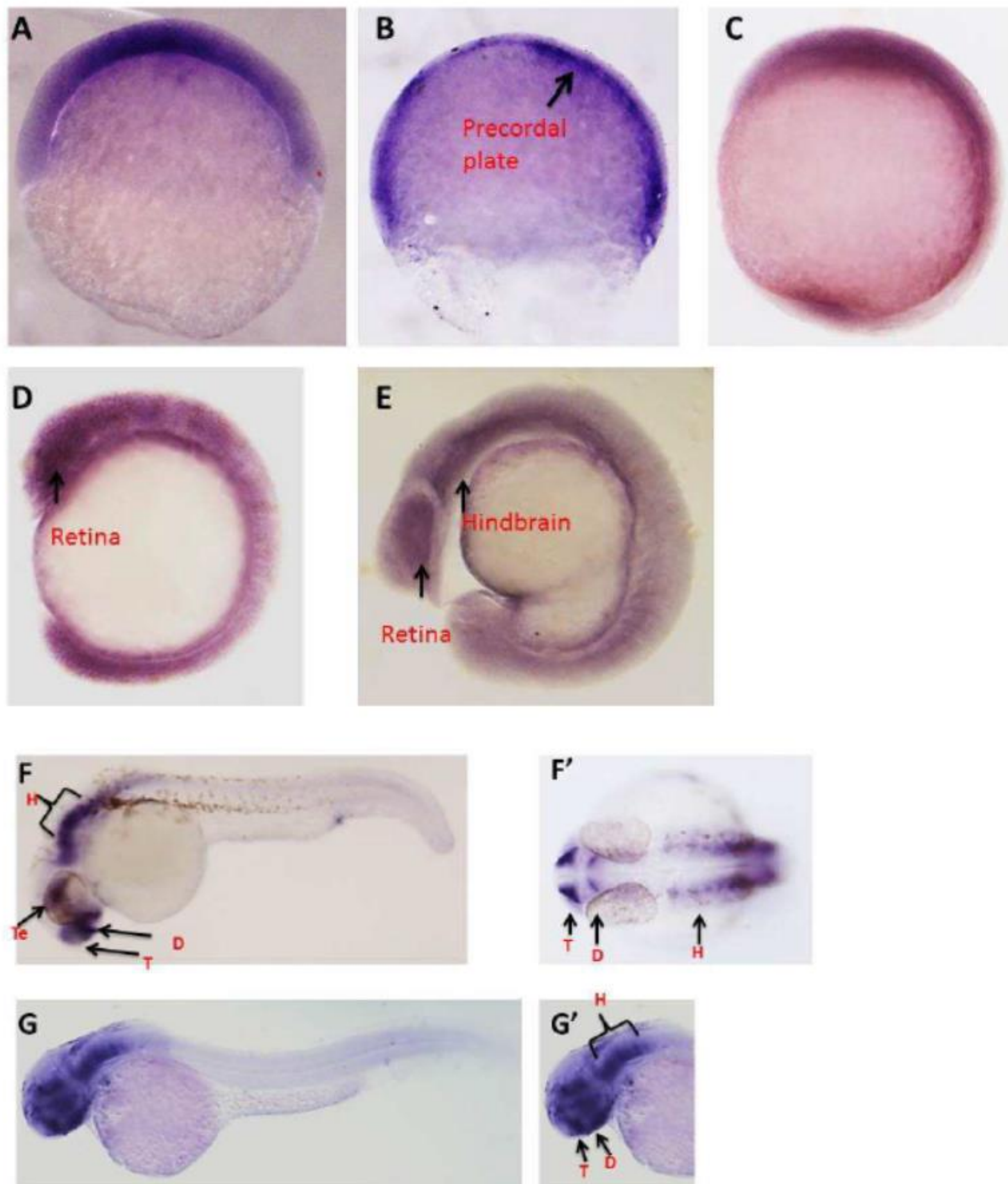

Figure 1.9: Id4 Expression During Wild Type Zebrafish Embryo Development
(A) Shield Stage, (B) 70% Epiboly Stage, (C) 2 Somite Stage, (D) 5 Somite Stage, (E) 18 Somite Stage, (F, F'-Ventral View) 24 hpf, (G, G') 36 hpf. T: Telencephalon, D: Diencephalon, H: Hindbrain, Te: Tegmentum (Taken from Dhanaseelan, 2016).

When Id4 expression was disrupted using translation blocking morpholinos (MOs), a reduction in brain size was observed and brain boundaries were absent at 24 hpf. Given that injection of MOs can sometimes cause non-specific upregulation of the p53 pathway that results in a similar phenotype (Robu *et al.,* 2007), p53-specific MOs were co-injected with Id4 MOs to eliminate potential off-target effects. Although slightly less severe, double morphants showed a similar phenotype as single Id4 morphants indicating the requirement of Id4 for normal brain development in zebrafish (Patlola, 2009; Dhanaseelan, 2011).

In addition, Id4 and Id4/p53 morphants exhibited a reduction of proliferating cells and an increase in apoptotic cells during tail bud stage. It is likely therefore that decreased cell proliferation and increased apoptosis lead to a reduced brain size due to the defects of the neural epithelium (Dhanaseelan, 2011).

TALEN-mediated Id4 knockout zebrafish (referred to as qmc803) were generated by Tamil Dhanaseelan (2016). A target site for the TALENs was chosen in exon 1 of the Id4 gene (Gene: ENSDARG00000045131) downstream of the ATG start codon and upstream of the region encoding the HLH domain (Figure 1.10). Any out-of-frame mutation would therefore disrupt the normal amino acid sequence and likely result in a premature stop codon and expression of an N-terminal truncated protein lacking the HLH domain. Left TALEN (5’-TTTATTACAATGAAGGCC-3’) and right TALEN (5’-TAGAAGGAAGCTTATGAG-3’) sequences were selected with a 16 bp spacer in between which contains a HpaII restriction site (5’-AGCGTG***CCGG***TTCGCC-3’).

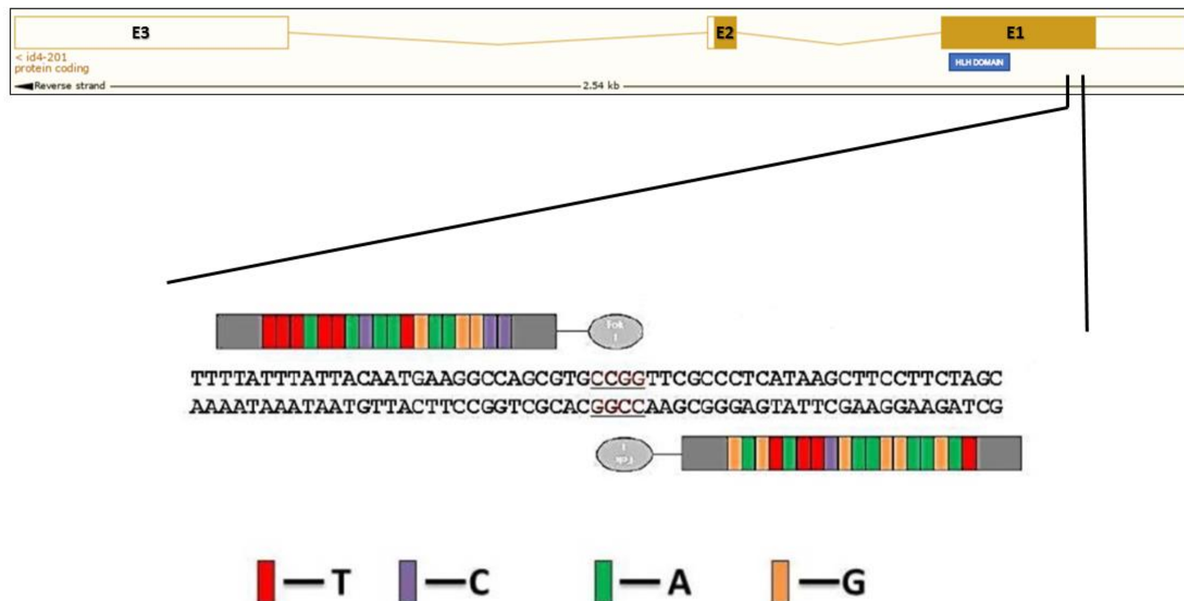

Figure 1.10: TALEN-Mediated Id4 Knockout in Zebrafish
First exon including the HLH domain of Id4 gene was targeted with a pair of TALENs separated by a 16 bp spacer that contains HpaII restriction site. E1: Exon 1, E2: Exon 2, E3: Exon 3, **CCGG:** HpaII Restriction Site (Adapted from Dhanaseelan, 2016).

In contrast to the phenotype observed in Id4 morphants as summarised above, TALEN-mediated Id4 knockout embryos at 24 hpf didn't show morphological abnormalities, neither in the brain nor trunk (Figure 1.11). However, whole mount *in situ* hybridisation using riboprobes for Ngn1 as a marker for differentiation of cortical progenitors into neurons and HuC/elavl3 for neuronal determination and differentiation indicated that Id4 knockout leads to premature neuronal differentiation (Figure 1.12-13). In line with the analysis of Id4 and Id4/p53 morphants, cell proliferation was decreased and apoptosis was increased in Id4 knockout zebrafish embryos (Dhanaseelan, 2016).

It seems therefore that Id4 function in the developing nervous system of both mouse and zebrafish is similar ensuring that the NSC and progenitor pool is maintained through balanced cell proliferation, death and timing of differentiation.

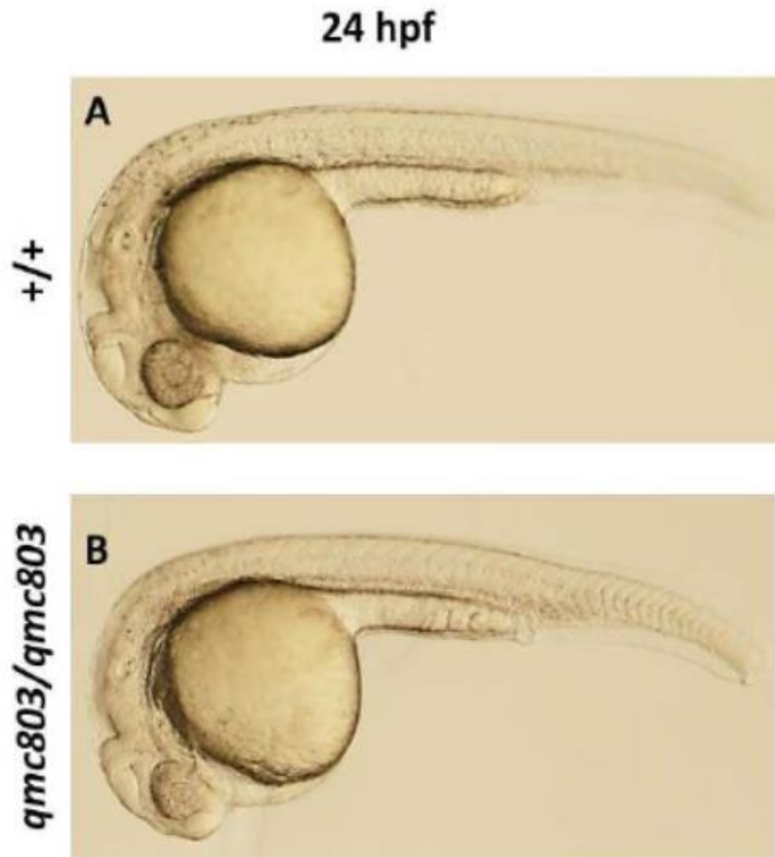

Figure 1.11: Homozygous Id4$^{qmc803/qmc803}$ embryos are morphologically normal
Crosses of heterozygous Id4$^{qmc803/+}$ zebrafish gave rise to homozygous offspring (B) that at 24 hpf appeared morphologically normal compared to wild type (A) (Adapted from Dhanaseelan, 2016).

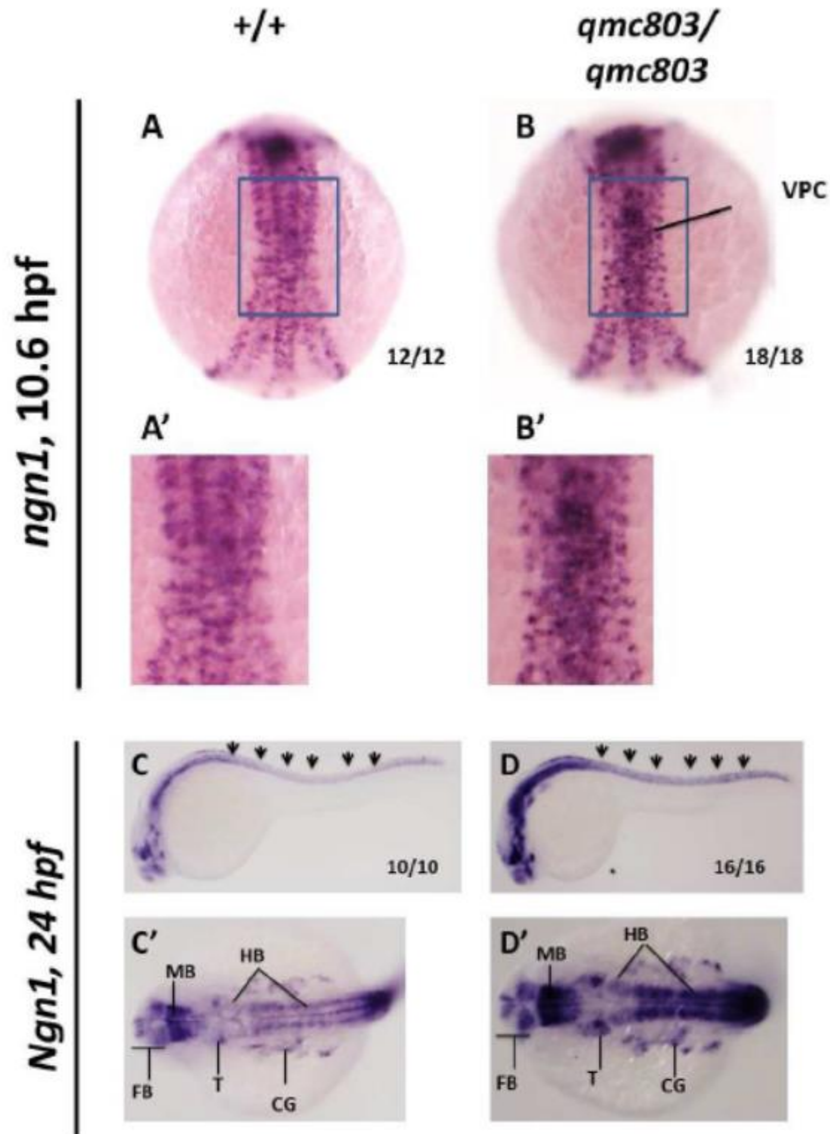

Figure 1.12: Elevated Expression of Ngn1 in Id4 Knockout Zebrafish Embryo at 10.6 and 24 hpf

Ngn1 is a bHLH protein which promotes differentiation of cortical progenitors into neurons. A slight difference of the expression pattern of ngn1 between wild type (A, A') and Id4 homozygous mutant (B, B') was apparent at 10.6 hpf. At 24 hpf ngn1 expression in the Id4 mutants (D, D') was markedly increased throughout the CNS compared to wild type (C, C'). VPC: Ventral Pro-Neural Clusters, FB: Forebrain, MB: Midbrain, HB: Hindbrain, T: Tectum, CG: Cranial Ganglia. Arrows indicate spinal cord (Taken from Dhanaseelan, 2016).

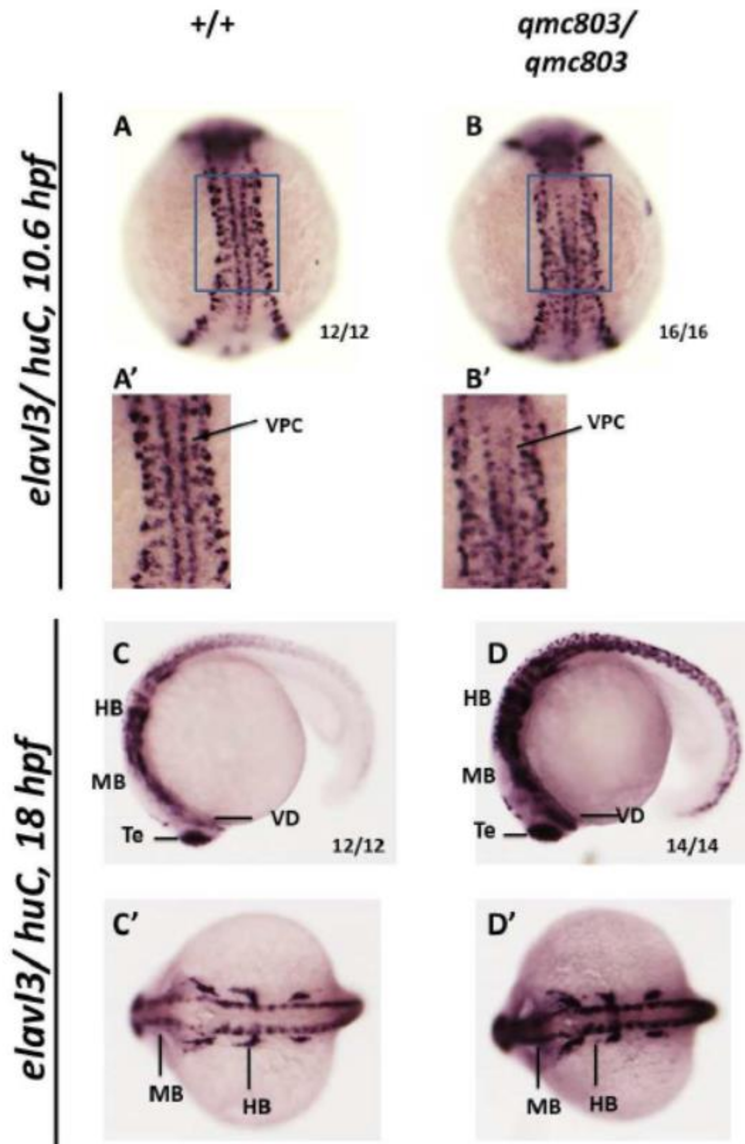

Figure 1.13: Elevated Expression of HuC/elavl3 in Id4 Knockout Zebrafish Embryos at 10.6 and 18 hpf

HuC/elavl3 is a marker for neuronal determination and differentiation. The expression pattern of HuC/elavl3 between wild type (A, A') and Id4 homozygous mutant (B, B') at 10.6 hpf is indistinguishable. At 18 hpf, a significant increase of HuC/elavl3 expression is apparent in homozygous Id4 mutants (D, D') throughout the CNS compared to wild type (C, C'). VPC: Ventral Pro-Neural Clusters, MB: Midbrain, HB: Hindbrain, Te: Telencephalon, VD: Ventral Diencephalon. Arrows indicate spinal cord (Taken from Dhanaseelan, 2016).

1.5 Notch Signalling Pathway

1.5.1 Discovery

First indication of the Notch signalling pathway was the observation of small notches at the tips of *Drosophila melanogaster* wings which was noticed by John S. Dexter as a heritable abnormality in 1914. The first alleles of the Notch gene were later identified by Thomas Hunt Morgan in 1917.

1.5.2 Structure

Notch receptors are transmembrane proteins which are involved in a signalling cascade that controls a variety of events such as cell fate determination via cell-cell communication and pattern formation, from invertebrates to humans. Notch receptors have 2 domains: the large extracellular domain which contains many epidermal growth factor (EGF)-like repeats, and the intracellular domain (Rebay *et al.,* 1991). In *C. elegans*, there are 2 Notch homologs named LIN-12 and GLP-1 while humans have 4 named as Notch1-4. Notch signalling pathway system contains 2 additional components other than Notch receptors: ligands and transcription factors. In *D. melanogaster*, 2 types of Notch ligands have been identified which are called Delta and Serrate. On the other hand, *C. elegans* has 4 and mammals have 5 types of Notch ligands. Transcription factors belong to CSL (CBF1/Su(H)/LAG1) family which are sequence-specific DNA-binding proteins and downstream targets of Notch (Table 1.2) CBF1 is found in mammals, Su(H) in *D. melanogaster* and LAG-1 in *C. elegans* (Lai, 2004).

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Core component	*C. elegans*	*D. melanogaster*	Mammals
Ligand	LAG-2 APX-1 ARG-2 F16B12.2	Delta Serrate	Delta-like1 (DLL1) Delta-like2 (DLL2) Delta-like3 (DLL3) Jagged 1 (JAG1) Jagged 2 (JAG2)
Receptor (Notch)	LIN-12 GLP-1	Notch	Notch1 Notch2 Notch3 Notch4
Transcription factor (CSL)	LAG-1	Suppressor of Hairless [Su(H)]	CBF1/RBPJκ RBPL

Table 1.2: Notch Signalling Pathway Components in Different Species
Ligand, receptor and transcription factor components of Notch Signalling Pathway (Adapted from Lai, 2004).

1.5.3 Action Mechanism

Interaction of ligands such as Delta-like 1 (DLL1) or Jagged1 with notch trigger two cleavage events. First cleavage (S2) results in the release of the majority of the Notch extracellular domain (NECD). Cleaved Notch becomes a substrate for γ-secretase which is a multicomponent complex that creates the second cleavage (S3) and as a result release the Notch intracellular domain (NICD). Subsequently, the released NICD is transferred to the nucleus and forms a complex with CSL such as Rbpj and transcriptional co-activators like Maml (Mastermind-like). When the NICD-Rbpj-Maml complex is formed, the expression of bHLH transcriptional repressors will be induced. In the absence of NICD, co-activators will be replaced by co-repressors which leads to the inhibition of Notch target repressor genes (Figure 1.14) (Artavanis-Tsakonas *et al.,* 1999; Lai, 2004; Kageyama *et al.,* 2007).

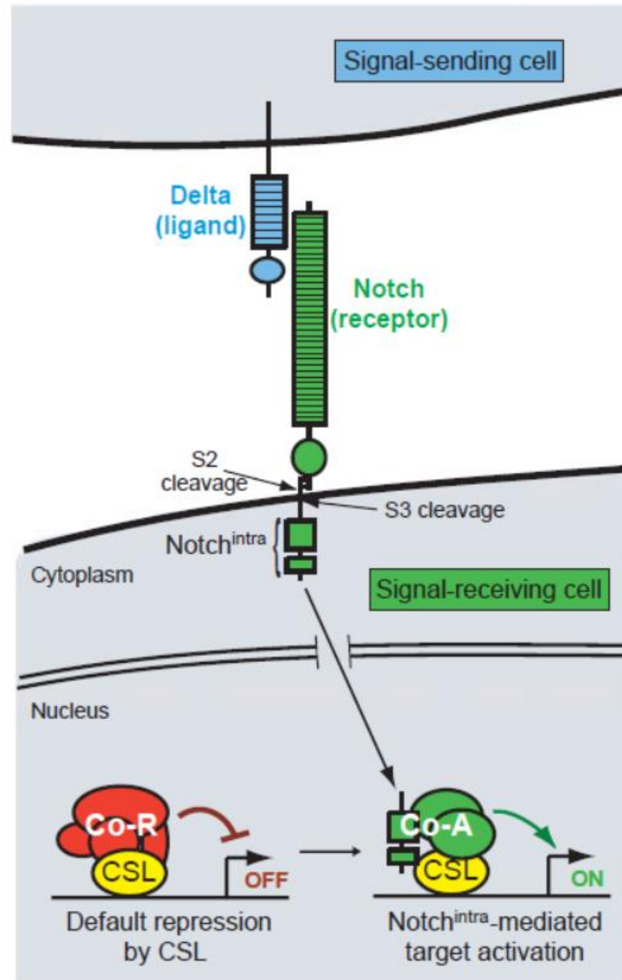


Figure 1.14: Mechanism of Notch Signalling
The interaction between Notch and its ligand triggers S2 and S3 cleavages. While S2 cleavage leads to the release of the majority of the extracellular domain (NECD), S3 results in intracellular domain (NICD) separation. NICD then translocates to the nucleus forming the NICD-CSL-CoA complex that activates expression of Notch target genes. In the absence of NICD, co-activators will be replaced by co-repressors which leads to the repression of Notch target genes. Co-R: Co-Repressor, Co-A: Co-Activator (Adapted from Lai, 2004).

1.5.4 Lateral Inhibition

Notch signalling restricts neural cell fates via lateral inhibition in order to prevent the differentiation of cells into the same cells types therefore provides cell specification (Artavanis-Tsakonas *et al.,* 1999). Inhibition is induced by a neuroblast, which has a committed neuronal cell fate as a result of proneural gene activation. Proneural genes such as Mash1 and Neurogenin2 (Ngn2) not only induce neuronal differentiation but also Notch ligands such as Dll1 and Jagged1 (Bertrand *et al.,* 2002; Castro *et al.,* 2006). Expression of Notch ligands activates Notch signalling in neighbouring cells therefore Notch target repressor genes such as Hes1 and Hes5 is expressed. Proneural gene expression will then be inhibited by respressor proteins and eventually lead to the maintenance of neural stem/progenitor cells (Figure 1.15) (Artavanis-Tsakonas *et al.,* 1999; Kageyama *et al.,* 2007; Shimojo *et al.,* 2011).

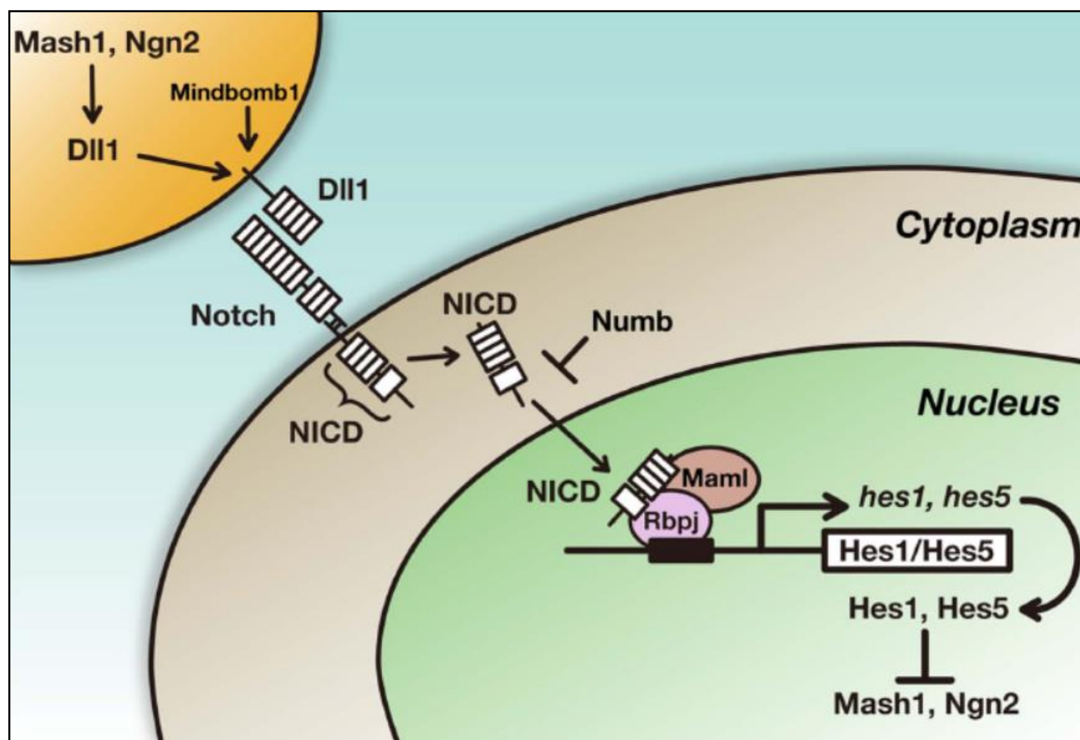


Figure 1.15: Lateral Inhibition Mediated by Notch Signalling
Proneural proteins Mash1 and Ngn2 induce DLL1 expression thereby promoting neuronal differentiation. Induced DLL1 expression activates Notch signalling in the neighbouring cells and as a result Hes 1 and Hes 5 are expressed which inhibits both the expression of proneural genes and DLL1. Mind bomb is a required ligase for the DLL1-induced activation of Notch signalling. Numb, a phosphotyrosine binding (PTB) domain adaptor protein, inhibits Notch signalling and induces neuronal differentiation (Adapted from Shimojo *et al.,* 2011).

1.5.5 Downstream Targets of Notch Signalling

Notch signalling pathway controls cell fate determination, proliferation and apoptosis via its target genes during both development and adult life. There are plenty of known target genes but two of them, Hes (Hairy/Enhancer of Split) and Hey families of bHLH transcriptional repressors are best known downstream targets of Notch signalling pathway (Iso *et al.,* 2003; Leimeister *et al.,* 1999). When Hes/Hey transcription is activated via Notch signalling, their target genes which are tissue-specific transcriptional activators that induce differentiation are repressed (Iso *et al.,* 2003).

Studies with an activated Notch1 system in murine embryonic stem cells (ESCs) and in mesodermal cells revealed several more target genes which are Sox9, Pax6, Runx1, Myf5 and Id proteins. While Sox9 has a role in all three germ layers, Pax6 is essential in neuro-ectodermal development. Myf5 and Runx1 are required for lineage specification of mesodermal derived tissues, skeletal muscle development and hematopoietic stem cell lineage respectively (Meier-Stiegen *et al.,* 2010).

Notch1 activation and signalling had a differential effect on Id gene expression. While Id2 expression displayed a downregulation, expression of other Ids, especially Id4, was upregulated. The significant upregulation of Id4 correlates with the fact that Id4 and Notch share similar roles in terms of proliferation, differentiation and apoptosis of neural stem cells during development (Meier-Stiegen *et al.,* 2010). A positive regulation was also shown with XId3, an Id member in Xenopus by Notch signalling through a Su(H)-dependent pathway (Reynaud-Deonauth *et al.,* 2002).

1.5.6 Inhibition of Notch Signalling Pathway

1.5.6.1 γ-Secretase Inhibition

One of the key elements within Notch signalling pathway is the γ-secretase which is a membrane protein complex. NICD needs to be released from the receptor by γ-secretase cleavage, in order to activate Notch target genes. Notch signalling can be blocked by inhibiting γ-secretase via specific inhibitors such as DAPT and DAPM. These inhibitors prevent the formation of γ-secretase complex, therefore inhibit Notch signalling (Crawford and Roelink, 2007). Although γ-secretase inhibition is a widely used method for blocking Notch effectively in vertebrate systems (Geling *et al.,* 2002), some studies revealed only partial inhibition (Al Khamees, 2016).

Inhibition of γ-secretase during zebrafish embryogenesis resulted in impaired somite formation with misshapen somatic boundaries at 24 hpf in line with the fact that under normal circumstances, Notch controls somite anteroposterior polarity. In addition, γ-secretase inhibition triggers a neurogenic phenotype by preventing lateral inhibition and neuroblast selection (Geling *et al.,* 2002).

1.5.6.2 Mind Bomb Mutants

Mib (Mind Bomb) is an E3 ubiquitin ligase coding gene required for efficient Delta-Notch interaction which is an essential step for Notch signalling (Figure 1.16). The experiments that has been done with a disrupted mib gene within zebrafish embryos resulted in severe neurogenic phenotype as well as impaired formation of somites, neural crest and vasculature (Jiang *et al.,* 1996; Lawson *et al.*, 2001).

Zebrafish mib mutants displayed premature neuronal differentiation and a deficiency of late differentiating neurons in the spinal cord and other CNS regions. Reduced Notch signalling resulted in reduced selection of neural progenitors via lateral inhibition and in turn increased differentiation into neurons (Itoh *et al.,* 2003). A disruption in the development of hind brain motor neurons was also observed (Bingham *et al.*, 2003).

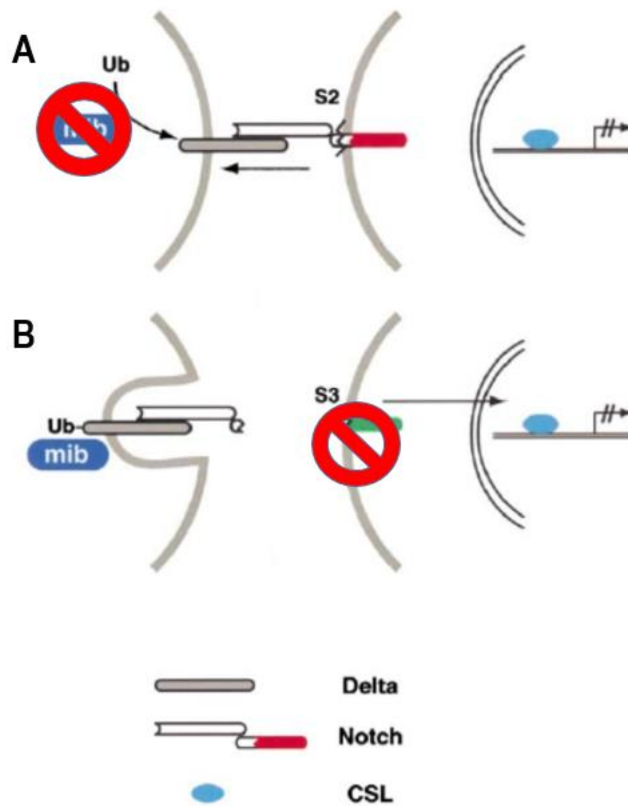


Figure 1.16: Inhibition of the Notch Signalling Pathway
A: In the absence of mib, both ubiquitination of Delta and endocytosis which facilitates S2 cleavage is inhibited.
B: DAPM and/or DAPT treatment inhibits the formation of γ-secretase complex, therefore neither S3 cleavage nor NICD translocation can take place (Adapted from Itoh *et al.,* 2003).

1.6 Aims & Objectives

- Determine the role of Id4 in neurogenesis utilising TALEN-mediated Id4 mutant zebrafish (Id4^{qmc803})

  The initial phenotypic analysis of Id4^{qmc803} mutant zebrafish revealed that lack of Id4 resulted in premature neural differentiation during embryogenesis. To test whether Id4 has also a role in neuronal stem and progenitor cell maintenance and/or glial cell differentiation, expression of genes marking theses cell populations was determined at various stages of development using whole-mount *in situ* hybridisation.

- Determine whether Id4 expression in zebrafish is regulated through notch signalling.

  Neuronal stem cell proliferation and differentiation is regulated through Notch signalling and inhibition or lack of notch signalling disrupts neurogenesis. To test whether Id4 is a downstream target of notch signalling, Id4 expression was determined in wild type zebrafish embryos, in wild type embryos treated with the notch inhibitor DAPM or in Mind bomb mutant embryos that are deficient in notch signalling.

2. MATERIALS & METHODS

2.1 DNA Preparation

2.1.1 Transformation of Competent Cells

2.1.1.1 Materials

Technical Equipment	
Name	**Source & Details**
Orbital Incubator	SI 50; Stuart Scientific
Heat Block	DRI-BLOCK DB3; Jencons Techne
Chemical Reagents & Buffers	
Name	**Source & Details**
Competent Cells	NEB 5α (New England Biolabs)
LB (Luria-Bertani) Medium	10g Bactotryptone; 5g Yeast Extract; 5g NaCl; distilled H_2O
LB Agar	LB Agar with 1.5% Bacto-Agar
Ampicillin Antibiotics	100µg/ml; Sigma-Aldrich

2.1.1.2 Method

10-100ng of plasmid DNA was added to 50µl of NEB 5α competent cells, after they had been thawed on ice, mixed gently and incubated on ice for 30 minutes. Right after incubation, cells were heat-shocked at 42°C for 45 seconds and immediately incubated on ice again for 2 minutes. 500µl of SOC medium was added to the cells that were incubated for an hour at 37°C with continuous shaking. 100-150µl of the cells were spread out onto ampicillin containing LB agar plates and left overnight at 37°C. Following day, single colonies were picked from the agar plate and inoculated into falcon tubes containing 5ml LB medium with ampicillin. The tubes left overnight (16-18 hours) in 37°C with maximum agitation.

2.1.2 Plasmid DNA Preparation

2.1.2.1 Materials

Technical Equipment	
Name	**Source & Details**
Microfuge	SciQuip; Sigma
Chemical Reagents & Buffers	
Name	**Source & Details**
Mini Prep DNA Kit	Resuspension Buffer, Lysis Solution, Neutralization Buffer, Column Preparation Buffer, Wash Solution, Elution Buffer; Sigma-Aldrich

2.1.2.1 Method

Sigma Mini Prep DNA Kit was used to extract plasmid DNA. The transformed bacteria were aliquoted into eppendorf tubes and centrifuged at 12.000 rpm for a minute. The supernatant was discarded and the pellet resuspended in 200µl of Resuspension Buffer. The pellet was gradually dissolved by gentle pipetting. Then 200µl of Lysis Solution was added and the tubes inverted gently 6-8 times and waited for 3-4 minutes. Finally, 350µl of Neutralization Buffer was added, gently inverted 6-8 times and centrifuged for 10 minutes at maximum speed. Meanwhile, spin column tubes were prepared. 500µl of Column Preparation Buffer was added to each spin column, centrifuged at 12.000 rpm for a minute and the flow-through was discarded. After lysed cells were pelleted, the supernatant was transferred to the column, centrifuged at the highest speed for a minute and the flow-through was discarded. Then 750µl of Wash Solution was added to the column, centrifuged at 12.000 rpm for a minute and the flow-through discarded. After discarding the flow-through, the column tubes were centrifuged at maximum speed for a minute to ensure all residual buffer was eliminated. Then the spin column was transferred into an Eppendorf tube and 100µl of Elution Buffer was added. After 30 seconds at room temperature, spin columns in the Eppendorf tubes were centrifuged at 12.000 rpm for a minute. Inner column was removed and plasmid DNA was obtained within the remaining liquid.

2.1.3 Quantification of DNA

2.1.3.1 Materials

Technical Equipment	
Name	**Source & Details**
Nanodrop	ND-1000; Thermo Scientific

2.1.3.2 Method

The concentration of the plasmid DNA was determined using Nanodrop. The ratio of absorbance at 260nm was used in order to determine DNA concentration. Nuclease-free water was used as blank and 1.5µl of DNA was used for the measurement.

2.1.4 Restriction Enzyme Digestion

2.1.4.1 Materials

Technical Equipment	
Name	**Source & Details**
Orbital Incubator	SI 50; Stuart Scientific
Chemical Reagents & Buffers	
Name	**Source & Details**
Restriction Enzymes	New England Biolabs
Digestion Buffers	10X Concentration; New England Biolabs

2.1.4.2 Method

The reaction was set up based on the 1 unit of enzyme requirement for 1µg of DNA ratio. Each reaction tube contained 1µg of DNA, 8µl of 10X Digestion Buffer, 1µl of each Restriction Enzyme and the final volume was made up to 80µl with nuclease-free water. The tubes were kept in 37°C incubator for an hour.

2.1.5 Agarose Gel Electrophoresis

2.1.5.1 Materials

Technical Equipment	
Name	**Source & Details**
Horizontal Gel Electrophoresis Apparatus	Horizon 11.14; Thermo Fisher Scientific
Molecular Imager GelDoc System	Universal Hood II; BIO-RAD
Chemical Reagents & Buffers	
Name	**Source & Details**
Agarose	Sigma-Aldrich
TBE Buffer	10X Stock Buffer; 55g Boric Acid, 108g Trizma Base, 40ml 0.5M EDTA, distilled H_2O (final volume 1litre), Working Concentration; 1X
SafeView; Nucleic Acid Stain	NBS Biologicals
DNA Ladder	100bp, 1kb; New England Biolabs
Gel Loading Dye	6X Concentration; New England Biolabs

2.1.5.2 Method

DNA fragments were separated by agarose gel electrophoresis according to their sizes. Depending on the size of the DNA fragment different concentrations of agarose gels were prepared. For plasmid DNA, an agarose gel with 1% concentration was prepared. For RNA and small DNA fragments, the concentration was increased to 1.5%. Appropriate amount of agarose in accordance with the concentration was added to 1X TBE Buffer, melted until the mixture was homogenized, cooled at room temperature and then SafeView was added with a proportion of ⅙ and poured into gel casting tray. After the gel was solidified, molecular weight ladder was prepared by adding 1µl into 9µl of sterile distilled water and 6X gel loading dye was added to all samples. Samples were electrophoresed for one hour at 130V. The DNA fragments were visualized using a molecular imager gel system.

2.1.6 Purification of the Digested Plasmid

2.1.6.1 Materials

Technical Equipment	
Name	**Source & Details**
Micromax RF Centrifuge	IEC
Micro Centrifuge	Sigma
Chemical Reagents & Buffers	
Name	**Source & Details**
Phenol-Chloroform-Isoamyl Alcohol Mix	Sigma-Aldrich
Sodium Acetate Buffered Solution	pH: 5.2

2.1.6.2 Method

Equal volume (1 volume) of phenol-chloroform-isoamyl alcohol mix was added to the digested DNA sample, vortexed and centrifuged at 12.000 rpm for 5 minutes. Then aqueous (upper) layer was transferred to a new tube and 1 volume of chloroform was added, vortexed and centrifuged for 5 minutes at 12.000 rpm. The aqueous layer again transferred to a new tube. 2.5 volume of ice-cold EtOH and 1/10 volume of Sodium Acetate was then added and kept at -20°C for an hour. The sample was centrifuged at 13.000 rpm for 30 minutes at 4°C centrifuge. After observing a tiny pellet, the Sodium Acetate-EtOH solution was carefully discarded without touching the pellet. Then the plasmid DNA pellet was washed with 100µl of 70% EtOH and centrifuged again at 13.000 rpm for a few minutes at 4°C centrifuge. Finally, EtOH was discarded and the pellet was left to air dry for 10 minutes, dissolved in 10µl of nuclease-free water and stored at -20°C. Plasmid DNAs were analysed by gel electrophoreses and their concentration was determined using Nanodrop.

2.2 mRNA Probe Synthesis for Whole-Mount *in situ* Hybridisation

2.2.1 *In Vitro* Transcription

2.2.1.1 Materials

Technical Equipment	
Name	**Source & Details**
Orbital Incubator	SI 50; Stuart Scientific
Chemical Reagents & Buffers	
Name	**Source & Details**
DIG RNA Labelling Mix	10X Concentration; Roche
Transcription Buffer	5X Concentration; Thermo Scientific
Recombinant RNasin	Promega
DTT	100mM; Promega
T7 RNA Polymerase	Thermo Scientific
T3 RNA Polymerase	Promega

2.2.1.2 Method

In order to obtain mRNA probes for Whole-Mount *In Situ* Hybridisation, 1,5µg of purified DNA template was used to set up a 20µl reaction mixture along with 2µl of DIG RNA Labelling Mix, 4µl of Transcription Buffer, 0,5µl of RNasin, 2µl of DTT, 1µl of RNA Polymerase and nuclease-free water. The mixture was kept at 37°C incubator for 2 hours.

2.2.2 Purification of mRNA Probes

2.2.2.1 Materials

Technical Equipment	
Name	**Source & Details**
Micromax RF Centrifuge	IEC
Micro Centrifuge	Sigma
Chemical Reagents & Buffers	
Name	**Source & Details**
TURBO DNase	Ambion
Ammonium Acetate	Ambion
EDTA	0.5M, pH:8; VWR

2.2.2.2 Method

After *in vitro* transcription process was completed, 1µl of DNase was added to the reaction mixture and incubated at 37°C for 20 minutes to digest residual plasmid DNA. The DNase reaction was stopped by adding 1µl of EDTA. Then 78µl of nuclease-free water was added to complete the total volume to 100µl. ⅓ volume (~33µl) of Ammonium Acetate and 2,5 volume (250µl) of EtOH was added afterwards. The sample was incubated on ice for 10 minute, centrifuged at 13.000 rpm for 15 minutes at 4°C centrifuge. After observing a tiny pellet, the Ammonium Acetate-EtOH solution was carefully discarded without touching the pellet. Then the pellet was washed with 100µl of 70% EtOH and centrifuged again at 13.000 rpm for a few minutes at 4°C centrifuge. Finally, EtOH was discarded and the pellet was left to air dry for 10 minutes and dissolved in 40µl of nuclease-free water. Aliquots of the RNA was analysed by gel electrophoreses using a 1.5% agarose gel. Some amount was used for preparing the probes for *in situ* hybridisation with a dilution of 1:100 and 1:200. The rest was stored at -80°C.

2.3 Manipulation of Zebrafish Embryos

All the zebrafish were maintained at 28.5°C in system water. After collection of embryos following natural spawning; they were raised at 28°C in E3 Buffer. All the experiments were performed under the Home Office Project License 30/3378 and Personal Licence I4B3BEDA0.

2.3.1 Fin-Clipping

2.3.1.1 Materials

Chemical Reagents & Buffers	
Name	**Source & Details**
MS-222	0.03% (V/V), pH:7; Sigma

2.3.1.2 Method

MS-222 was used to anaesthetise the zebrafish. After the movement of both fish and the gills slowed down, the zebrafish were placed on a paper towel and a small piece from tail fin was clipped using scissors. The small piece of the fin was then put into an eppendorf tube and kept on ice until the DNA extraction took place. The fin-clipped fish was kept in a separate tank for a week to prevent any infection.

2.3.2 DNA Extraction From Zebrafish Fin Tissues and Embryos

2.3.2.1 Materials

Technical Equipment	
Name	**Source & Details**
Heat Block	DRI-BLOCK DB3; Jencons Techne
Microfuge	SciQuip; Sigma
Chemical Reagents & Buffers	
Name	**Source & Details**
Base Buffer	50X Stock Buffer; 1.25M NaOH, 10mM EDTA(pH: 12), Working Concentration; 1X
Neutralization Buffer	50X Stock Solution; 2M Tris-HCl (pH: 5), Working Concentration; 1X

2.3.2.2 Method

Both stock solutions were diluted to 1X concentration with sterile distilled water. First; 50µl of Base Buffer was added each fin tissue/embryo, then kept at 98°C until completely dissolved fin tissues/embryos in the buffer were observed which took 40-50 minutes. Then the eppendorf tubes were placed on ice for a few minutes and 50µl of Neutralization Buffer was added, vortexed and centrifuged for 5 minutes at 3.000 rpm to precipitate debris. Extracted DNA samples were stored at 4°C.

2.3.3 DNA Extraction From Embryos After *in situ* Hybridisation

2.3.3.1 Materials

Technical Equipment	
Name	**Source & Details**
Heat Block	DRI-BLOCK DB3; Jencons Techne
PCR	PCR Thermal Cycler; Takara
Microfuge	SciQuip; Sigma
Chemical Reagents & Buffers	
Name	**Source & Details**
PBST	pH:7.4, 0.1% Tween 20 (1ml Tween 20 in 1lt PBS)
Glycerol	Dilutions were made with PBST
NaCl	300mM
Base Buffer	50X Stock Buffer; 1.25M NaOH, 10mM EDTA(pH: 12), Working Concentration; 1X
Neutralization Buffer	50X Stock Solution; 2M Tris-HCl (pH: 5), Working Concentration; 1X

2.3.3.2 Method

Embryos were washed with 50% and 20% glycerol and then 3 times washed with PBST to eliminate the glycerol. 100μl of NaCl was added and the embryos were kept at 65°C for 3 hours in the heat block. Then NaCl was discarded and 30μl of Base Buffer was added to each embryo and kept at 98°C in the PCR machine for an hour. Subsequently the tubes were placed on ice for a few minutes, 30μl of Neutralisation Buffer was added and centrifuged for 5 minutes at 3.000 rpm to precipitate debris. Extracted DNA samples were stored at -20°C.

2.3.4 PCR (Polymerase Chain Reaction)

2.3.4.1 Materials

Technical Equipment	
Name	**Source & Details**
PCR	PCR Thermal Cycler; Takara
Chemical Reagents & Buffers	
Name	**Source & Details**
Q5 Reaction Buffer	5X; New England Biolabs
dNTP	10mM; New England Biolabs
Id4 Forward Primer	10µM; Invitrogen
Id4 Reverse Primer	10µM; Invitrogen
Q5 Hot Start High-Fidelity DNA Polymerase	New England Biolabs
Primer Sequences (5'-3')	
Id4 Forward Primer	TGTGACCAACAATAACTCATCCG
Id4 Reverse Primer	TTGACTATATTCTGGACCTGCAGC

2.3.4.2 Method

PRC reactions were set up with a total volume of 50µl: 10µl of 5X Reaction Buffer, 1µl of dNTP, 1,5µl of each primer, 8µl of extracted DNA, 0,5µl of DNA Polymerase and nuclease-free water. Annealing temperature set up for 55°C with 30 cycles.

2.3.5 Restriction Enzyme Digestion

2.3.5.1 Materials

Technical Equipment	
Name	**Source & Details**
Orbital Incubator	SI 50; Stuart Scientific
Chemical Reagents & Buffers	
Name	**Source & Details**
Restriction Enzyme	HpaII; New England Biolabs
Digestion Buffers	10X Concentration; New England Biolabs

2.3.5.2 Method

TALEN-induced Id4 mutation resulted in a deletion of the HpaII restriction site. Therefore, HpaII restriction enzyme was used for genotyping. 17μl of PCR products, 1μl of HpaII and 2μl of Digestion Buffer was used for digestion reaction with a total volume of 20μl at 37°C for 2 hours. After digestion, each DNA sample was analysed by agarose gel electrophoresis to determine the genotype.

2.3.6 DAPM and DMSO Treatment of Embryos

2.3.6.1 Materials

Technical Equipment	
Name	**Source & Details**
Incubator	LMS
Chemical Reagents & Buffers	
Name	**Source & Details**
DAPM	Stock Concentration: 12.5mM, Working Concentration: 102.5µM; Calbiochem
DMSO	Sigma
E3 Buffer	60X Stock Solution; 5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2.2H_2O$, 0.3mM $MgSO_4.7H_2O$

2.3.6.2 Method

Embryos were obtained by crossing wild type fish and placed in petri dishes which contains E3 fish medium. They were left to grow in 28.5°C incubator. On the other hand, 2 separate petri dishes were prepared for DAPM and DMSO treatment. 205µl was used from both DMSO and DAPM stock within 25ml of E3 Buffer. The petri dishes were shaken gently after adding DMSO and DAPM into E3 Buffer to ensure they mixed homogeneously. Once the fish grew up to 5.5 hours, they were transferred into DMSO and DAPM mixed E3 Buffer containing dishes and left at 28.5°C for development.

2.3.7 Fixation and Storage of Embryos for Whole-Mount *in situ* Hybridisation

2.3.7.1 Materials

Technical Equipment	
Name	**Source & Details**
Orbital Shaker	Stuart Scientific
Chemical Reagents & Buffers	
Name	**Source & Details**
PFA	4% (W/V), Sigma
PTU	0.03% (W/V), Sigma
PBST	pH:7.4, 0.1% Tween 20 (1ml Tween 20 in 1lt PBS)

2.3.7.2 Method

After the embryos reached to the required stage, they were fixed overnight with PFA at 4°C. Fixation process differs based on the stage of the embryos. The embryos younger than 22 hours directly exposed to PFA and left overnight. Next day, they were washed 3 times for 5 minutes with PBST using a shaker and then dechorionated. Embryos older than 22 hours were first exposed to PTU when they were around 19-20 hours old, to stop pigmentation. After they reached the required stage, their chorions were removed and embryos fixed overnight with PFA. The next day embryos were washed 3 times with PBST using a shaker. Subsequently, embryos were dehydrated incubating them in (I) 25% MeOH / 75% PBST, (II) 50% MeOH / 50% PBST, (III) 75% MeOH / 25% PBST and (IV) 100% MeOH for 5 minutes each on a shaker. After the final wash, MeOH was refreshed and stored at -20°C.

2.3.8 Whole-Mount *in situ* Hybridisation

2.3.8.1 Day 1

2.3.8.1.1 Materials

Technical Equipment	
Name	**Source & Details**
Heat Block	DRI-BLOCK DB3; Jencons Techne
Orbital Shaker	Stuart Scientific
Chemical Reagents & Buffers	
Name	**Source & Details**
PBST	pH:7.4, 0.1% Tween 20 (1ml Tween 20 in 1lt PBS)
Hybe+ Buffer	Hybridisation Buffer Mix
Hybe- Buffer	Hybridisation Buffer Mix
Hybe+ Buffer	
Formamide	Sigma
SSC	Working Concentration: 20X; 3M Sodium Chloride, 300mM Trisodium Citrate
tRNA	50mg/ml; Roche
Heparin	100mg/ml; Sigma
Citric Acid	1M; Fisher Chemicals
Tween 20	20% (V/V); Sigma
sdH_2O	Sterile Distilled Water
Hybe- Buffer	
Formamide	Sigma
SSC (Saline Sodium Citrate)	Working Concentration: 20X; 3M Sodium Chloride, 300mM Trisodium Citrate
Citric Acid	1M; Fisher Chemicals
Tween 20	20% (V/V); Sigma

sdH_2O	Sterile Distilled Water

2.3.8.1.2 Method

All washings were performed with a volume of 500µl in 1.5ml eppendorf tubes. All the solutions with Hybe+ and Hybe- were pre-heated at 68°C before application. Embryos were rehydrated incubating them in (I) 75% MeOH / 25% PBST, (II) 50% MeOH / 50% PBST, (III) 25% MeOH / 75% PBST and (IV) 3 times 100% PBST for 5 minutes each on a shaker. Then the embryos were washed with 50% Hybe-/50% PBST on the shaker for 5 minutes. Normally Hybe- doesn't contain Heparin but within this wash; 0.5µl of Heparin was added into 500µl of Hybe- buffer. Then Hybe- was replaced by Hybe+ and incubated for 3 hours at 68°C in the heat block. After 3 hours; Hybe+ buffer was replaced by sense and antisense mRNA probes with an amount of 500µl at 68°C in the heat block and left overnight. The tubes were placed laying down on their side for both Hybe+ incubation and probe hybridisation to ensure equal exposure of each embryo to buffer and probe.

2.3.8.2 Day 2

2.3.8.2.1 Materials

Technical Equipment	
Name	**Source & Details**
Heat Block	DRI-BLOCK DB3; Jencons Techne
Orbital Shaker	Stuart Scientific
Chemical Reagents & Buffers	
Name	**Source & Details**
Hybe- Buffer	Same Contents
SSC	Working Concentration: 2X, 0.2X
MAB	10X Stock Buffer: 1M Maleic Acid, 1.5M NaCl, dH_2O, pH adjusted to 7.5 with NaOH
MABT	0.1% Tween 20 (1ml Tween 20 in 1lt MAB)

Blocking Reagent	2% (V/V); Roche
Anti-DIG	Roche

2.3.8.2.2 Method

All washings were performed with a volume of 500µl in 1.5ml eppendorf tubes. All the solutions with Hybe- and SSC were pre-heated at 68°C before application. On day 2, probes were recovered and stored at -20°C. Embryos were washed with (I) 100% Hybe-, (II) 75% Hybe- / 25% 2X SSC, (III) 50% Hybe- / 50% 2X SSC, (IV) 25% Hybe- / 75% 2X SSC and (V) 2 times with 100% 2X SSC for 10 minutes each at 68°C in the heat block with the tubes laying down on their side. Embryos were then washed 3 times for 15 minutes at 68°C in 0.2X SSC to remove excess probes. Embryos were then washed for 5 minutes each at room temperature on a shaker with (I) 75% 0.2X SSC / 25% MABT, (II) 50% 0.2X SSC / 50% MABT, (III) 25% 0.2X SSC / 75% MABT and finally (IV) 100% MABT. In order to block non-specific binding of the antibody, embryos were treated with 500µl of 2% Blocking Reagent for 2 hours on the shaker at room temperature. 10% Stock Blocking Reagent was diluted with MAB to 2%. Blocking process was followed by Anti-DIG treatment. Blocking reagent was removed and Anti-DIG which was diluted with a proportion of 1/5000 within blocking reagent, was then added and left on the shaker for 3 hours at room temperature. After 3 hours, Anti-DIG was removed and embryos were washed 3 times with MABT for 15 minutes each at room temperature on a shaker to remove excess antibody. After final wash, MABT was refreshed and embryos were kept at 4°C overnight.

2.3.8.3 Day 3

2.3.8.3.1 Materials

Technical Equipment	
Name	**Source & Details**
Orbital Shaker	Stuart Scientific
Chemical Reagents & Buffers	
Name	**Source & Details**
MABT	0.1% Tween 20 (1ml Tween 20 in 1lt MAB)
BCL Buffer	Buffer Mix
BM Purple	Roche
EDTA	20mM
PFA	4% (W/V), Sigma
PBST	pH:7.4, 0.1% Tween 20 (1ml Tween 20 in 1lt PBS)
Glycerol	Dilutions were made with PBST
BCL Buffer	
Tris-HCl	1M, pH: 9.5
NaCl	5M
$MgCl_2$	0.5M
Tween 20	20% (V/V); Sigma
sdH_2O	Sterile Distilled Water

2.3.8.3.2 Method

All washings were performed with a volume of 500µl in 1.5ml eppendorf tubes at room temperature. Embryos were again washed with MABT 3 times for 15 minutes and then incubated twice in freshly prepared BCL Buffer for 5 minutes on the shaker. BM Purple was diluted with BCL Buffer. 250µl of BM Purple was used for 250µl of BCL Buffer; 1:1 dilution. A total of 500µl of BM Purple-BCL Buffer mix was used for each tube. After adding the mix, all the tubes were protected from light and staining of the embryos

checked regularly. When the desired staining level was obtained, the reaction was stopped by washing the embryos with 20mM EDTA 3 times for 5 minutes each on the shake. Embryos were fixed with PFA for 10 minutes on the shaker and subsequently washed with PBST 3 times for 5 minutes each on the shaker to eliminate PFA. Embryos were washed in 50% glycerol and finally stored at 4°C in 90% glycerol.

2.3.9 Imaging of Zebrafish Embryos

2.3.9.1 Materials

Technical Equipment	
Name	**Source & Details**
Microscope	Nikon SMZ1500
Camera	Nikon ACT-2U
Software	NIS-Elements

3. RESULTS

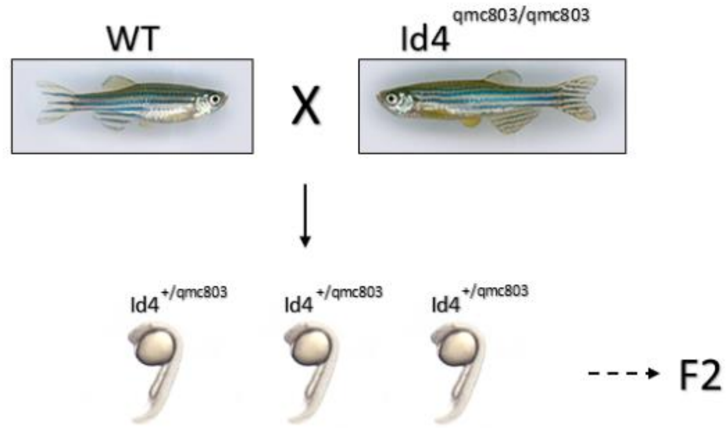
3.1 TALEN-mediated mutagenesis of the Id4 gene in zebrafish

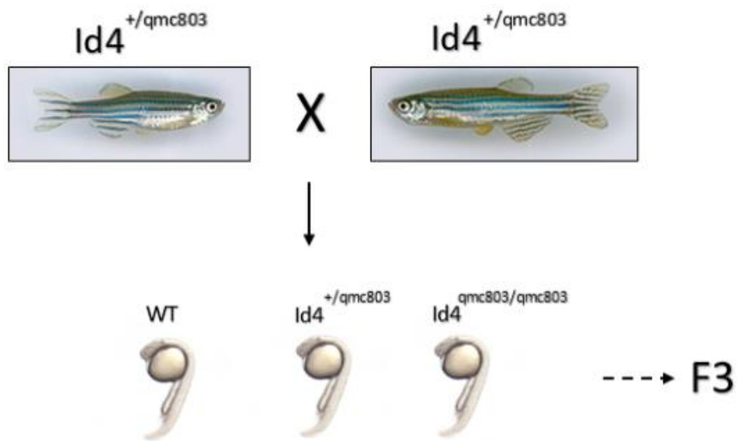
To create a non-functional Id4 gene in zebrafish, a suitable region in the 1st exon just downstream of the ATG start codon was chosen as a target site which contained a HpaII enzyme restriction sequence (Figure 3.1). TALENs were designed and made in Keith Joung's research group (Joung *et al.,* 2013) and purchased from addgene (addgene.org). TALENs were injected into one-cell-stage embryos and offspring of founders crossed with wild type zebrafish screened for mutations using PCR and subsequent restriction enzyme analysis using HpaII (Dhanaseelan, 2016). Several mutations were observed, and subsequent sequence analysis revealed that one of the founders exhibited a 8 bp deletion resulting in an out-of-frame mutation and premature stop codon. Crossing this founder with wild type zebrafish and subsequent screening as above resulted in the identification of heterozygous mutant zebrafish (F0) called **qmc803** (Dhanaseelan, 2016). Homozygous mutants (F1) were established that looked morphologically normal and survived for over a year without any obvious phenotype.

5'TGTGACCAACAATAACTCATCCGCTGTTTTGCACTGTGCGTTCACACTCAGAGAGGAGATGTG
GTCAGGATAGATCTGCGCTCTTTTTCTTCGTGCACCATACTACGTTTTTTTTTTACTAAACGTATTG
TTGCGTCCGTGTTTTTTTTTTTCCTTTTTCACGTTTTTCTTTTTGTAGTTTTATTTATTACAATGAAG
GCCAGCGT/G**C CGG**TTC/GCCCTCATAAGCTTCCTTCTAGCTGCAGTCAGCTCTCCTTG CGTTA
TTTGTCGGAGAGCAGCCGATGCAAAATGGAAGATGAGGATCTTTTCTGTCTGCAGTACGACATGA
ACGACTGCTACAGCCGACTCAAACGCTTGGTGCCCACTATTCCGCAGGATAAGAAAGTCAGTAA
AGTGGAAATCCTCCAGCATGTCATTGACTATATTCTGGACCTGCAGC**3'**

Figure 3.1: Id4 Exon 1 Sequence
Yellow-highlighted sequence: forward primer; pink-highlighted sequence: reverse primer; green-highlighted sequences: TALEN target sequences; red dashes: indicates the location of the 8bp deletion; blue sequence: indicates the deleted region containing the HpaII cutting site.

However, when crosses were set up with homozygous Id4$^{qmc803/qmc803}$ that were a year old, female zebrafish failed repeatedly to lay eggs. Therefore, male Id4$^{qmc803/qmc803}$ were backcrossed with wild type female fish to obtain heterozygous mutants (F2) (Figure 3.2). When they reached adulthood, a pair from F2 heterozygous fish were incrossed to create F3 generation and left to grow up until they were old enough (~3 month) to be genotyped using genomic DNA isolated from fin clips.


A
WT
Id4 qmc803/qmc803
X
Id4 +/qmc803
Id4 +/qmc803
Id4 +/qmc803
F2


B
Id4 +/qmc803
Id4 +/qmc803
X
WT
Id4 +/qmc803
Id4 qmc803/qmc803
F3

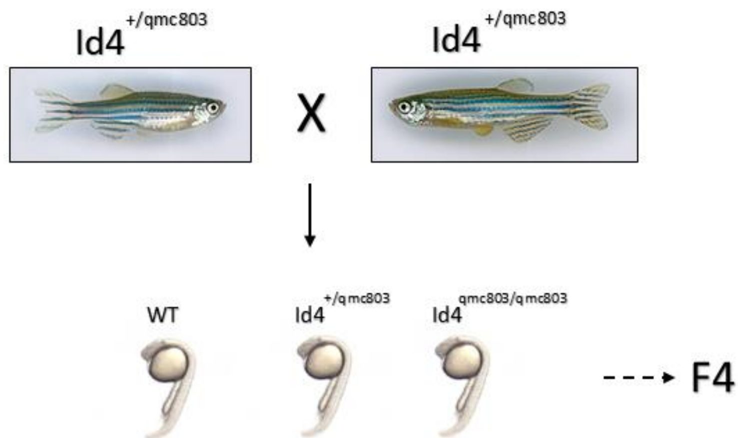

C
Id4 +/qmc803
Id4 +/qmc803
X
WT
Id4 +/qmc803
Id4 qmc803/qmc803
F4

Figure 3.2: Production of each Generation of Id4 Zebrafish
A: Homozygous mutant male crossed with a wild type female and offspring left for development to create F2 heterozygous generation.
B: Heterozygous mutant male and female fish incrossed and offspring left for development to create F3 generation which contains 3 genotypes including homozygous mutants, heterozygous mutants and wild types.
C: Three zebrafish of the F3 generation, identified as heterozygous through sequence analysis, were crossed to obtain embryos (F4) for *in situ* hybridisation and subsequent genotyping.

To detect homo/heterozygous mutants, the genomic DNA of 9 fish was amplified (primer sequences see Figure 3.1) and PCR products analysed by gel electrophoresis. Subsequently, PCR products were digested with HpaII and the DNA again analysed by gel electrophoresis. As seen in Figure 3.3, genotyping was ambiguous because none of the expected pattern of DNA bands was observed. PCR fragments derived from wild type fish should have been fully digested with HpaII resulting in two fragments (208 and 228 bp length) whereas PCR products from mutant fish should have resulted in fragments of either 436 bp plus 208 and 228 bp length (heterozygous) or in just one undigested fragment of 436 bp. However, neither of the expected results were obtained (Figure 3.3). Nevertheless, two fish were chosen that exhibited a pattern resembling most closely the pattern expected for homozygous fish (Figure 3.3; sample number 3 and 4). Offspring (25 embryos at 2dpf) of these two presumed homozygous fish were analysed as described above (Figure 3.4). If the parents were both homozygous, all offspring should have been homozygous. The result obtained was different however: while many embryos seemed to be indeed homozygous, others appeared as either heterozygous or wild type suggesting that the parents were heterozygous (Figure 3.4).

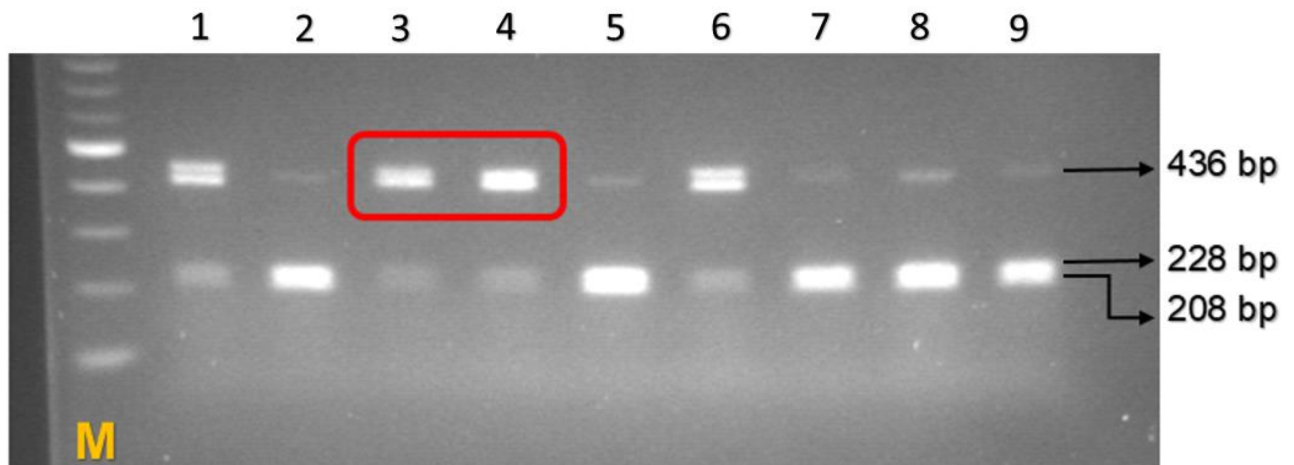


Figure 3.3: Detection of Homo/Heterozygous Mutant Fish from Heterozygous Incross, First Batch.

Genotyping of 9 fish was ambiguous because none of the expected pattern of DNA bands was observed. While samples in lanes 2 and 5 predominately contained the 228 and 208 bp fragments, a residual band at 436 bp was also present. Similarly, samples in lanes 3 and 4 (red box) predominantly contained the 436 bp fragment, residual bands at 228 and 208 bp were also visible. Furthermore, the additional fragment in some samples (lanes 1, 3, 4 and 7) just above the 436 bp fragment was completely unexpected. Nevertheless, female in lane 3 and male in lane 4 were chosen for further breading. Yellow M: DNA size marker.

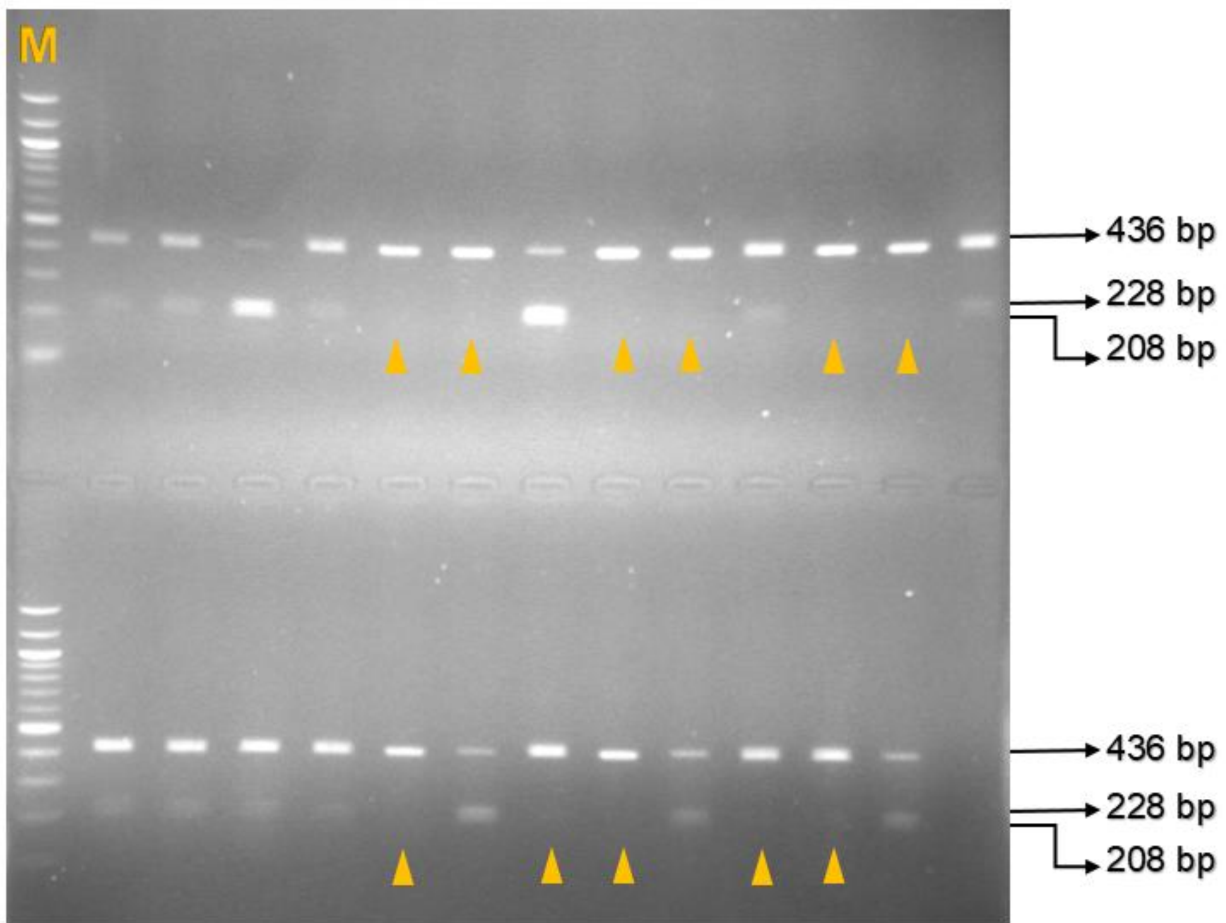


Figure 3.4: Possible Mutant Parents' Embryos
25 random embryos from the cross with putative homozygous fish were genotyped. While some samples appeared to be indicating homozygosity (yellow arrow head), the other samples were again ambiguous not exhibiting the expected band pattern.
Yellow M: DNA size marker; Δ: Indicates homozygous mutants.

Therefore 16 more F3 fish were fin-clipped and the genomic DNA analysed as before (Figures 3.5 and 3.6). Not all PCR reactions were successful and digestion of the remaining PCR products produced again ambiguous results (Figure 3.6). However, one sample (red box in Figure 3.6) appeared to be undigested indicative of homozygous genotype. Given that this fish was male, it was incrossed with the female of the pair used before.

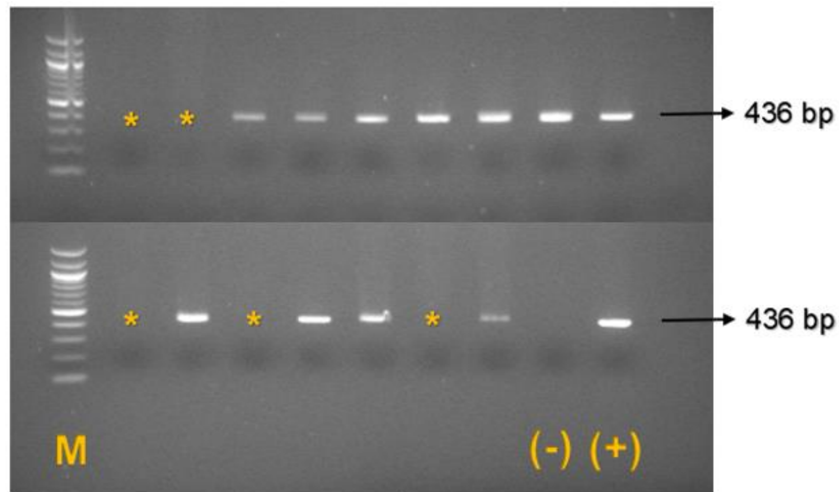


Figure 3.5: PCR amplification of genomic DNA isolated from fin-clips of 16 zebrafish
PCR fragments obtained were analysed by restriction digest with HapII (Figure 3.6). (*): PCR amplification failed; yellow M: DNA size Marker; (-): negative control; (+): positive wild type control.

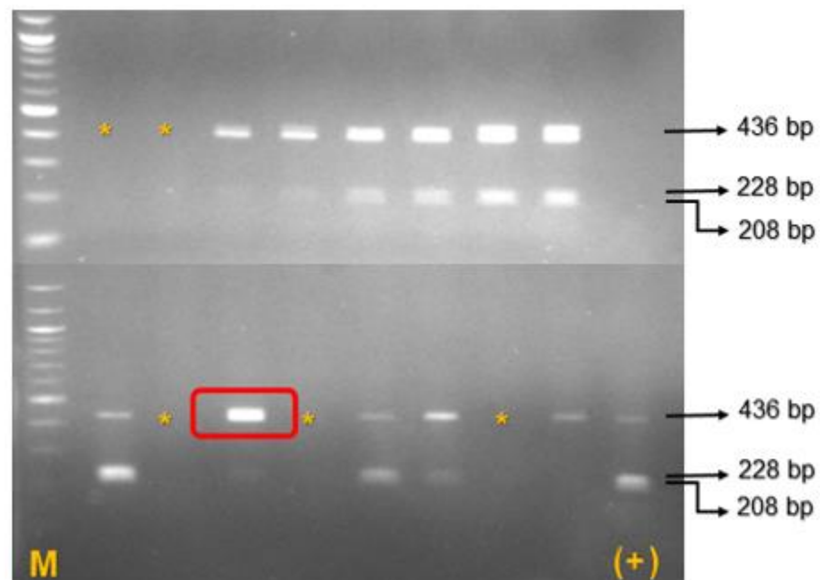


Figure 3.6: Genotype analysis using HpaII digestion of PCR fragments
Ambiguous fragment pattern in most samples including the wild type control (+) was likely due to partial digest. Nevertheless, one sample (red box) showed a pattern indicative as homozygous. Yellow M: DNA size marker; (+): positive wild type control, (*): no PCR product.

Genotyping of 20 2-day-old embryos obtained from F4 cross is shown in Figures 3.7 and 3.8. While again 8 sample out of 18 exhibited a single fragment of 436 bp indicative for homozygous embryos, 4 samples not only showed the expected bands for wild type (228 and 208 bp) but also exhibited a faint residual undigested fragment of 436 bp. A feature that was also observed for the wild type control (Figure 3.8).

Given that so far the genotyping using PCR and restriction enzyme digest was clearly not providing clear-cut results, the strategy was changed. Instead, the PCR products from the 3 zebrafish used in the F3 and F4 crossing as described above were **sequenced.**

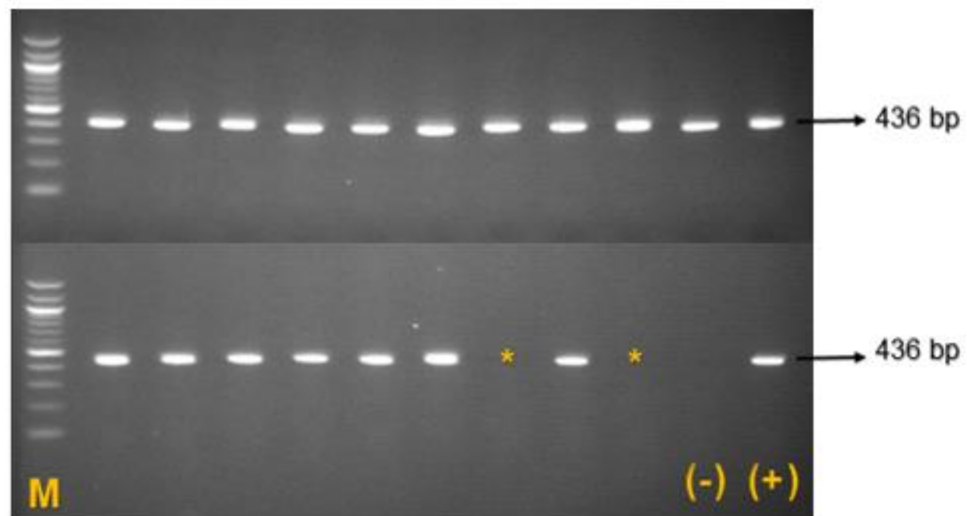


Figure 3.7: PCR amplification of genomic DNA isolated from embryos obtained from F4 cross
20 random embryos were picked for analysis. 2 samples failed to amplify (*). Yellow M: DNA size marker; (-): negative control; (+): positive wild type control.

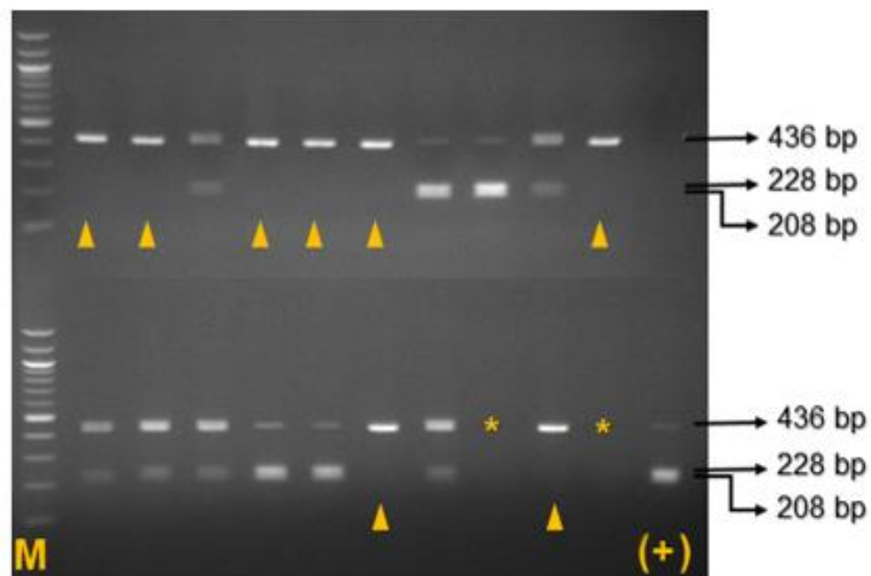


Figure 3.8: Genotyping of embryos obtained from F4 cross
While 8 PCR samples showed a single undigested fragment of 436 bp (yellow arrow heads) indicative of homozygous genotype, the other sample including the wild type control (+) were again ambiguous. Yellow M: DNA size marker; (*): no PCR product.

The obtained sequences from the 3 parent fish were compared to the wild type sequence and it was obvious that from the 8 bp deletion onwards two sequences overlapped clearly showing that all 3 parent fish were heterozygous (Figure 3.9).

Due to time constrains, no further attempts were made to identify homozygous mutants. Instead, the 3 parent fish clearly identified as heterozygous were crossed and offspring analysed by *in situ* hybridisation using different gene markers. Embryos that exhibited a different expression pattern other than wild type embryos were selected as possible mutants and genotyped by PCR and sequencing.

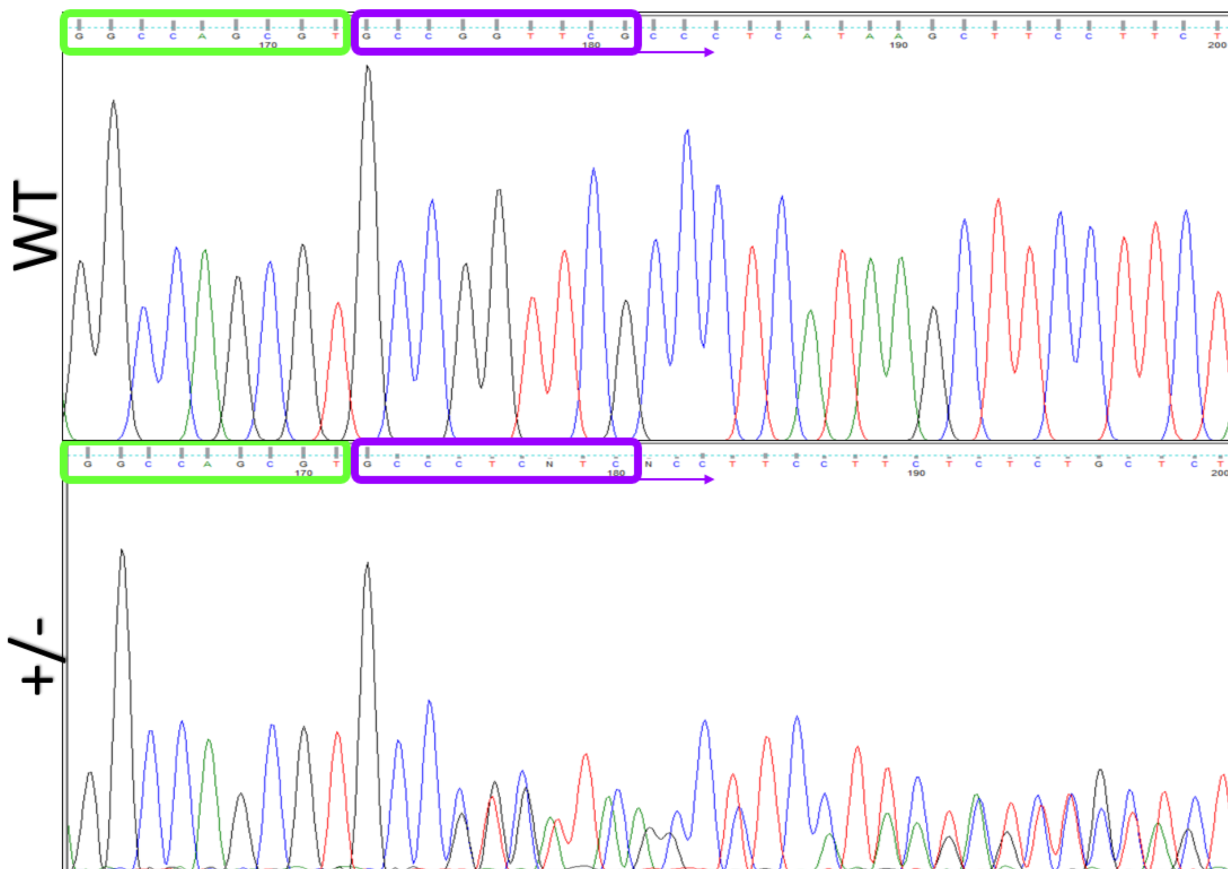


Figure 3.9: Sequence Comparison of Wild Type and Heterozygous Mutant Zebrafish

The regions indicated with green frames are the overlapping sequences between wt and heterozygous mutant fish. The purple frames indicate the sequence difference where TALEN induced 8 bp deletion starts. As the figure examined in the direction of the purple arrow, further differences between sequences can be seen. Shown heterozygous mutant sequence belongs to one of the 3 parent fish.

3.2 Analysis of gene expression in embryos obtained from crosses with Id4$^{qmc803/+}$ parents by *in situ* hybridisation

3.2.1 Sox2 Expression is Downregulated in Id4 Homozygous Mutant Fish at 24 hpf.

Sox2 is a transcription factor which works cooperatively with other transcription factors such as Oct4 and Nanog to maintain the regulatory networks responsible for self-renewal in embryonic stem cells (ESCs) (Boyer *et al.,* 2005). Therefore Sox2 antisense probe was used *in situ* hybridisation to mark self-renewing stem cells. Sox2 is expressed within the central nervous system including immature eye, all brain regions, spinal cord as well as otic vesicle (Thisse *et al.,* 2001). As shown in Figure 3.10, Sox2 expression in Id4$^{qmc803/qmc803}$ embryos at 24 hpf was markedly decreased compared to wild type embryos. Heterozygous embryos exhibited also a decrease in Sox2 expression albeit less severe. It seems therefore that the number of self-renewing NSCs is decreased in Id4$^{qmc803/qmc803}$ embryos.

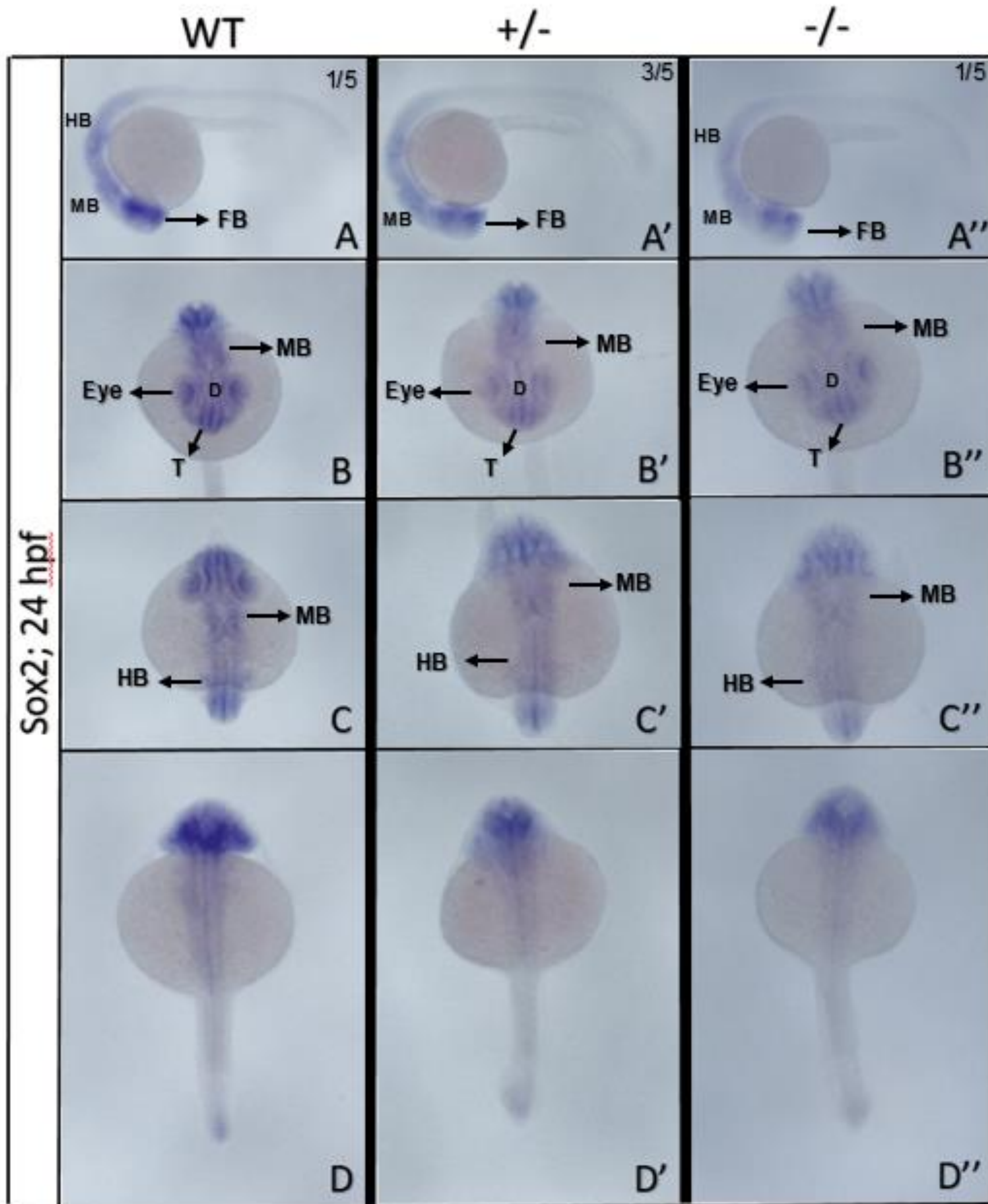


Figure 3.10: Reduced Sox2 Expression in Id4 Heterozygous and Homozygous Zebrafish at 24 hpf

Sox2 expression appears somewhat reduced in heterozygous and markedly reduced in homozygous Id4 embryos. A,A',A'': Lateral View, B,B',B'': Frontal View, C,C',C'': Anterior View, D,D',D'': Dorsal View. T: Telencephalon, D: Diencephalon, FB: Frontal Brain, MB: Mid Brain, HB: Hind Brain. 5/18 embryos obtained from *in situ* hybridisation were genotyped by sequencing based on possible candidates for the three different genotypes (3 shown). Images of the remaining 13 embryos presented in the Appendix.

3.2.2 No Significant Alteration of HuC Expression was Observed in Id4 Homozygous Mutants at 24 hpf.

HuC is a RNA binding protein and one of the earliest markers for early neuronal cell fate determination in zebrafish. During zebrafish embryogenesis, first HuC expression appears in scattered cells in the neural plate immediately after gastrulation. HuC expression is widespread within the nervous system including brain regions and spinal cord during development. As neurogenesis proceeds, HuC expressing cells increase within the nervous system, as a possible result of the elevation of newly born postmitotic neurons (Kim *et al.,* 1996). We used HuC antisense probe in order to understand the early neuronal activity in the absence of Id4 via *in situ* hybridisation. Heterozygous and homozygous mutant embryos didn't display a significantly altered expression of HuC when compared to wt embryos at 24 hpf which suggests an unimpaired neurogenesis in the absence of Id4.

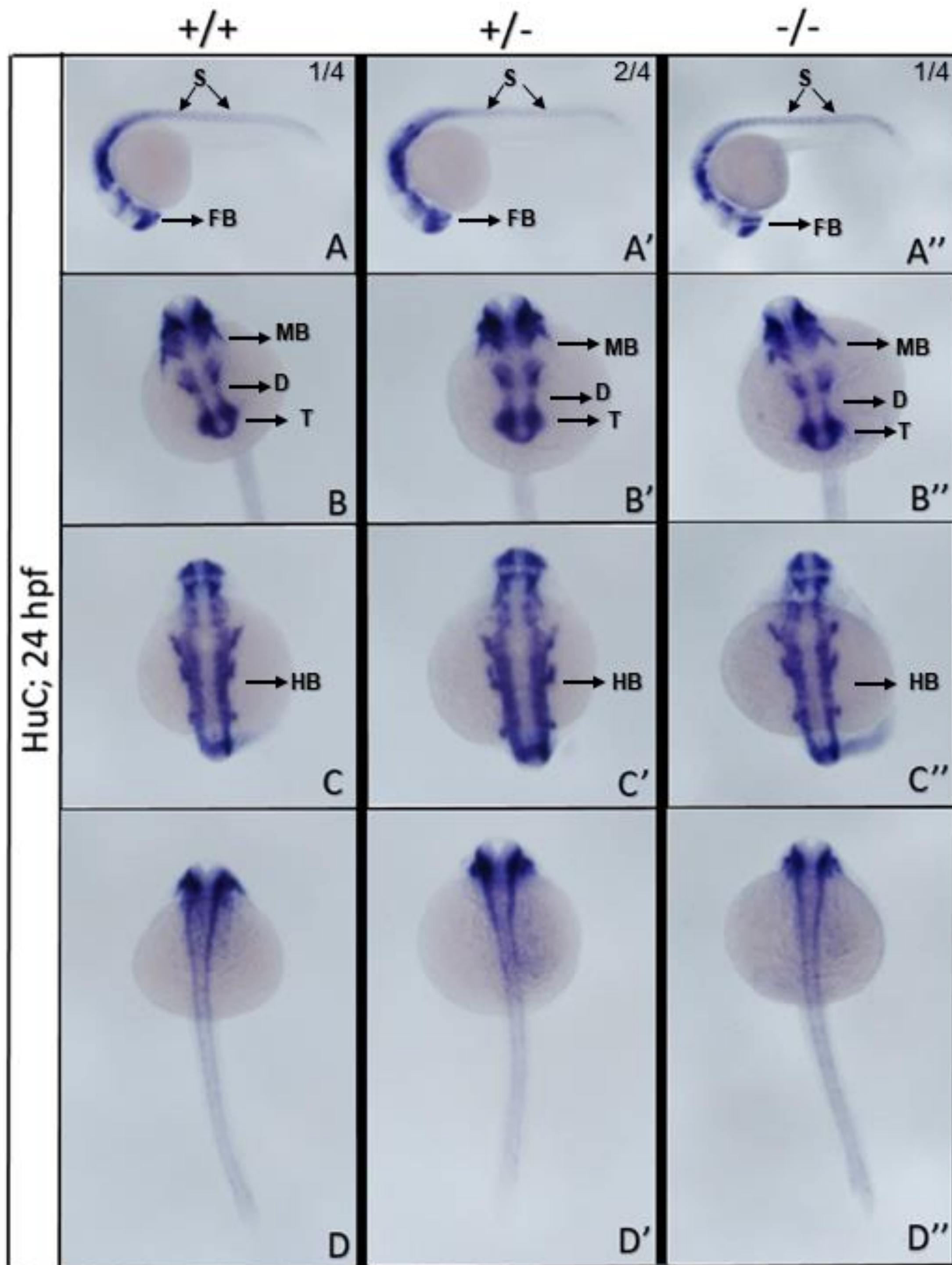


Figure 3.11: Unvaried Expression of HuC within Id4 Heterozygous and Homozygous Mutants at 24 hpf.

A,A',A'': Lateral View, B,B',B'': Frontal View, C,C',C'': Anterior View, D,D',D'': Dorsal View. T: Telencephalon, D: Diencephalon, FB: Frontal Brain, MB: Mid Brain, HB: Hind Brain, S: Spinal Cord. 4/21 embryos obtained from *in situ* hybridisation were genotyped by sequencing based on possible candidates for the three different genotypes (3 shown). 9 images of the remaining embryos presented in the Appendix. Remaining 8 embryos not shown since their expression were indistinguishable from the ones shown in the Appendix.

3.2.3 GFAP (Glial Fibrillary Acidic Protein) Expression is Upregulated in Id4 Homozygous Mutant Fish at 24 hpf.

GFAP is an intermediate filament protein which is widely used as an antigen marker specific to astrocytes in a wide range of species, including so distantly related species as humans, fish and snails (Nielsen *et al.,* 2003). Earliest GFAP expression detected in zebrafish is around 15 hpf (Marcus and Easter, 1995) and maintains within the CNS glia as the development proceeds to adulthood (Tomizawa *et al.,* 2000). We used GFAP antisense probe with 24 hpf embryos to understand the astrocyte activity in the absence of Id4 via *in situ* hybridisation. We observed a consistent upregulation of GFAP expression in accordance with the heterozygous and homozygous genotype in the absence of Id4 at 24 hpf.

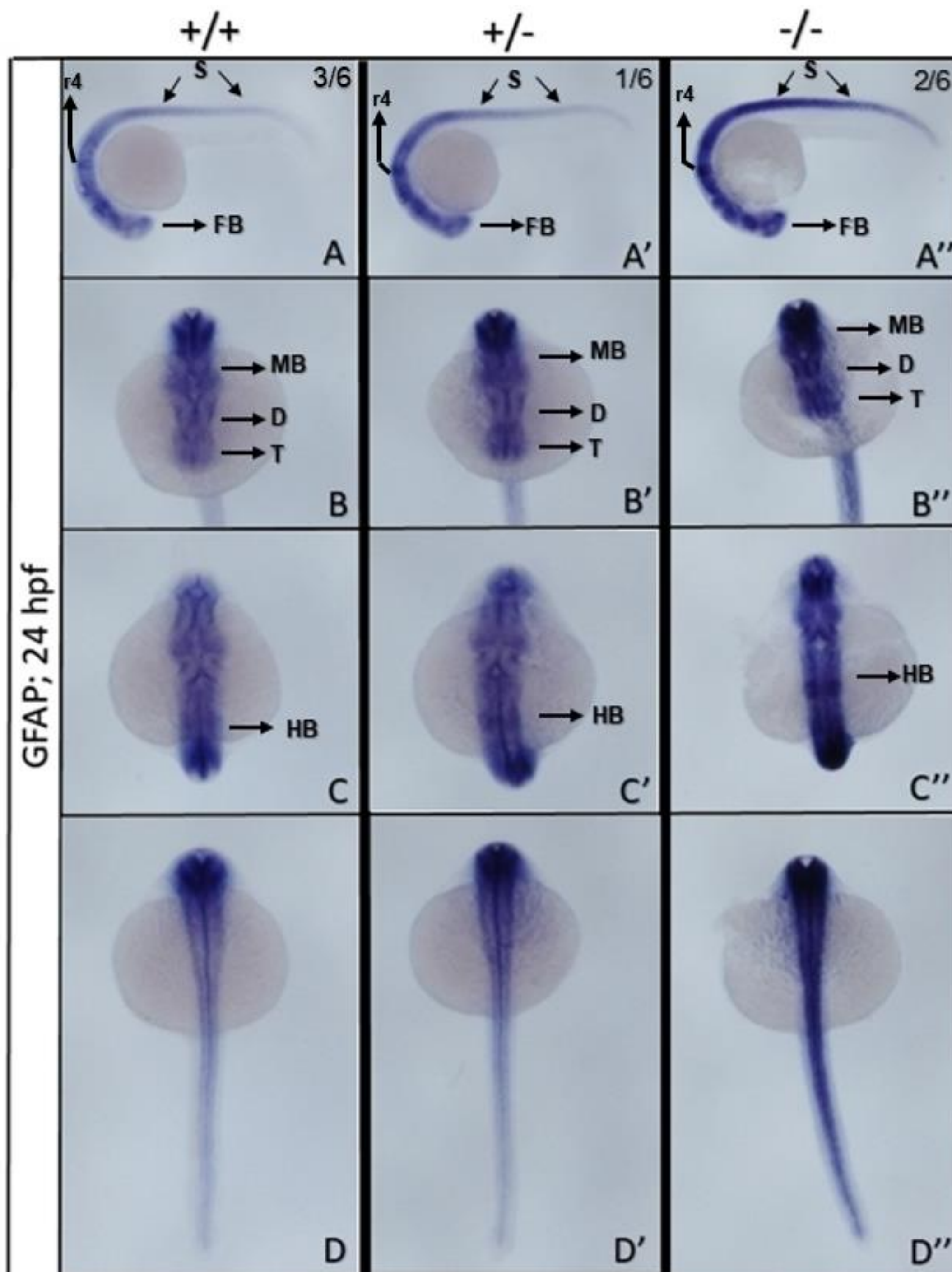


Figure 3.12: Elevated GFAP Expression in Id4 Heterozygous and Homozygous Zebrafish at 24 hpf

A,A',A'': Lateral View, B,B',B'': Frontal View, C,C',C'': Anterior View, D,D',D'': Dorsal View. T: Telencephalon, D: Diencephalon, FB: Frontal Brain, MB: Mid Brain, HB: Hind Brain, r4: Rhombomere 4, S: Spinal Cord. 6/22 embryos obtained from *in situ* hybridisation were genotyped by sequencing based on possible candidates for the three different genotypes (3 shown). Images of the remaining 16 embryos presented in the Appendix.

3.2.4 Analysis of Olig2 and Ascl1a Expression was Inconclusive

Olig2 is a bHLH transcription factor which plays an essential role in the development of primary motor neurons and oligondendrocytes (Park *et al.,* 2002). In zebrafish it is mainly expressed within forebrain, midbrain and primary motor neurons within the spinal cord at 24 hpf (Wang *et al.,* 2013). Ascl1a is a proneural gene and encodes a bHLH transcription factor which is required not just for the differentiation of CNS neurons but also peripheral neurons and sensory cells (Pogoda *et al.,* 2006). In zebrafish, Ascl1a is expressed in the ventral forebrain at 24 hpf (Kudoh *et al.,* 2001). Unfortunately, none of the embryos analysed by *in situ* hybridisation with Olig2 turned out to be homozygous and only one of the embryos was heterozygous that appeared to express less Olig2 (Figure 3.13). Furthermore, one embryo that exhibited an expression pattern similar the heterozygous one turned out to be wild type. *In situ* hybridisation with ascl1a antisense probes resulted in variation of gene expression within wild type as well as mutant embryos (Figure 3.14) with no correlation of expression pattern and genotype making it impossible to interpret the data.

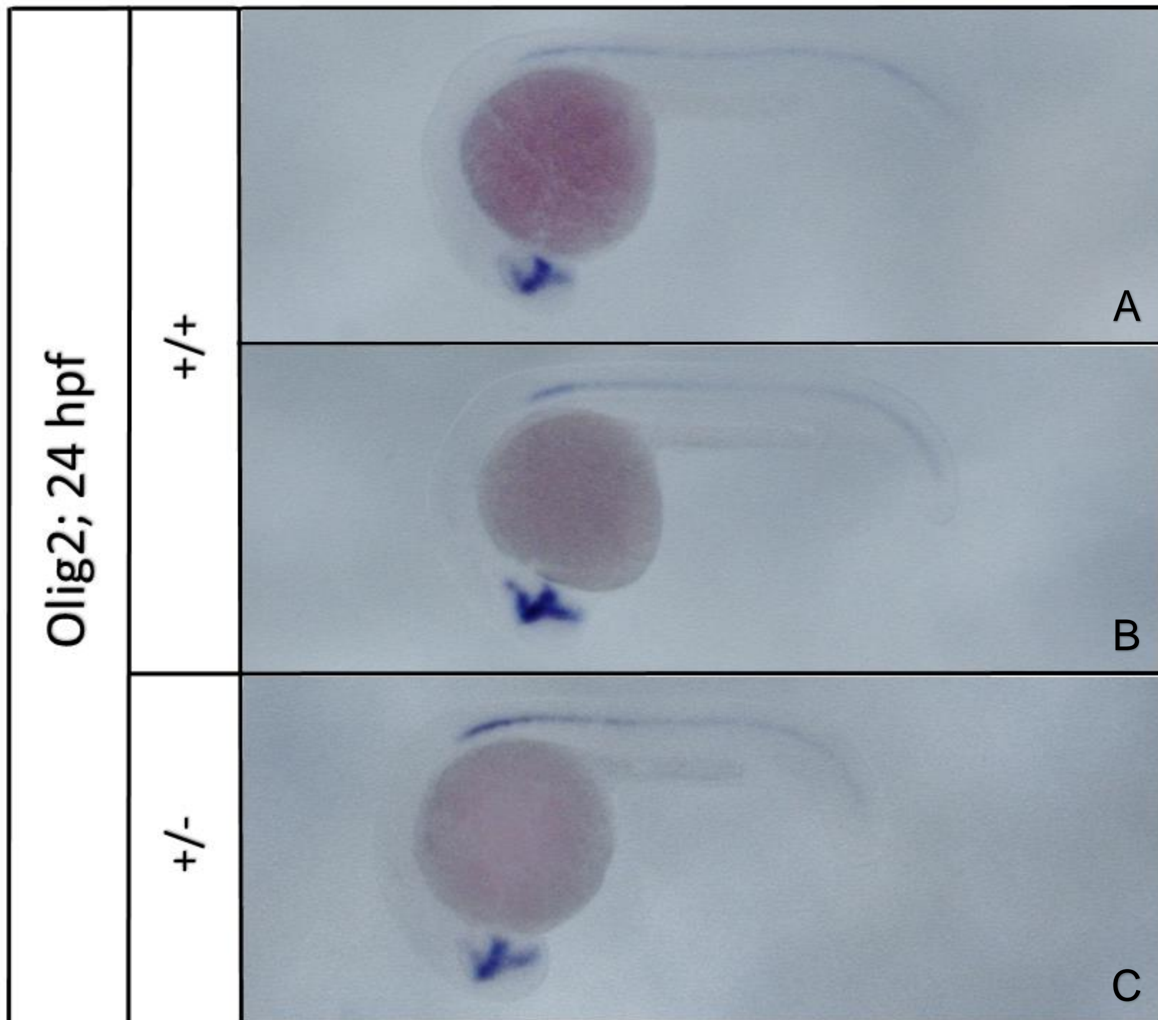


Figure 3.13: No Correlation between Olig2 Expression Pattern and Genotype with 3 Sequenced Embryos

20 embryos were used from F3 generation for Olig2 hybridisation, 18 of them displayed same patterning and intensity of signal as shown in Figure B which correlated with a separate wt control group. Therefore embryos of this group were chosen as the wt control of the mixed batch. Embryos shown in Figure A and C displayed a somewhat different Olig2 expression pattern and the signal was reduced. They were chosen as potential mutants. Sequence analysis confirmed that the embryo in B was indeed wt but one of the presumed mutant embryos (A) was also wt and the otherone (C) was heterozygous. 3/19 embryos obtained from *in situ* hybridisation were genotyped by sequencing based on possible candidates for the three different genotypes (3 shown). 6 images of the remaining embryos presented in the Appendix. Remaining 10 embryos not shown since their expression were indistinguishable from the ones shown in the Appendix.

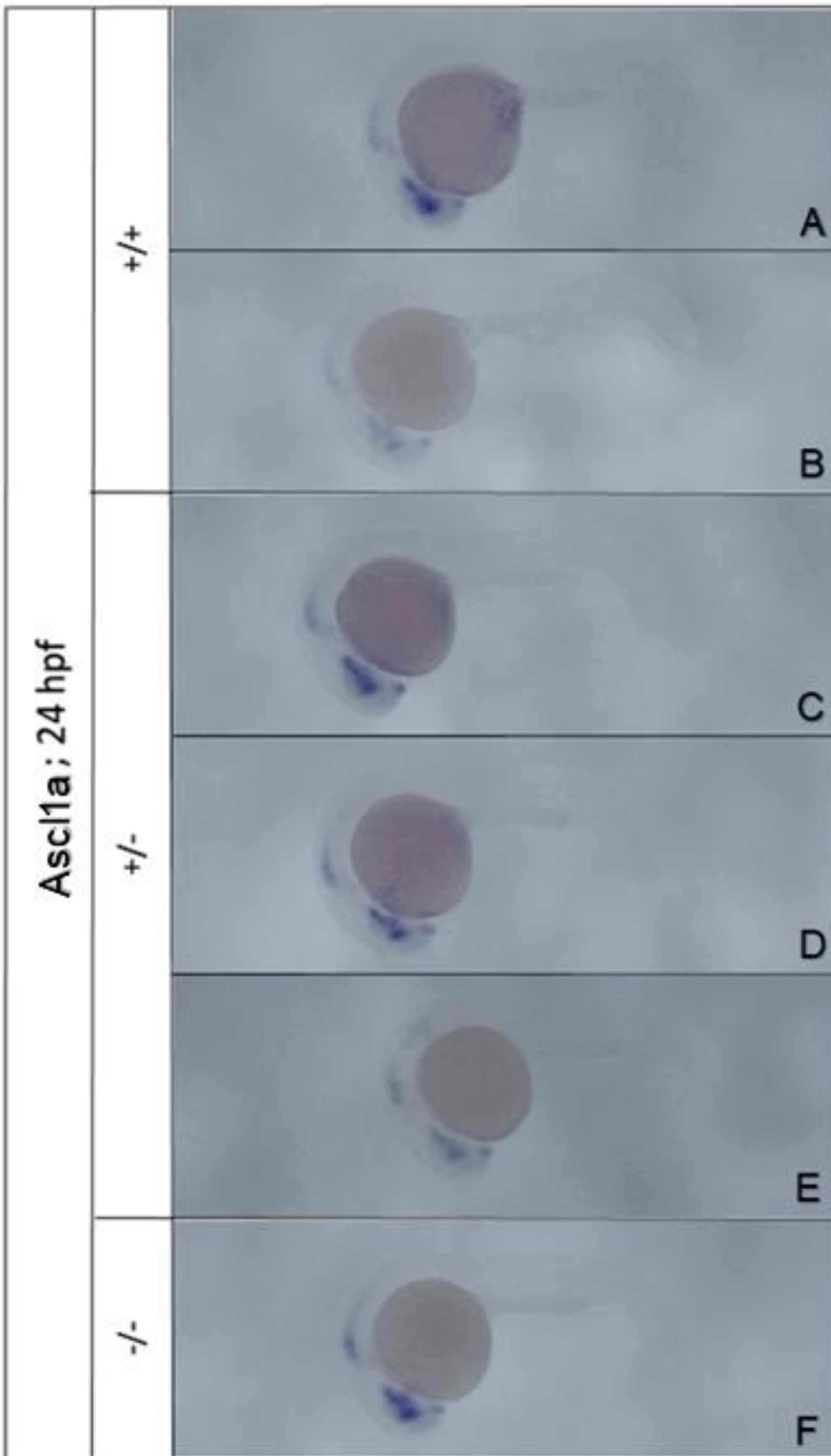

Ascl1a ; 24 hpf
+/+
+/-
-/-
A
B
C
D
E
F

Figure 3.14: No Correlation between Ascl1a Expression Pattern and Genotype with 6 Sequenced Embryos

20 embryos were used from F3 generation for Ascl1a hybridisation. They were grouped based on their expression pattern. Embryos that appeared to be similar to a separate Wt group were chosen as putative wt (A). The embryos shown in B and E were chosen as potential mutants because of the significant downregulation of Ascl1a expression. The ones shown in C, D, F were chosen as potential heterozygous mutants. Sequence analysis after *in situ* hybridisation revealed that there was no clear cut correlation with the expression pattern observed and genotype obtained. While embryos in A, C and D were correctly predicted as wt and heterozygous, genotype of embryos in B, E and F were different from prediction. Variability in the hybridisation and lack of sufficient numbers of homozygous mutants making it impossible to interpret this data set. 6/18 embryos obtained from *in situ* hybridisation were genotyped by sequencing based on possible candidates for the three different genotypes (3 shown). Images of the remaining 12 embryos presented in the Appendix.

3.3 Inhibition of Notch signalling results in increased Id4 expression during neurogenesis

3.3.1 Id4 Expression in Zebrafish

In developing zebrafish embryos, Id4 is expressed in the central nervous system (Thisse *et al.,* 2001) and the earliest expression was detected at 6 hpf (shield stage) by RT-PCR (Bashir, 2010) as well as *in situ* hybridisation (Dhanaseelan, 2016). Zebrafish full length Id4 gene was cloned by Tamil Dhanaseelan and cDNA was used to synthesise RNA probes for *in situ* hybridisation (see Materials & Methods). Id4 sense and antisense RNA probes were used to determine Id4 expression at 3 different developmental stages via *in situ* hybridisation (Figure 3.15-16). In all 3 stages, Id4 expression was mainly restricted to the CNS including telencephalon, diencephalon, midbrain, hindbrain and spinal cord.

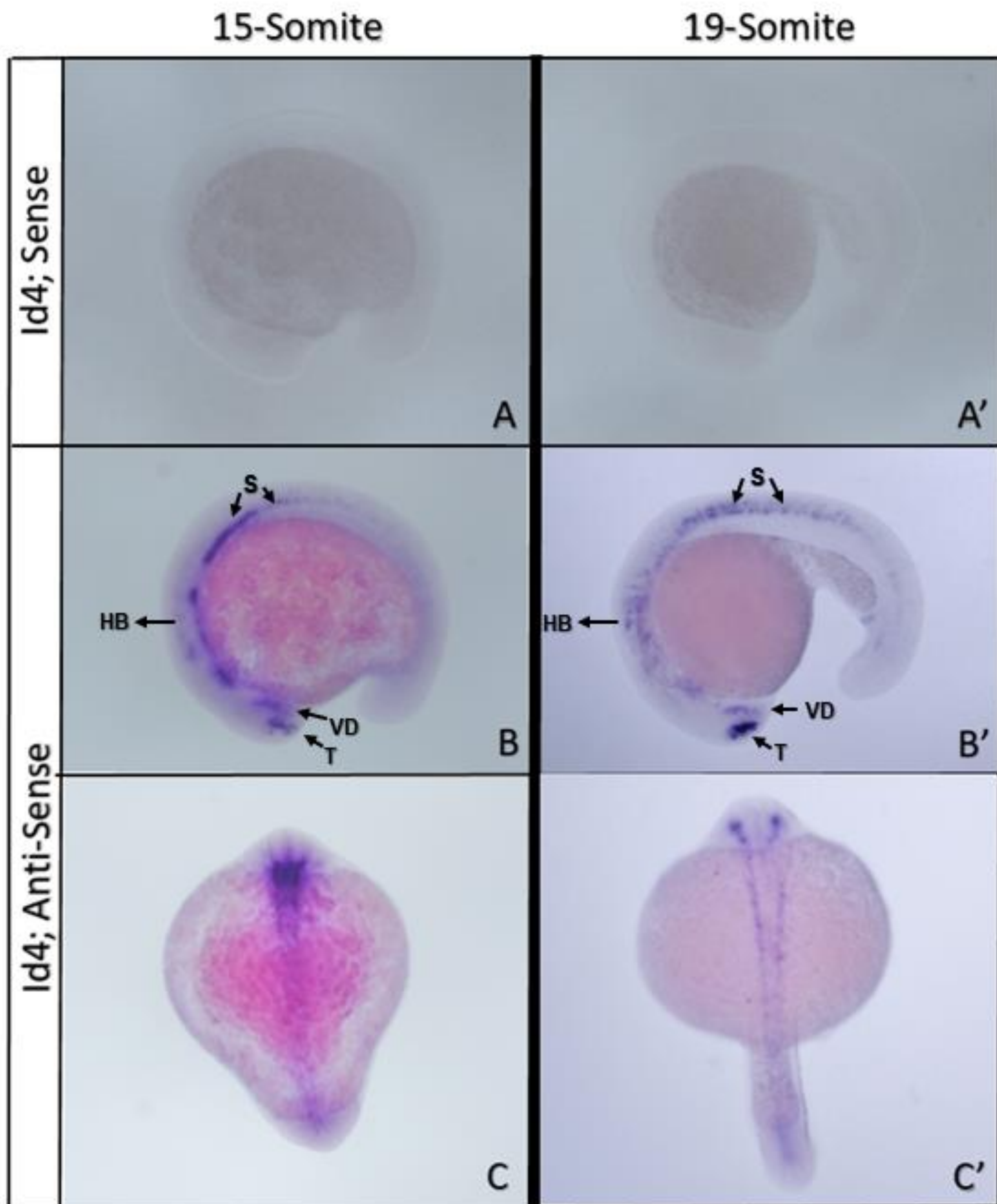


Figure 3.15: Id4 Expression at 15 and 19-Somite Stages
A,A': No expression detected with the Id4 sense probe. B,B': Lateral View, C,C': Dorsal View. Id4 expression is observed throughout the CNS with strong expression within telencephalon. T: Telencephalon, VD: Ventral Diencephalon, MB: Midbrain, HB: Hindbrain, S: Spinal Cord.

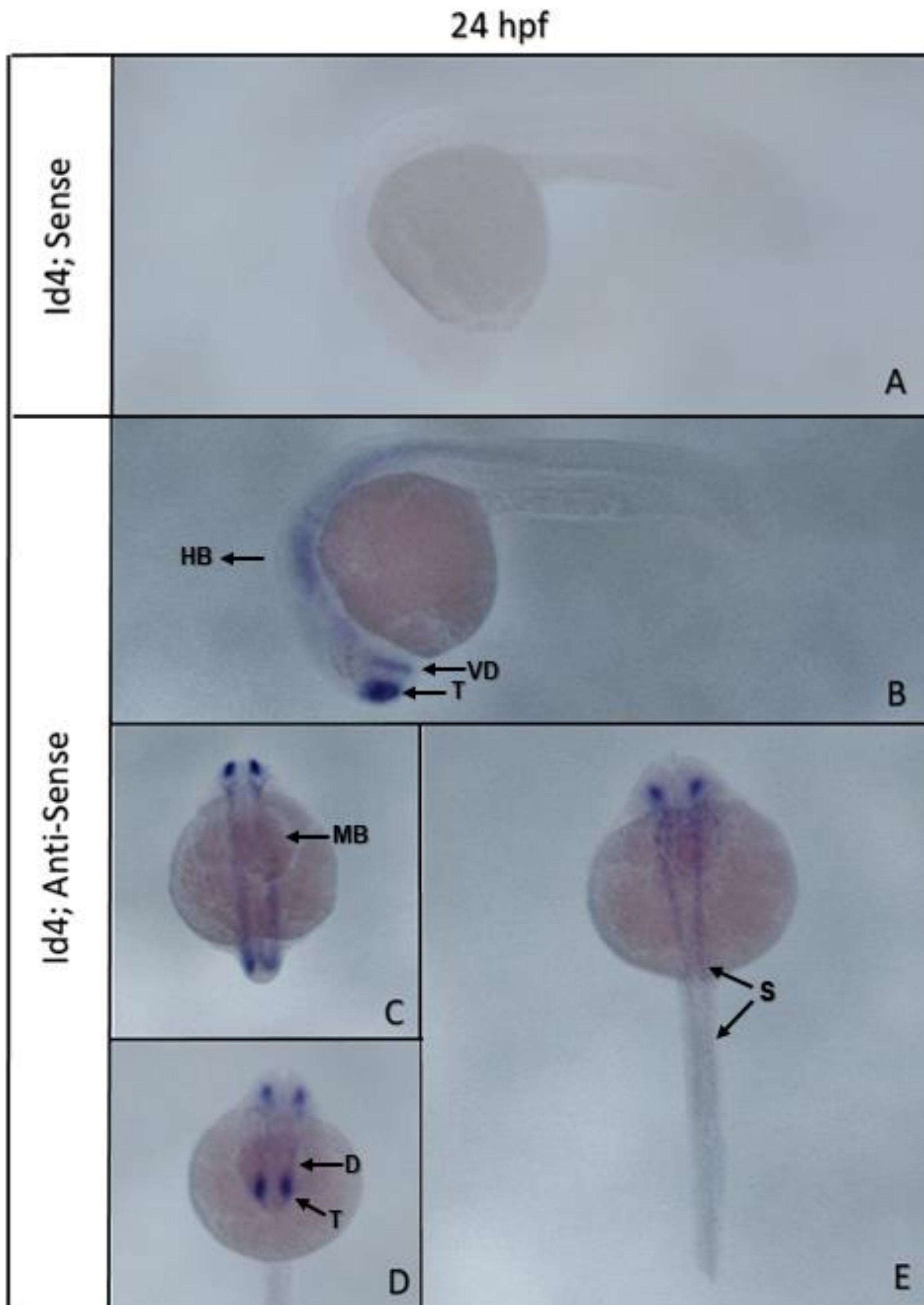


Figure 3.16: Id4 Expression at 24 hpf Stage

A: No expression detected with the Id4 sense probe. B: Lateral View, C: Anterior View; Fore-Mid-Hindbrain can be seen as a whole, D: Frontal View, E: Dorsal View. Id4 expression is observed throughout the CNS with again strong expression within telencephalon as the embryo reaches 24 hpf. T: Telencephalon, D: Diencephalon, VD: Ventral Diencephalon, MB: Midbrain, HB: Hindbrain, S: Spinal Cord.

3.3.2 DAPM Treatment of zebrafish embryos results in increased Id4 expression

Notch signalling regulates cell fate specification via lateral inhibition in many developmental mechanisms. Studies done with murine embryonic stem cells showed that Id4 is upregulated within an Notch1-activated system (Meier-Stiegen *et al.,* 2010) suggesting a positive correlation between Id4 and Notch1. However, γ-secretase inhibition by DAPM inhibiting Notch signalling caused a clear upregulation Id4 expression in zebrafish at 25 hpf (Dhanaseelan, 2016) which suggests a negative correlation between Notch activity and Id4 expression. In other words, Notch signalling may inhibit Id4 expression in zebrafish rather than driving it (Dhanaseelan, 2016). To substantiate the previous observation, wild type embryos were treated with DAPM and Id4 expression was observed via *in situ* hybridisation at 15-somite (Figure 3.17), 19-somite (figure 3.18) and 24 hpf stages (Figure 3.19). Notch inhibition by DAPM resulted in a consistent and clear upregulation of Id4 expression throughout the CNS in zebrafish embryos at all stages tested (Figures 3.17-18-19).

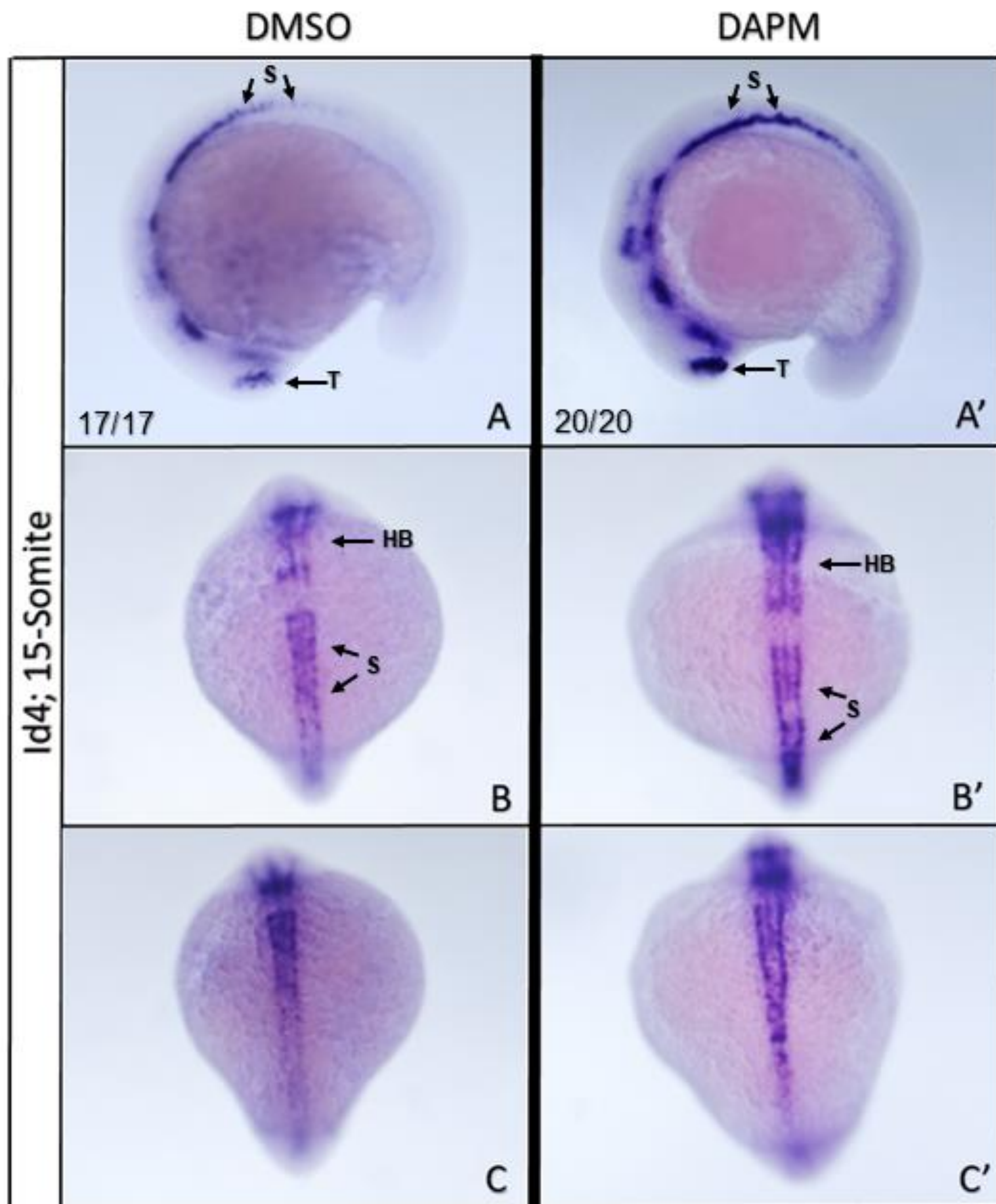


Figure 3.17: Elevated Expression of Id4 in DAPM-Treated Embryos at 15-Somite Stage

A,A': Lateral View, B,B': Anterior-Dorsal View, C,C': Dorsal View. HB: Hindbrain, T: Telencephalon, S: Spinal Cord

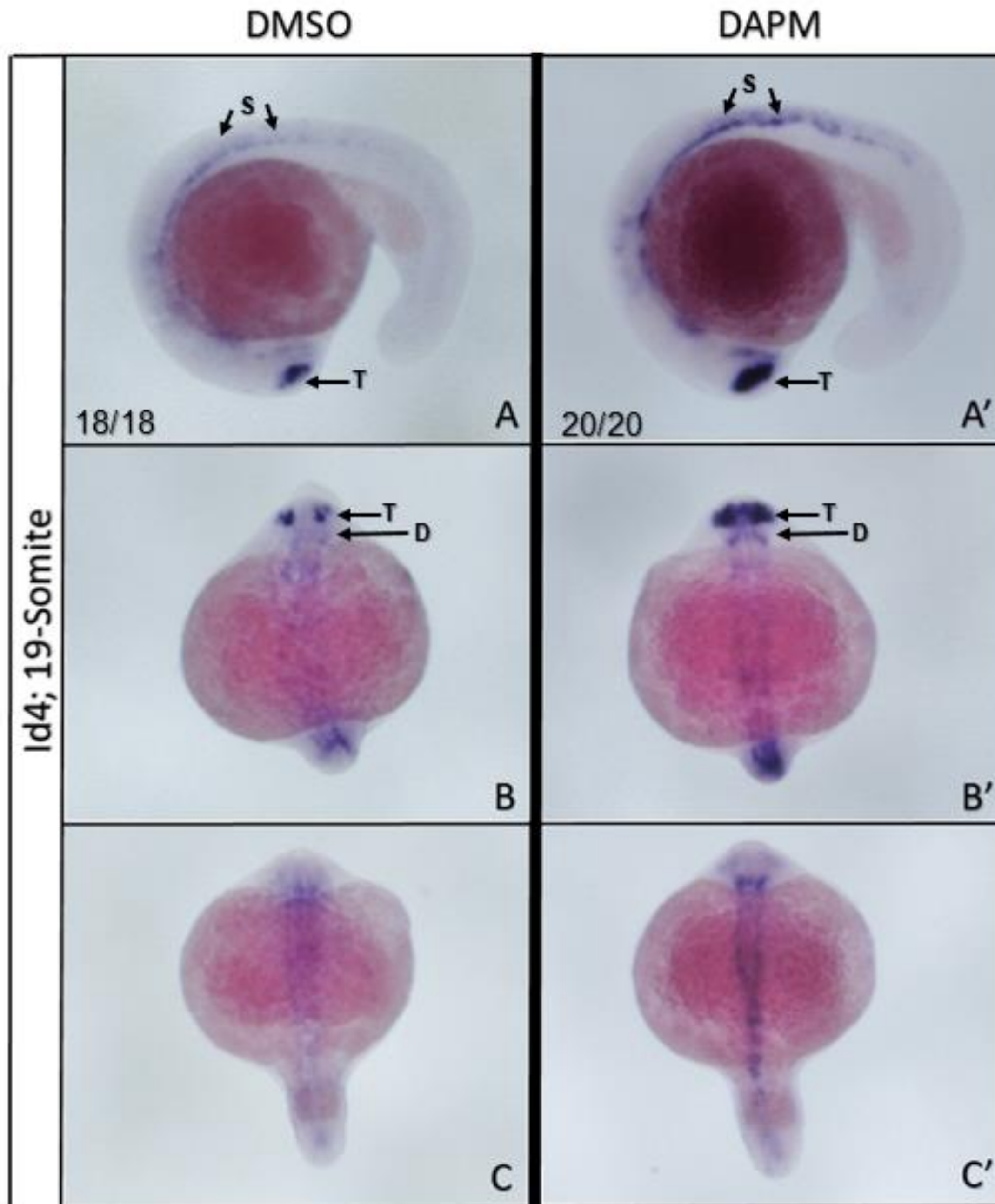


Figure 3.18: Elevated Expression of Id4 in DAPM-Treated Embryos at 19-Somite Stage

A,A': Lateral View, B,B': Anterior View, C,C': Dorsal View. HB: Hindbrain, T: Telencephalon, D: Diencephalon, S: Spinal Cord

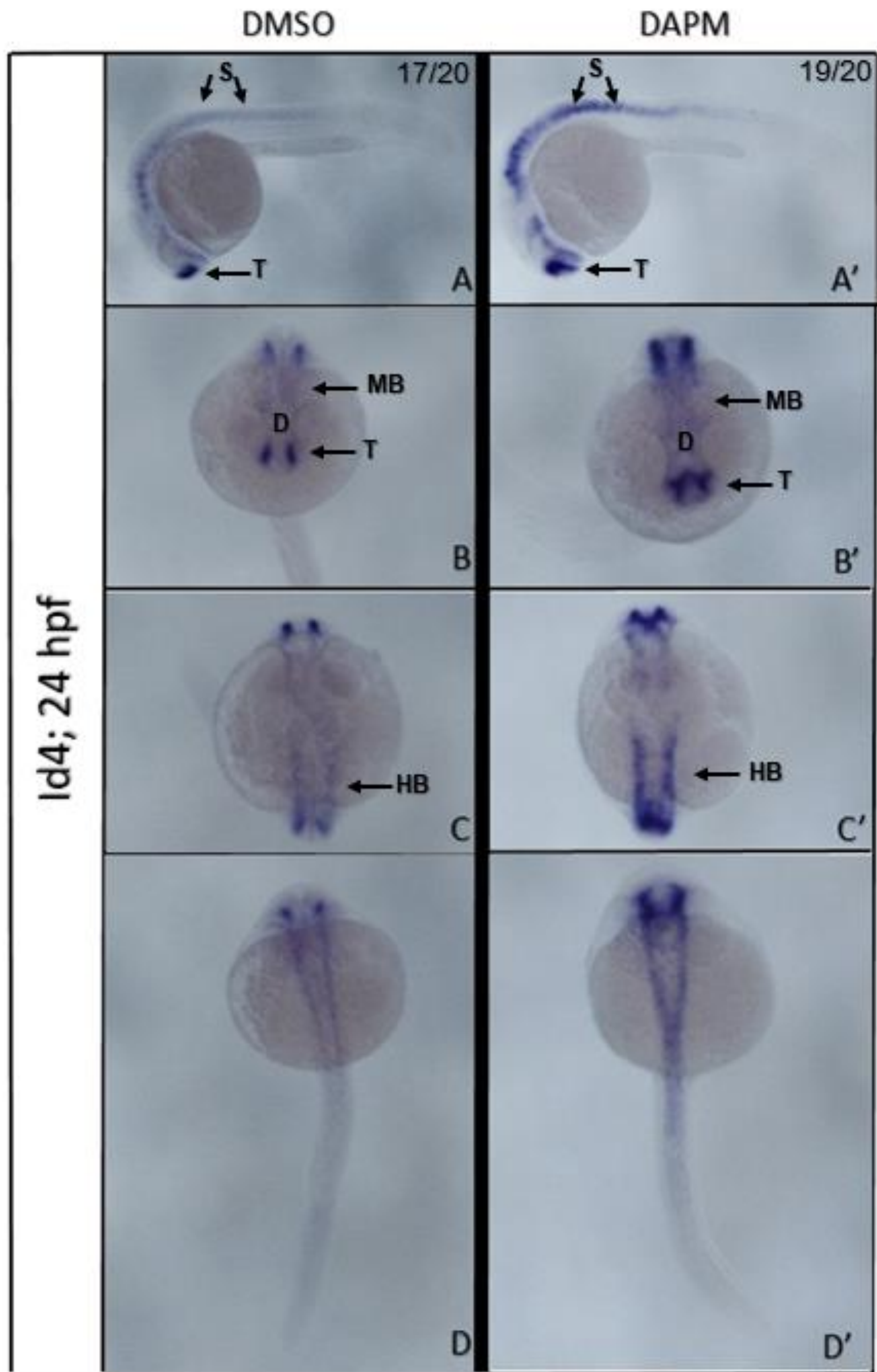


Figure 3.19: Elevated Expression of Id4 in DAPM-Treated Embryos at 24 hpf Stage

A,A’: Lateral View, B,B’: Frontal View, C,C’: Anterior View, D,D’: Dorsal View. MB: Midbrain, HB: Hindbrain, T: Telencephalon, D: Diencephalon, S: Spinal Cord.

Inhibiting Notch signalling pathway by DAPT also created somite boundary defects. Previous studies using DAPT as Notch inhibitor showed that depending on the starting time point of DAPT treatment, defective somites formed at different time points. When embryos were treated with DAPT at 3 hpf, earliest defective somite observed around 8-somite stage. Treatment at later stages resulted in the formation of the first defective somites at later stages (Özbudak and Lewis, 2008). Since DAPT and DAPM cause same effects in terms of Notch inhibition (Dang, *et al.,* 2008), DAPM treatment is comparable with DAPT. DAPM treatment started around 5,5 hpf, right before gastrulation and defects in somite boundaries were apparent around 14-somite stage. Since it was also shown that Notch signalling is required for the correct formation of somites, the segmented precursors of the vertebral column and skeletal muscle, older embryos exhibited a frizzled body shape (Figure 3.20-21).

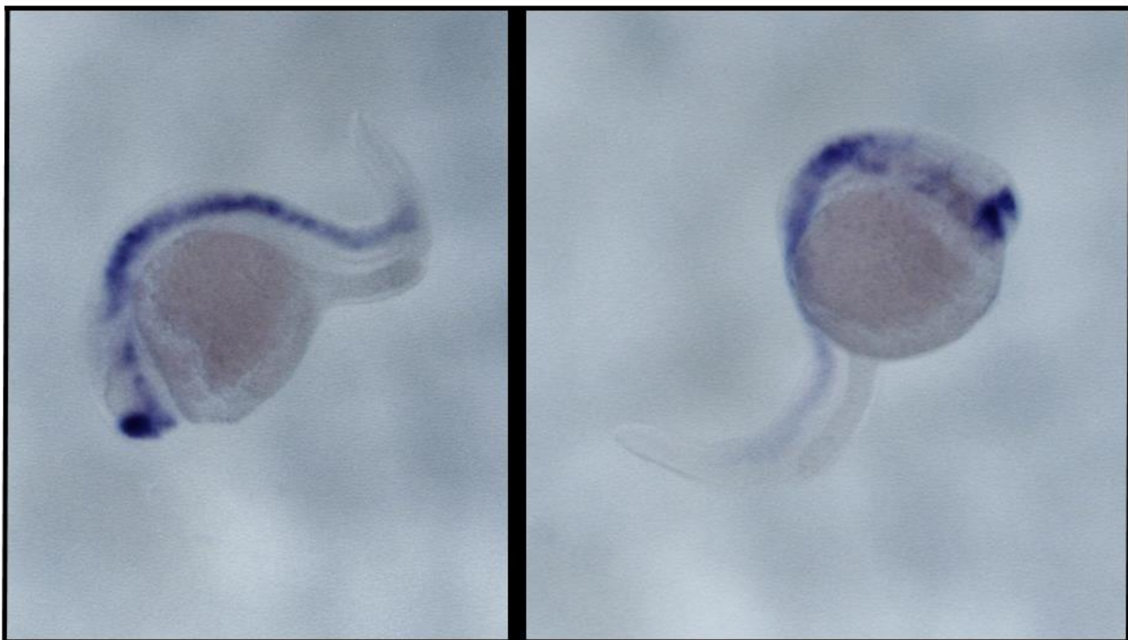


Figure 3.20: Frizzled Body Shape Observed after DAPM Treatment
Id4 expression at 24 hpf in the developing CNS of frizzled shape embryos.

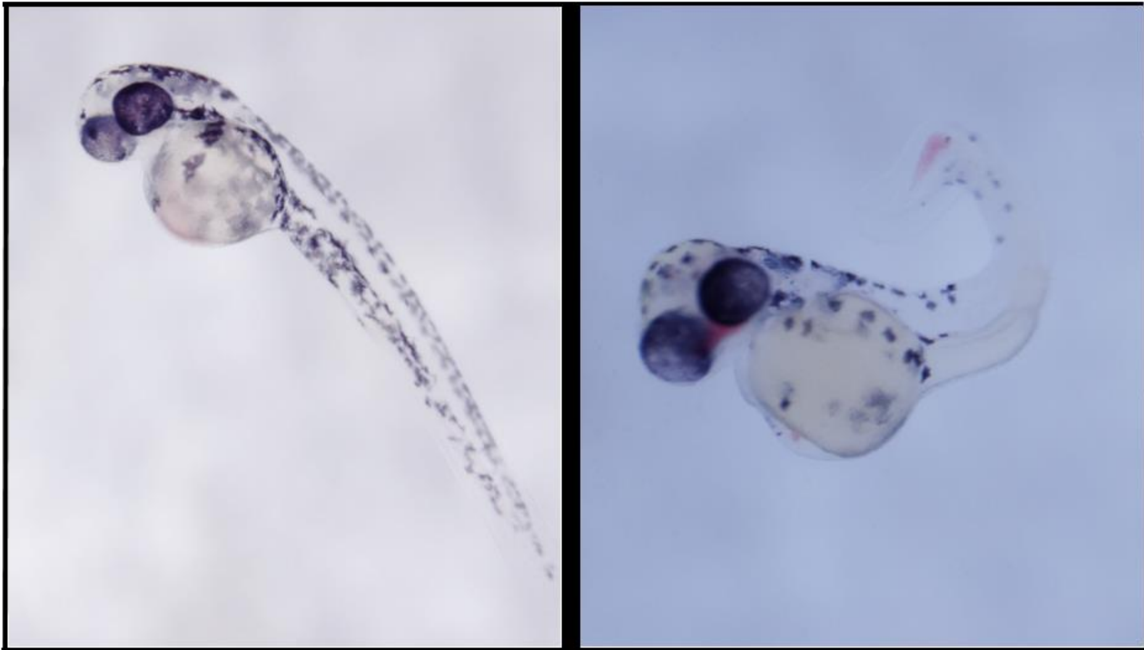


Figure 3.21: 2-Day-Old Zebrafish Embryos Treated with DMSO and DAPM
20 embryos left to grow for 2 days exhibited the expected body shape and lack of pigmentation confirming DAPM treatment.

3.3.3 Id4 expression is markedly elevated in Mind Bomb Mutants

Mind bomb encodes an E3 ubiquitin ligase which is essential for the proper function of Delta and therefore Notch signalling. Mind bomb mutants show a strong neurogenic phenotype with primarily premature neuronal differentiation (Jiang *et al.,* 1996; Itoh *et al.,* 2003).

Hortopan and Baraban (2011) showed that mind bomb mutation resulted in a downregulation of bHLH and hes/hey genes in zebrafish. Previous analysis using RT-PCR suggested that Id4 is downregulated in mind bomb mutant zebrafish at 24 hpf, 48 hpf and 72 hpf (Ganguly, 2013). In contrast, *in situ* hybridisation revealed an upregulation of Id4 expression at 24 hpf (Dhanaseelan, 2016).

To clarify the situation, Id4 expression was determined in embryos obtained from crosses of heterozygous mind bomb (mib) mutants (Figure 3.22). Embryos were then grouped based on their expression pattern in accordance with their possible genotypes. Embryos that exhibited an Id4 expression pattern similar to wild type embryos analysed separately, were deemed to be wild type. All other embryos showed an elevated Id4 expression with some embryos exhibiting very strong upregulation of Id4 expression similar to the DAPM treated embryos shown above. Therefore, it is very likely that these embryos are indeed homozygous mib mutants (Figure 3.23).

Taken together, lack of Notch function results in upregulation of Id4 suggesting that Notch signalling negatively regulates Id4 expression.

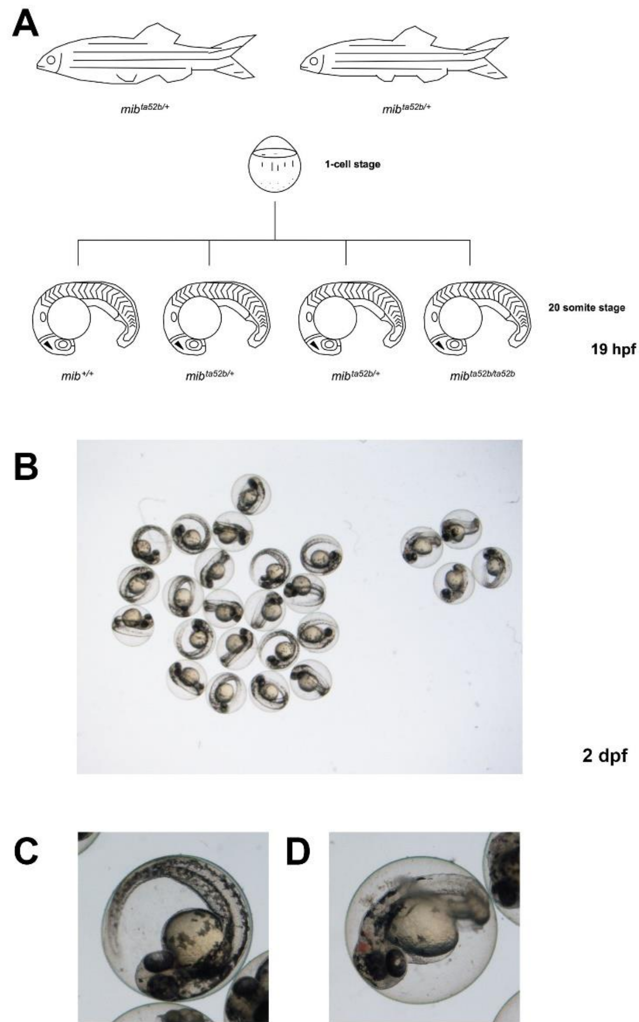


Figure 3.22: Preparation of mib Embryos
A: Heterozygous mib mutants incrossed and their embryos were collected at 19 hpf. B,C,D: Some of the embryos spared and left for development until 2 dpf in order to observe the existence of mib homozygous mutants with a 25% proportion of total embryos. Mib mutants were provided by Dr. Martin Gering and his research team.

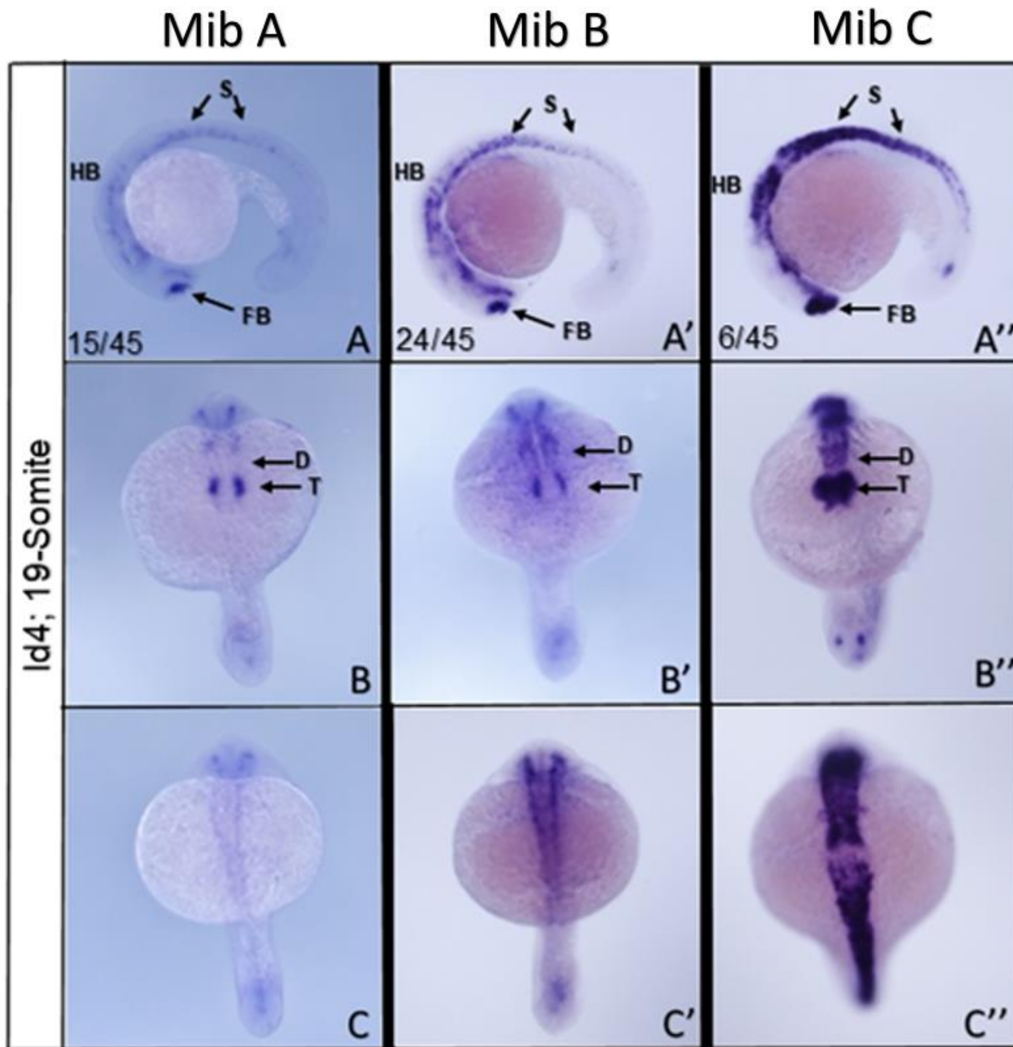


Figure 3.23: Elevated Expression of Id4 in Mind Bomb Mutants
Embryos obtained from Mib heterozygous incross were grouped based on the expression pattern of Id4 (Mib A, B, C). Left panel: 15/45 embryos exhibited weak Id4 expression as observed in a separate wild type control group (not shown). Middle panel: 24/45 embryos displayed an elevated Id4 expression. Right panel: in 6/45 embryos, Id4 expression was very strong. These data are reminiscent with the previous results (Figures 3.17-19), showing that inhibition of notch signalling resulted in upregulation of Id4 expression and suggest that upregulation of Id4 expression in Mib mutants is due to the lack of notch signalling.
A,A',A'': Lateral View, B,B',B'': Frontal View, C,C',C'': Dorsal View. T: Telencephalon, D: Diencephalon, FB: Frontal Brain, HB: Hind Brain, S: Spinal Cord.

3.4 Summary of the Main Findings

- Id4 mutant qmc 803 zebrafish embryos exhibited a reduced Sox2 expression suggesting a role for Id4 in the maintenance of neural progenitor cell population.

- Expression of the early neuronal marker HuC appeared unaltered but expression of the astrocyte marker GFAP was elevated in the absence of Id4 at 24 hpf indicating that Id4 is not required for neural nor glial cell differentiation but appears to prevent premature astrocyte differentiation.

- Inhibition or lack of Notch signalling pathway by either DAPM treatment of wild type embryos or in mib mutant embryos resulted in increased Id4 expression. Given that in the absence of Notch signalling the neural stem cell pool is depleted through premature neuronal differentiation, this result could indicate that elevated Id4 expression was due to an expansion of Id4 expressing progenitor cells. Alternatively, Notch signalling might directly or indirectly control Id4 gene expression negatively.

4. DISCUSSION

Despite numerous attempts to identify Id4 homozygous mutant zebrafish, only three heterozygous mutants could be identified unambiguously through sequence analysis that caused restrictions to this project. Therefore, embryos obtained from heterozygous parents had to be genotyped after *in situ* hybridisation. The main problem was that extraction of genomic DNA from embryos that had been processed during *in situ* hybridisation procedure was unreliable often resulting in lack of PCR amplification (Fig. 4.1). While the conditions described in Materials and Methods (2.3.2) created the adequate requirements to extract genomic DNA from fin tissues and untreated embryos (Figure 3.5; Figure 3.7), they were not sufficient to extract DNA from embryos after *in situ* hybridisation. Various attempts were undertaken to optimise the DNA extraction (see Materials and Methods; 2.3.3) but despite some improvement lack of PCR amplification was still a major problem (see Figures 3.5 and 3.7). In addition, restriction enzyme digestion of successfully amplified DNA was also often ambiguous further hampering the clear identification of the genotypes (Figures 3.3-4-6-8). Therefore, amplification products had to be sequenced to clearly identify the genotypes of the embryos and consequently, the number of embryos within each group analysed was small. Nevertheless, combined with previous results obtained the following can be concluded.

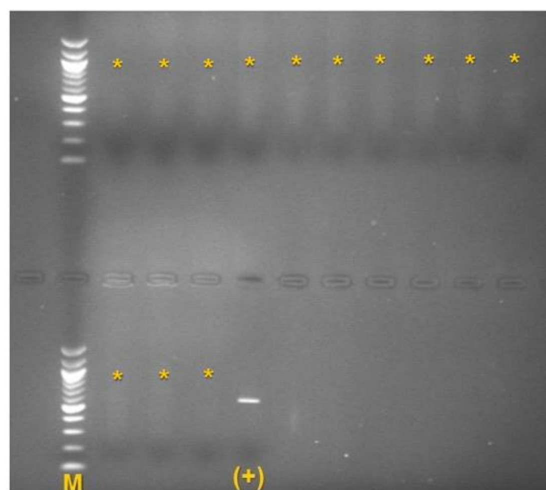


Figure 4.1: Lack of PCR amplification of genomic DNA extracted from embryos after *in situ* hybridisation.
Initial attempts to amplify genomic DNA isolated from embryos after *in situ* hybridisation using 50µl of both Base and Neutralisation Buffer failed completely (*) but the same conditions allowed amplification of wild type control DNA (+). M: DNA size Marker

4.1 Id4 morphants and Id4 knockout mutant zebrafish displayed a non-overlapping phenotype at 24 hpf.

Translation blocking morpholinos (MOs) were used to induce Id4 knockdown in the developing zebrafish embryos which resulted in reduced brain size and lack of brain boundaries at 24 hpf (Dhanaseelan, 2011). MO injection can sometimes cause artificial upregulation of the p53 pathway that results in a similar phenotype (Robu *et al.,* 2007), therefore p53-specific MOs were co-injected with Id4 MOs to eliminate potential off-target effects. Double morphants still showed a similar phenotype as single Id4 morphants suggesting the essential role of Id4 for the proper development of the brain (Figure 4.1) (Dhanaseelan, 2011). Another study demonstrated that, the severity of the phenotype depends on the amount of injected MO. While the injection of less than 10ng of MO didn't cause any phenotype, 20ng of Id4 MO created a severe phenotype with completely malformed brains without distinct boundaries (Figure 4.2) (Patlola, 2009).

Recent studies showed that morpholino-mediated knockdown experiments are usually (but not always) non-reproducible in knockout mutant fish (Kok *et al.,* 2015). To test the specificity of the Id4 morphants' phenotype, TALEN-mediated Id4 knockout was introduced to zebrafish embryos which resulted in a morphologically indistinguishable phenotype at 24 hpf compared to wild type embryos (Figure 1.11). Id4 mutant zebrafish embryos could reach adulthood, were fertile and morphologically normal (Dhanaseelan, 2016).

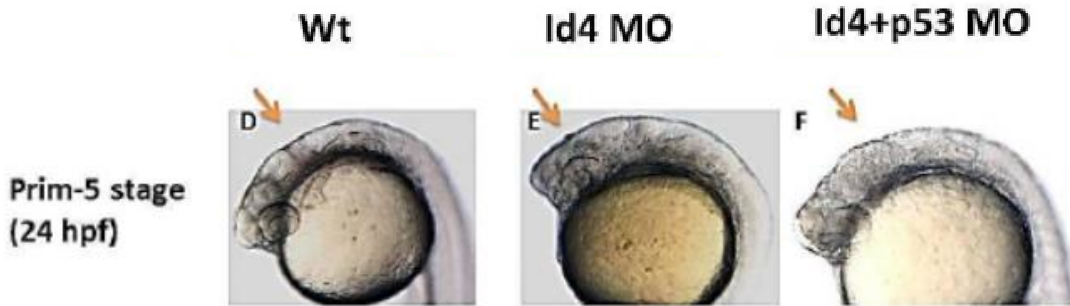


Figure 4.2: Morpholino-Mediated Id4 Knockdown in the Developing Brain of Zebrafish at 24 hpf.
In comparison with the wild type embryos (D), injection of Id4 MO (E) and co-injection of Id4+p53 MO (F) caused a reduction in the brain size and absent brain boundaries in the developing brain of zebrafish at 24 hpf. (Adapted from Dhanaseelan, 2011)

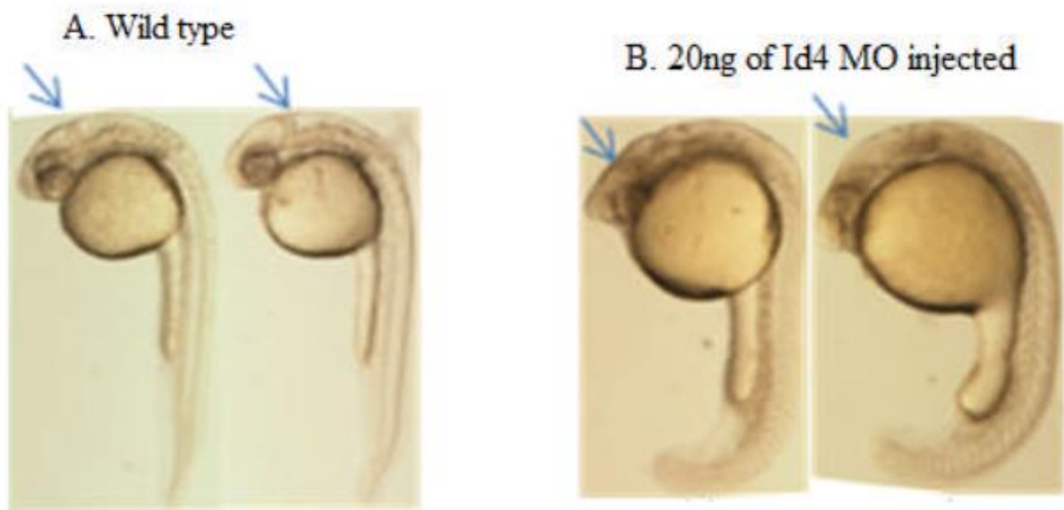


Figure 4.3: Severe Phenotype was Observed with Id4 Morphants at 24 hpf.
20ng of Id4 MO injection resulted in a severe phenotype (B) in the developing brain compared to wild type (A) at 24 hpf. (Adapted from Patlola, 2009)

4.2 Reduced Sox2 expression suggests a reduction within the stem cell pool in the absence of Id4.

Id4 is highly expressed in undifferentiated and proliferating cells, revealing its role for neural stem cell maintenance. To determine the situation of neural stem cell pool in the absence of Id4, Sox2 expression which is a marker for self-renewing stem cells was determined. (Figure 3.10)

Sox2 expression can be seen in the telencephalon, midbrain and hindbrain within wild type embryos at 2-somite stage. Morpholino-mediated Id4-deficient zebrafish displayed reduced Sox2 expression in the hindbrain and none in the midbrain. Id4/p53 double morphants also showed similar expression as Id4 morphants (Dhanaseelan, 2011). At 24 hpf, Sox2 is mainly restricted to the central nervous system including immature eye, brain regions and spinal cord. In line with the morpholino results, a downregulation of Sox2 expression throughout the CNS was observed indicating an exhaustion of the stem cell pool. Both results suggest that absence of Id4 cause a reduction in the number of self-renewing stem cells indicating the importance of Id4 in maintenance of the neural stem cell pool. The intensity of Sox2 expression in Id4 heterozygous and homozygous mutant fish displayed a reduction gradient in accord with the genotype.

4.3 Id4 impact on the timing of neurogenesis and gliogenesis varies between development stages.

Id4 is required for the correct timing of neural differentiation. HuC was used as an early neuronal marker and GFAP as a glial marker for astrocytes, in order to determine whether neuronal and/or glial cell differentiation was perturbed.

HuC is expressed in the CNS including some cell clusters of trigeminal ganglion neurons. Previous studies showed that in the absence of Id4, HuC expression displayed a slight increase at 2-somite stage (10,6 hpf) whereas a significant upregulation at 18 hpf suggesting premature neuronal differentiation at that stage (Dhanaseelan, 2016). In our study, no significant alteration of HuC expression was observed with Id4 mutants at 24 hpf suggesting that earlier premature differentiation of neurons was exhausted resulting in similar number of neurons in wild type and mutant CNS (Figure 3.11).

GFAP expression is widespread throughout the CNS, significantly in rhombomere 4. We observed a significant upregulation of GFAP in Id4 homozygous mutants at 24 hpf, suggesting premature glial differentiation. The intensity of GFAP expression in Id4 heterozygous and homozygous mutant fish displayed an elevation gradient in accord with the genotype (Figure 3.12).

In order to interpret these results, the development process of the CNS should be considered. The first rudiment of the CNS within the developing embryo is the formation of neural plate which is well delineated at the end of gastrulation, around 10 hpf (Kimmel *et al.,* 1995). Also, the onset of neurogenesis becomes apparent during late gastrulation in zebrafish via the induction of proneural genes like ascl1 and ngn1 (Appel and Chitnis, 2002). At 10.6 hpf, neurogenesis is newly started therefore it would be feasible to think of a relatively less impact of Id4 at that stage. At 18 hpf, neurogenesis is far more progressed and intense, yet more, the closure of the neural tube will be completed in 1-2 hours (20-somite stage). Thus, lack of Id4 caused a significant impact by spoiling the timing of neurogenesis eventually resulted in premature neuronal differentiation. At 24 hpf, the closure of the neural tube completed and expanded anteriorly, telencephalon, diencephalon, midbrain and hindbrain are formed. Therefore again, no-significant defect was observed in neurogenesis.

On the other hand, gliogenesis happens later than neurogenesis during development (Bertrand *et al.,* 2002). While neurogenesis seems not to be impaired at 24 hpf, gliogenesis was affected showing premature glial differentiation in the absence of Id4 indicating a role for Id4 in the timing of glial cell differentiation.

It is worth noting that, lack of Id4 created altered expressions of neural genes resulted in premature neural differentiation yet it didn't inhibit the formation of neither neurons nor glial cells, revealing the fact that it is not essential for neither of them. However, it is essential for correct timing of differentiation.

4.4 Id4 is not positively regulated by Notch signalling.

Notch signalling pathway controls a variety of events such as cell fate determination via cell-cell communication and pattern formation. Inhibition of Notch signalling causes the failure of lateral inhibition which creates a neurogenic phenotype due to premature neural differentiation and reduction of the progenitor cells (Jiang *et al.,* 1996; Lawson *et al.*, 2001; Geling *et al.* 2002; Bingham *et al.*, 2003).

γ-secretase protein complex which cleaves NICD in order to activate Notch signalling, can be inhibited by DAPM. DAPM treatment (see Materials & Methods; 2.3.6) resulted in an elevated expression of Id4 in zebrafish embryos at 15-somite, 19-somite and 24 hpf stages (Figure 3.17-18-19) within the central nervous system.

Mind bomb encodes an E3 ubiquitin ligase which is essential for the proper function of Delta and therefore Notch signalling. Mind bomb mutants display a strong neurogenic phenotype with primarily premature neuronal differentiation (Jiang *et al.,* 1996; Itoh *et al.,* 2003). Hortopan and Baraban (2011) observed a downregulation of bHLH and hes/hey genes in zebrafish mind bomb mutants. Previous RT-PCR analysis suggested that Id4 is downregulated in mind bomb mutant zebrafish at 24 hpf, 48 hpf and 72 hpf (Ganguly, 2013). In contrast, *in situ* hybridisation revealed an upregulation of Id4 expression at 24 hpf (Dhanaseelan, 2016). Mib embryos were obtained from heterozygous parents, therefore 3 different genotypes occurred. Since DNA extraction from young embryos is unreliable, genotyping of the mib embryos couldn't be done. Nevertheless, embryos were grouped based on Id4 expression pattern and intensity

of signal and compared to wild type embryos (Figure 3.23). In line with the DAPM treated embryos, some mib mutants also displayed an elevation of Id4 expression within the central nervous system. Together these results revealed that Id4 is upregulated in the absence of Notch in the developing zebrafish embryo.
Since Id4 is mainly expressed in undifferentiated and proliferating cells (Norton *et al.,* 1998), its upregulation in the absence of Notch (Figure 3.17-18-19-23) could be due to an exhaustion of the stem cell pool and excess proliferation of progenitor cells (transient amplification) resulting in an apparent increase of Id4 expression.

However, Id4 expression might be negatively regulated through notch as was shown for Rbf, the *Drosophila* homolog of the retinoblastoma factor (Boanza and Freeman, 2005). If so, Id4 upregulation would prevent neuronal differentiation in contrast to what was observed in notch mutants that displayed premature neuronal differentiation. Unless another mechanism leads to downregulation of Id4 later on to allow neurogenesis to occur.

On the other hand, in myogenic cell lines, it was also shown that functional Notch signalling is required for BMP4 (Bone Morphogenetic Protein-4)-induced inhibition of myogenic differentiation. Within a Notch inhibited system, BPM4 alone wasn't sufficient enough to inhibit myogenic differentiation (Dahlqvist *et al.,* 2003). Since recent studies showed Id4 is upregulated via BMP signalling (Ahuja *et al.,* 2016) it seems BMP signalling may also be capable of creating an upregulation of Id4 even though Notch was absent.

4.5 Id4 regulates cellular differentiation.

Id protein family members prevent the interactions between Class A (E proteins) and Class B (tissue specific proteins) bHLH proteins, therefore they are considered as dominant negative regulators of cellular differentiation and positive regulators of cellular proliferation (Massari and Murre, 2000). Id4 regulates differentiation via interacting with different binding partners such as Olig proteins Olig1 and Olig2 (Figure 4.4). Olig proteins are bHLH transcription factors that regulate primary motor neuron and oligondendrocyte differentiation (Park *et al.,* 2002). Interestingly, Id4 has the highest affinity to Olig1 and Olig2 among Id proteins, neither Id1 nor Id3 interact with Olig1/2 and Id2 interacts weakly compared to Id4 (Samanta and Kessler, 2004). In this scenario, almost no compensation by other Ids would be anticipated in the absence of Id4, resulting in premature differentiation driven by Olig1/2. Id4 also has a binding affinity to E47 (Table 1.1), therefore inhibit the formation of both E47 homodimers and E47/MyoD heterodimers (Riechmann *et al.,* 1994).

Recent studies revealed Hes gene expression is modulated via Id proteins. (Boareto *et al.,* 2017). Hes (Hairy and Enhancer of Split) proteins Hes1 and Hes5 are well known repressors of proneural activity targeted by Notch signalling pathway. During brain development, a regulatory network between Hes proteins and neural differentiation activators (proneural factors) like Ascl1 and Neurog2 is essential for NSC maintenance and differentiation (Artavanis-Tsakonas *et al.,* 1999; Kageyama *et al.,* 2007). Hes proteins have oscillatory mechanism via counteracting Notch, resulting in the inhibition of their own expression (Hirata *et al.,* 2002). Studies have revealed that Hes gene expression reaches maximum levels in the presence of both Notch signalling and Id proteins since it was observed that high levels of Ids are able to completely repress proneural gene activity via upregulating Hes (Boareto *et al.,* 2017).

Recent studies done with human prostate cancer cell lines have revealed that Id4 is able to form heterodimers with other Id proteins (Id1-2-3), thus repressing their dominant negative activity (Figure 4.4). In other words, Id4 can indirectly act as a differentiation inducer, differing from other Id proteins. Interestingly, Id4 seems to have a similar affinity of E47 and Id1 (Sharma *et al.,* 2015). Taken together with the study that showed Id4 interaction with Hes1 in order to promote osteoblast differentiation (Tokuzawa *et al.,* 2010), it can be said that the inhibitory action of Id4 on cell differentiation is context dependent.

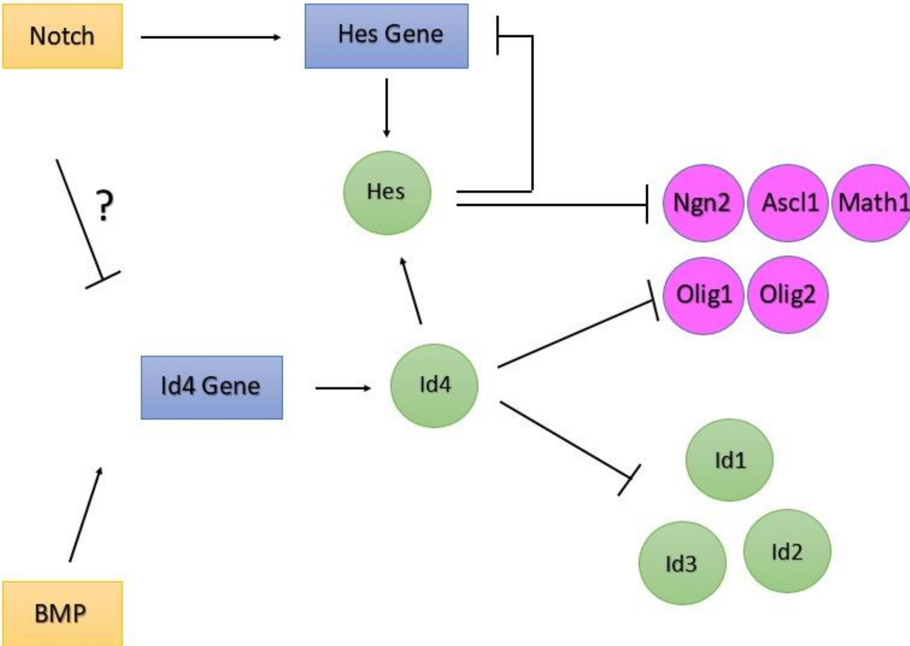


Figure 4.4: Components of Id4-related network
This scheme summarizes the interactions of Id4 with other components. Id4 inhibits the activity of tissue specific proteins such as Olig1/2 as an inhibitor of differentiation. It also inhibits other Ids therefore act as an inhibitor of inhibitors which makes it an indirect inducer of differentiation. Id4 interacts with Hes1 which is a downstream target of Notch signalling pathway, in order to promote osteoblast differentiation. Id4 is also upregulated via BMP signalling but seems to be downregulated by Notch. Yellow Boxes: Pathways, Blue Boxes: Genes, Green Circles: bHLH Proteins, Pink Circles: Proneural Proteins (also bHLH proteins)

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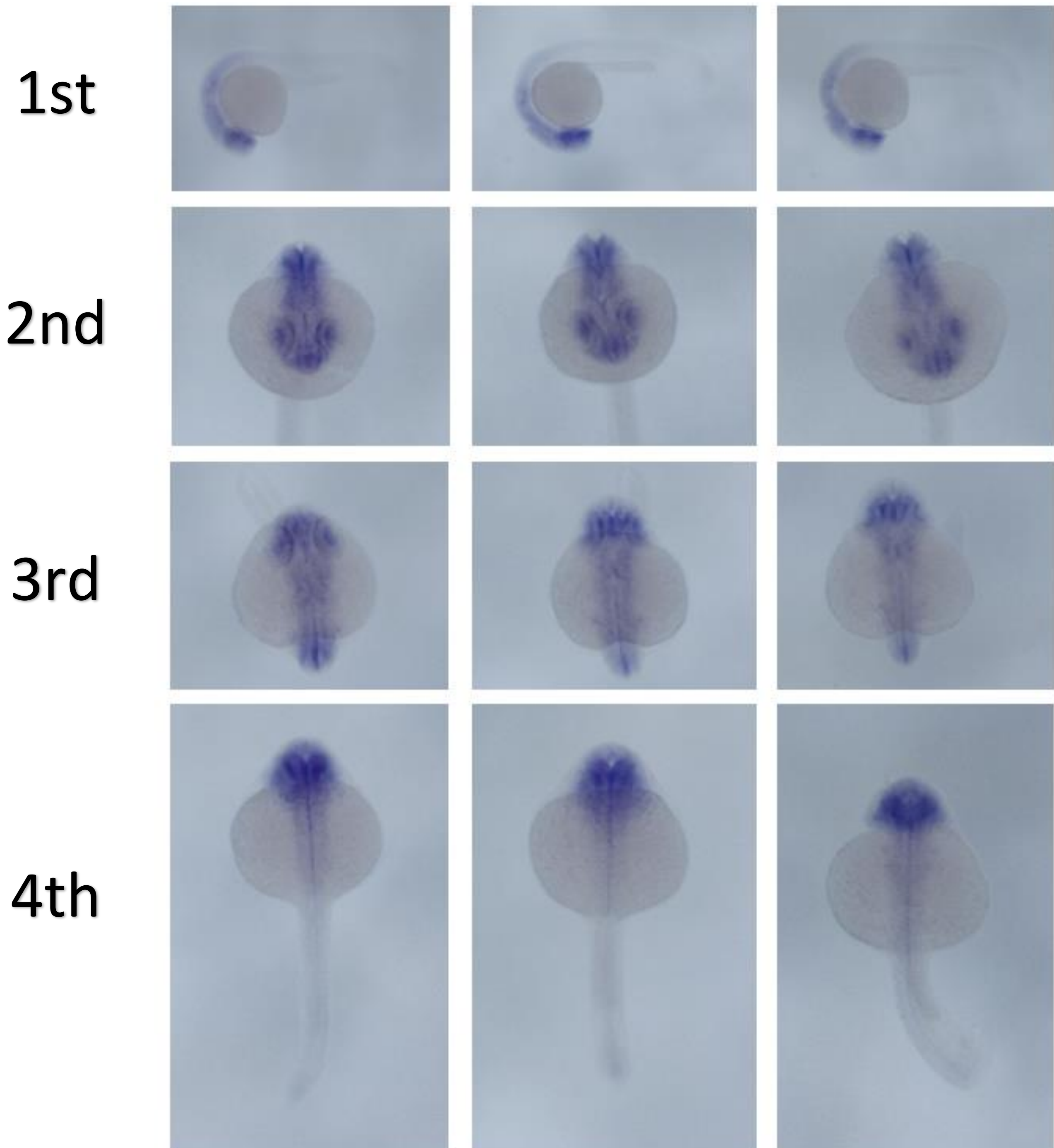
APPENDIX

1) Sox2 Hybridisation Results at 24 hpf

First Lane: Lateral View, Second Lane: Frontal View, Third Lane: Anterior View, Fourth Lane: Dorsal View. 5/18 embryos obtained from *in situ* hybridisation were genotyped by sequencing, chosen as possible candidates for three different genotypes. Here, images of the remaining not-sequenced 13 embryos presented.



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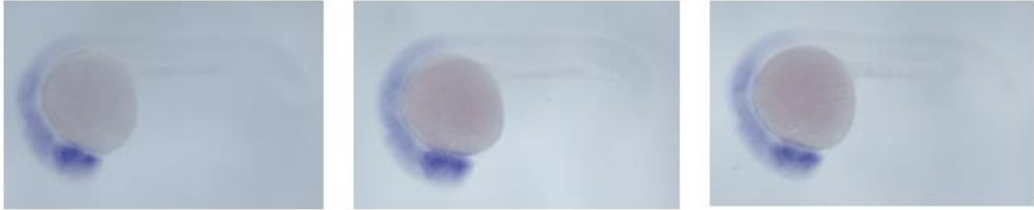

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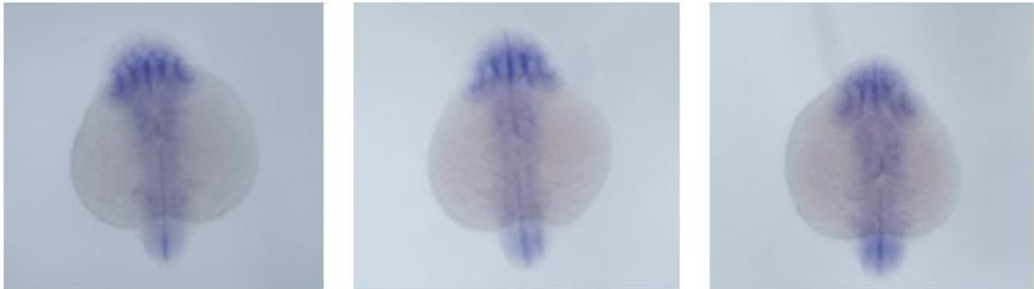

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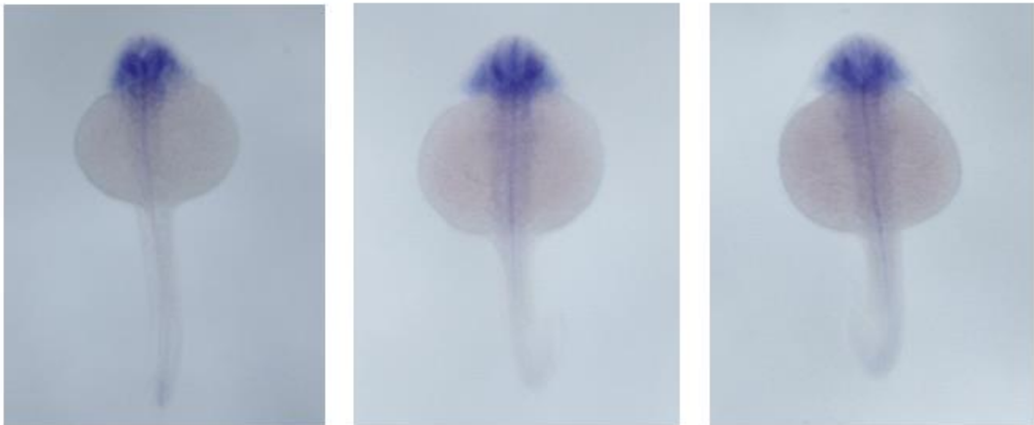

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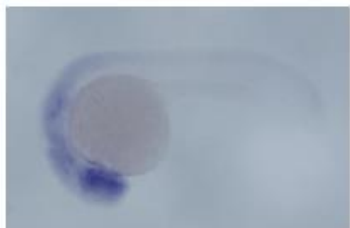
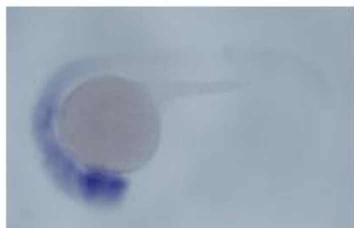
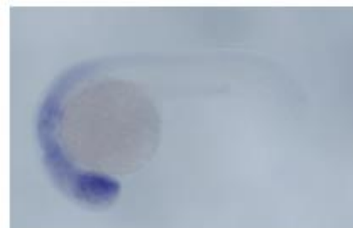

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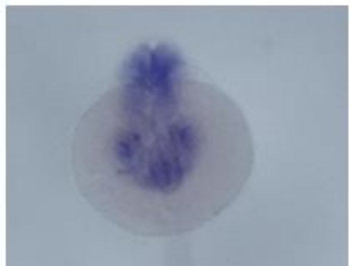
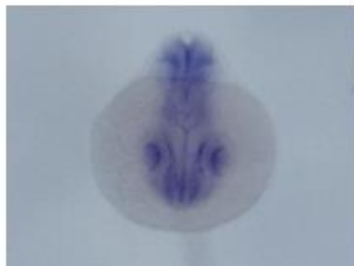
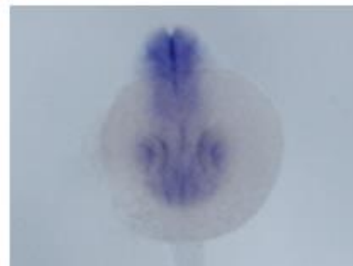

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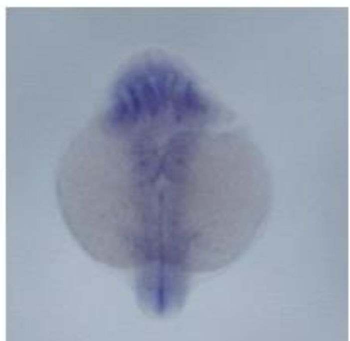
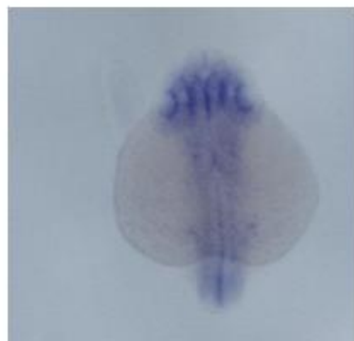
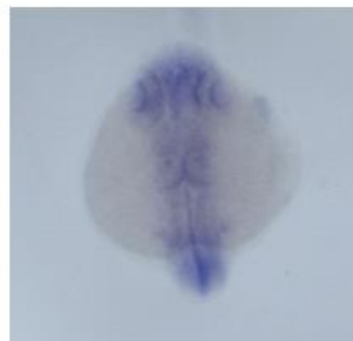

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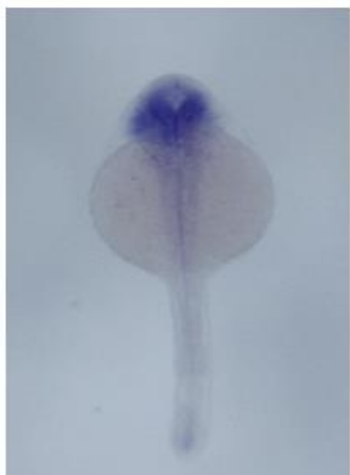
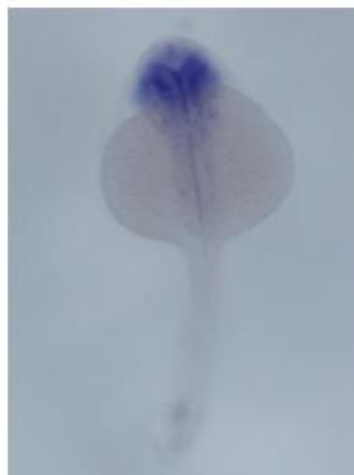
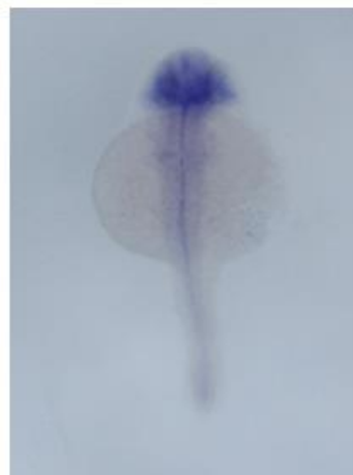

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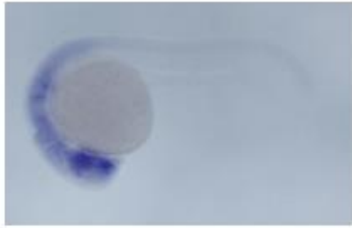
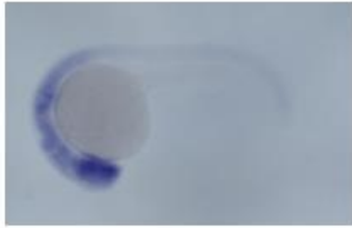
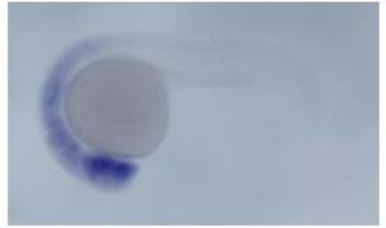

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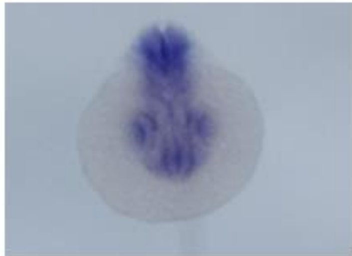
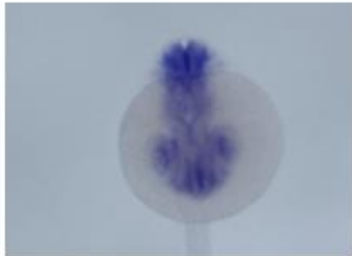
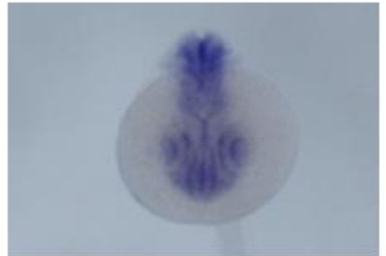

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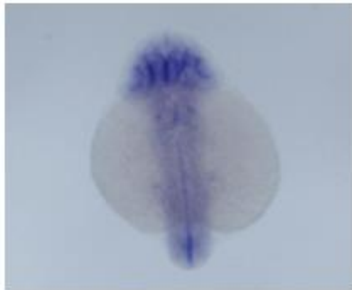
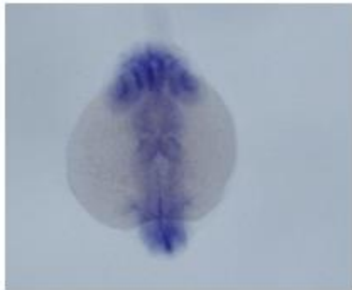
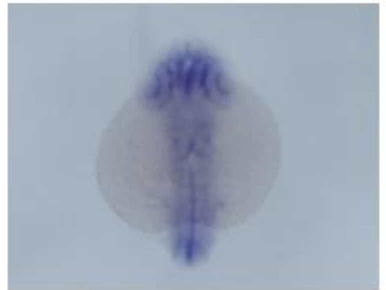

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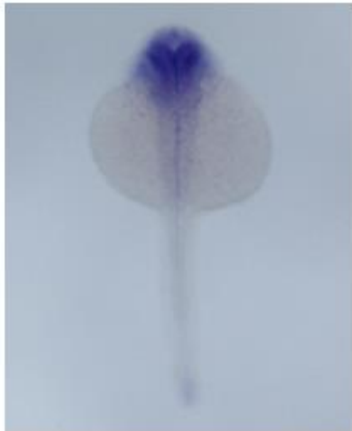
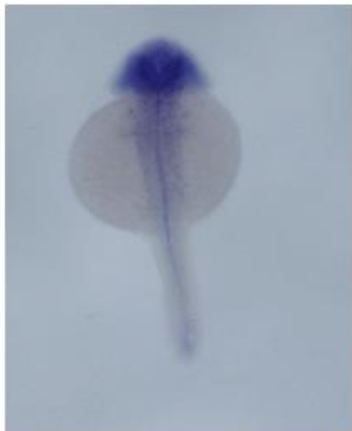
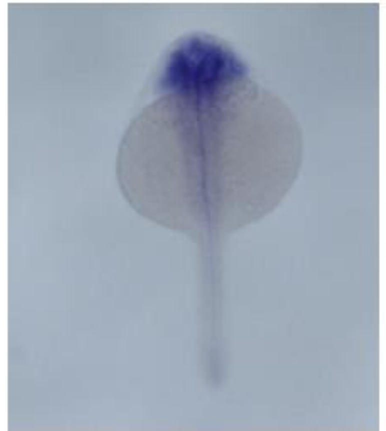

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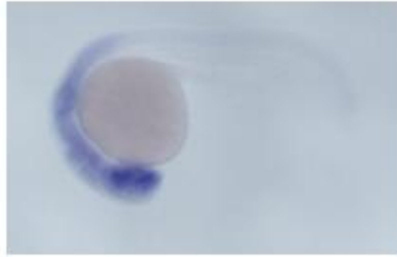

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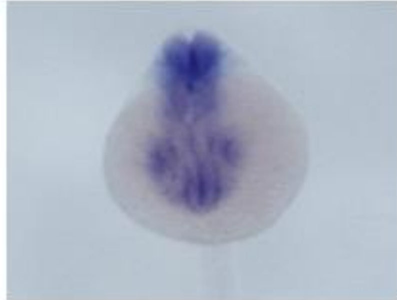

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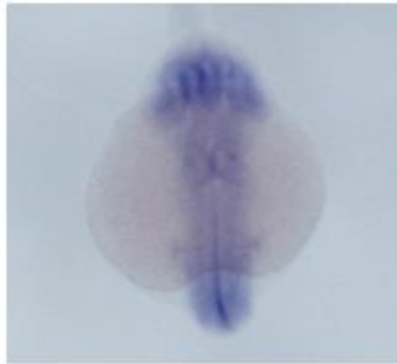

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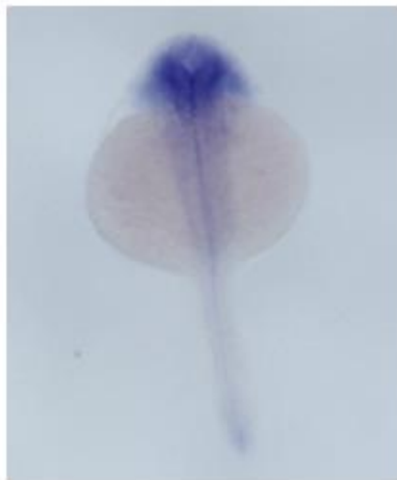
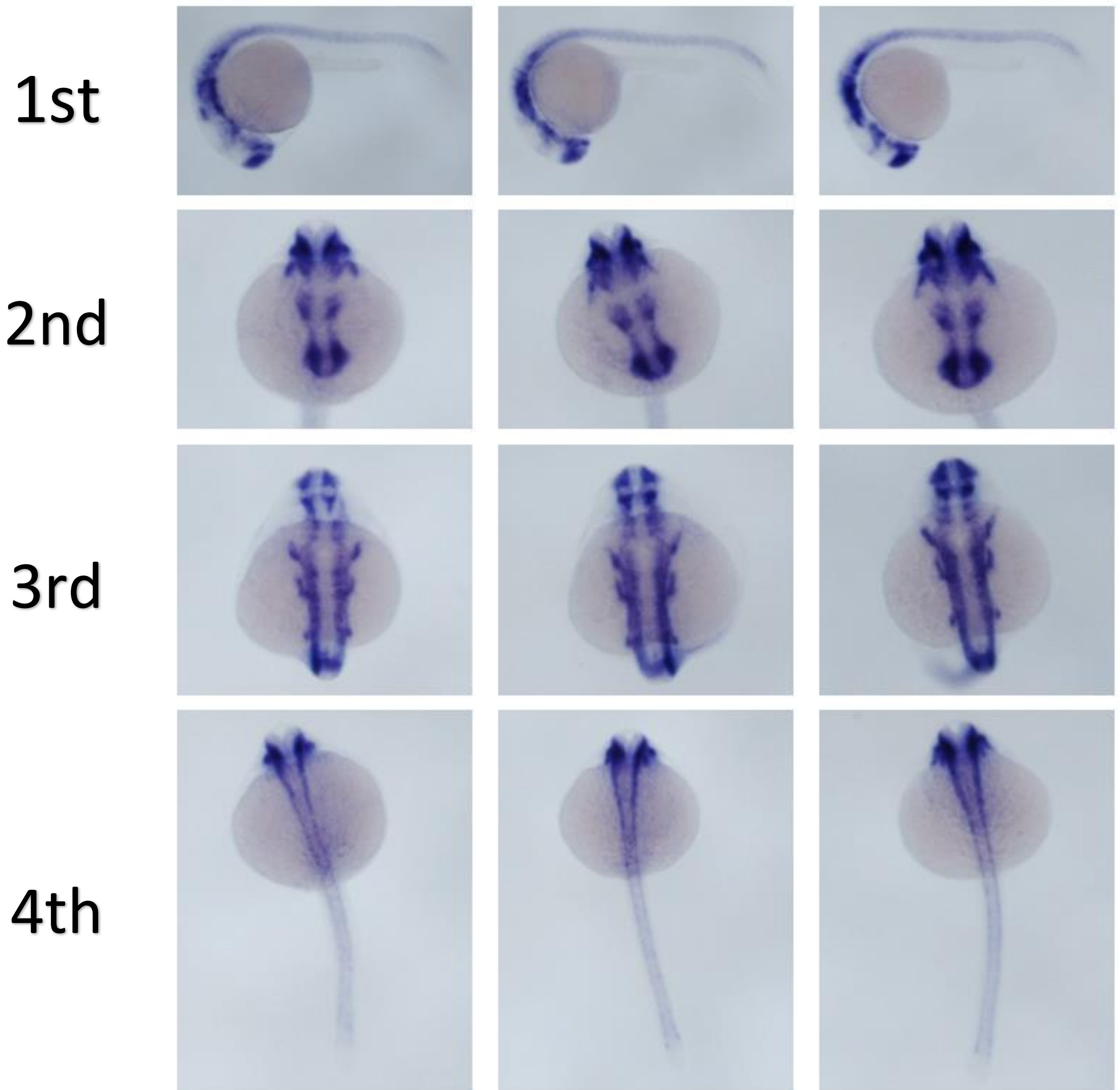


2) HuC Hybridisation Results at 24 hpf

First Lane: Lateral View, Second Lane: Frontal View, Third Lane: Anterior View, Fourth Lane: Dorsal View. 4/21 embryos obtained from *in situ* hybridisation were genotyped by sequencing, chosen as possible candidates for three different genotypes. Here, images of the remaining not-sequenced 9 embryos presented. Remaining 8 embryos not shown since their expression were indistinguishable from the ones shown in Appendix.



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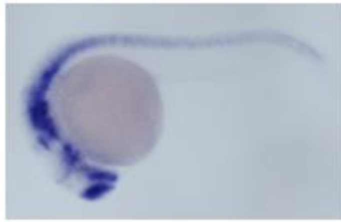
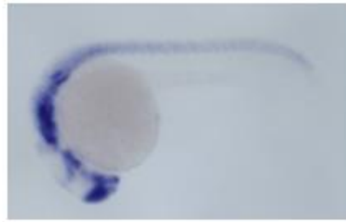
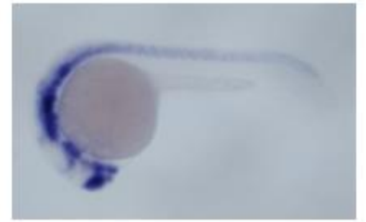

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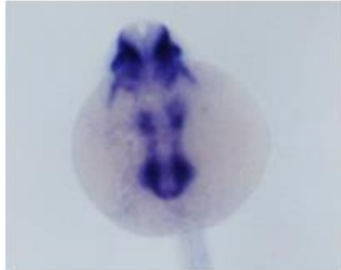
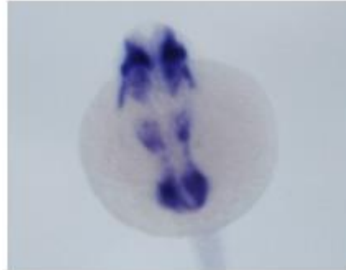
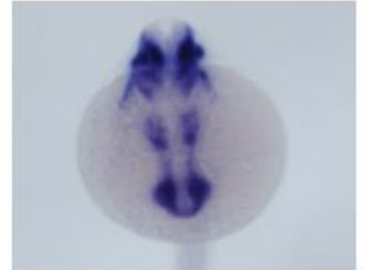

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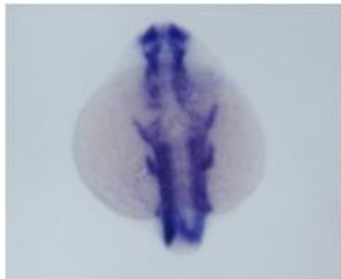
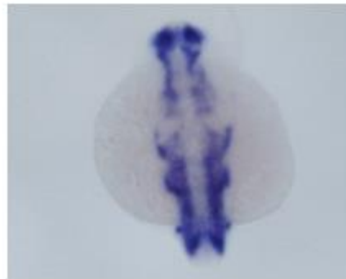
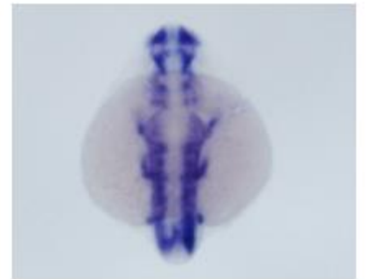

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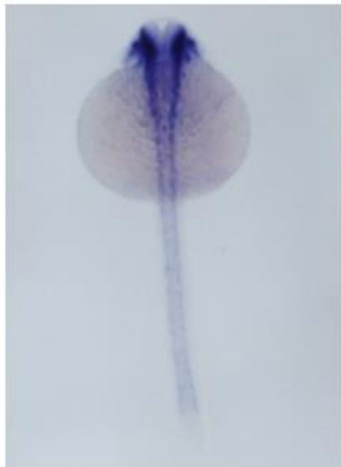
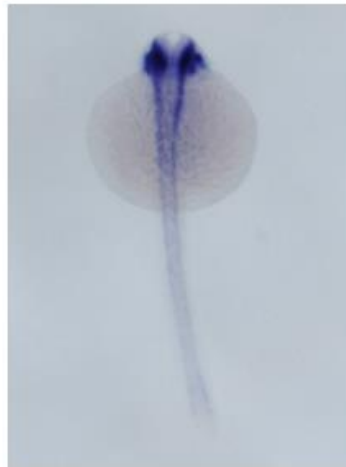

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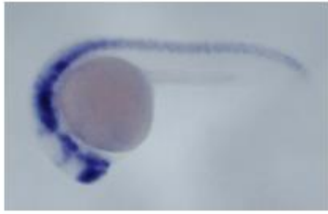
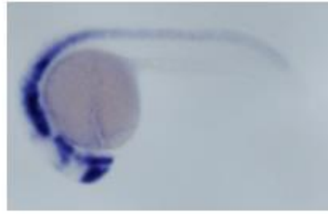
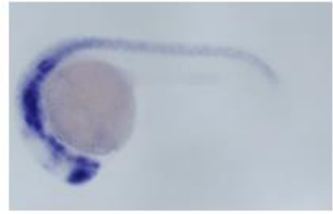

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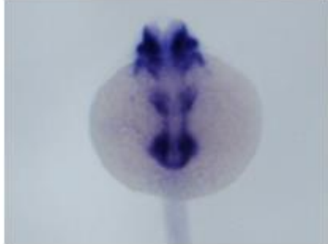
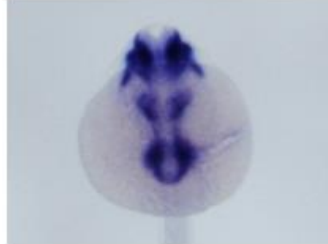
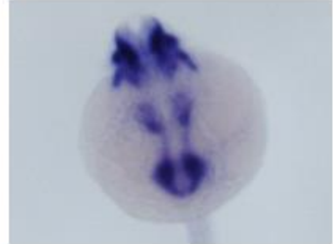

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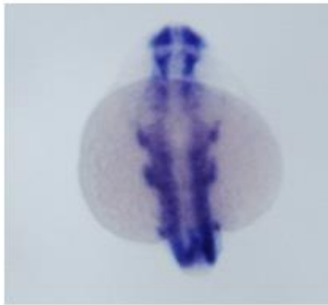
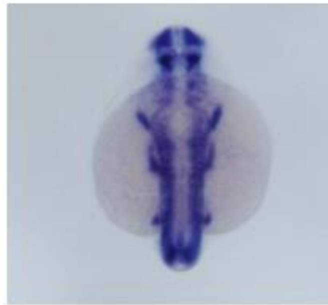
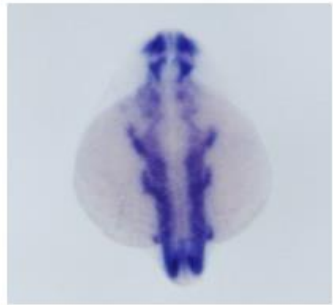

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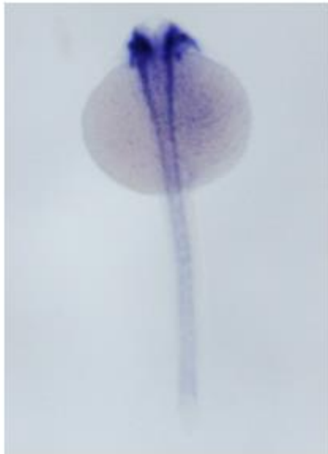
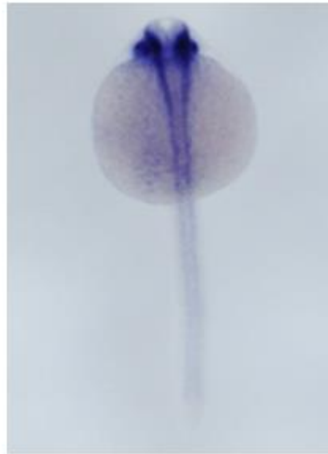
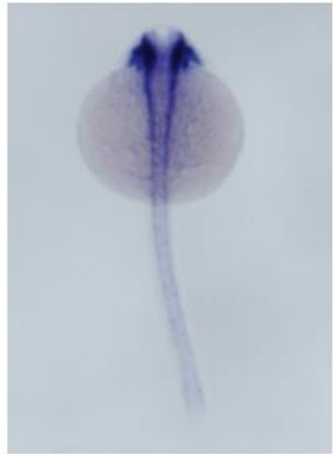
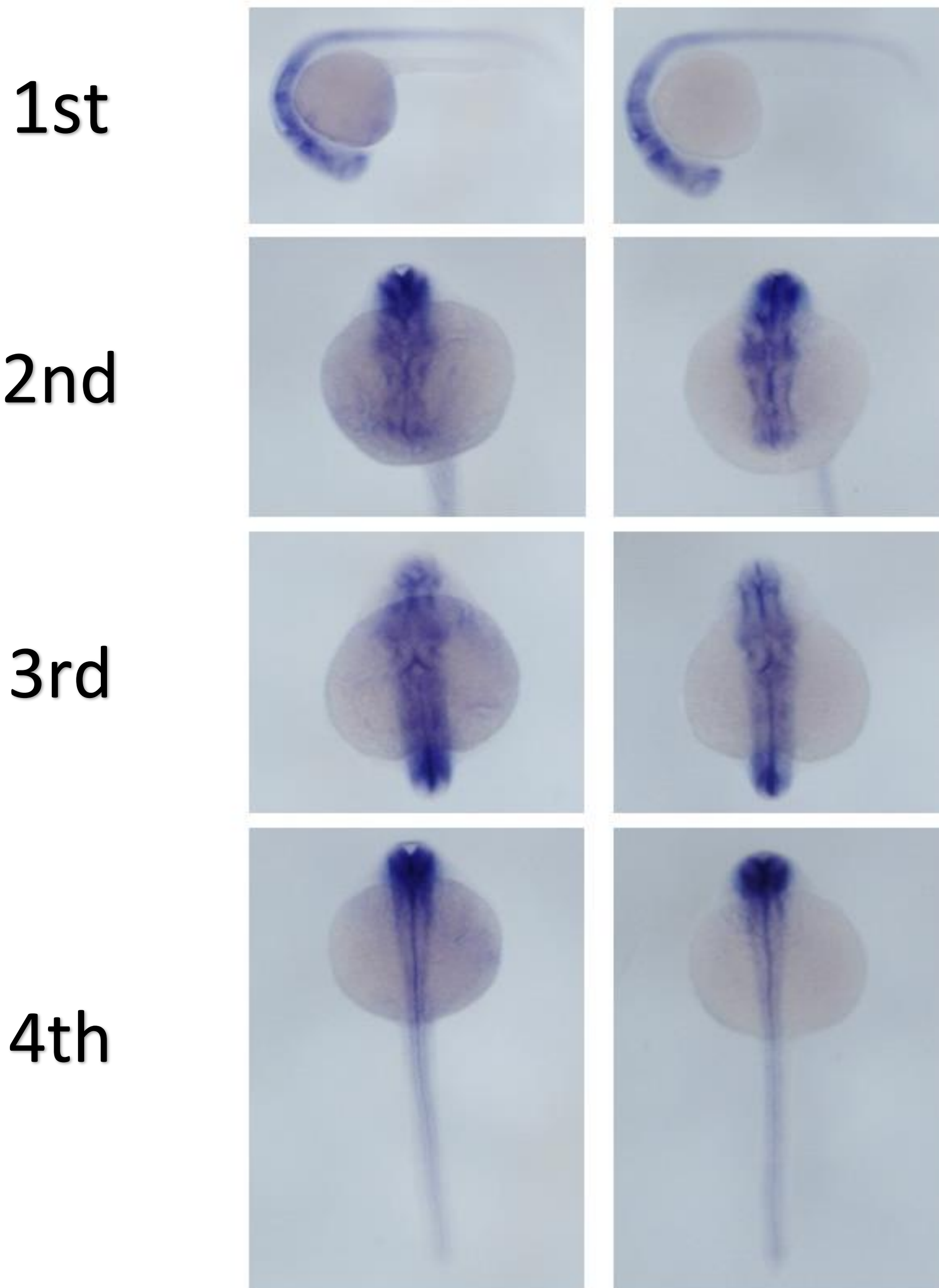




3) GFAP Hybridisation Results at 24 hpf

First Lane: Lateral View, Second Lane: Frontal View, Third Lane: Anterior View, Fourth Lane: Dorsal View. 6/22 embryos obtained from *in situ* hybridisation were genotyped by sequencing, chosen as possible candidates for three different genotypes. Here, images of the remaining not-sequenced 16 embryos presented..



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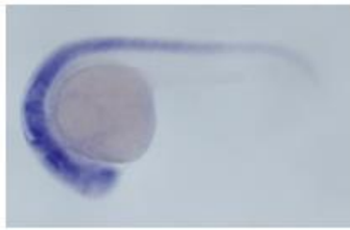
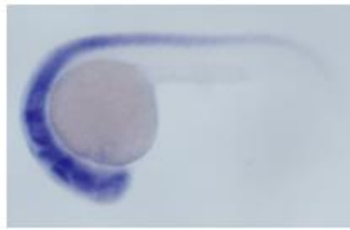
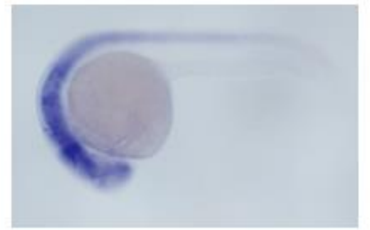

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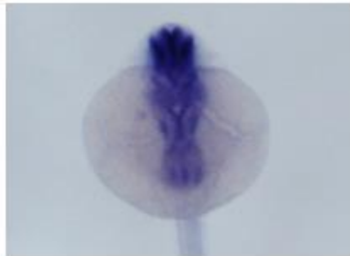
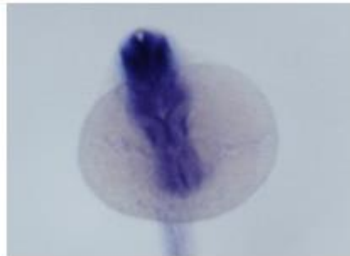
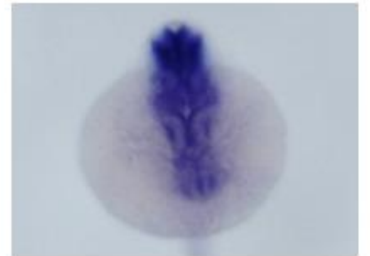

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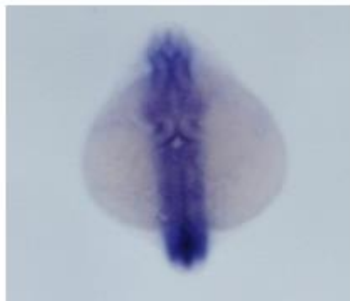
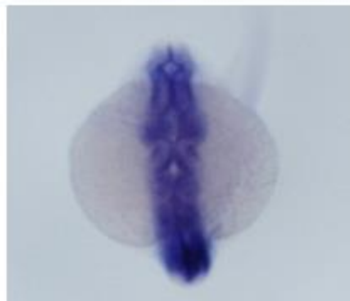
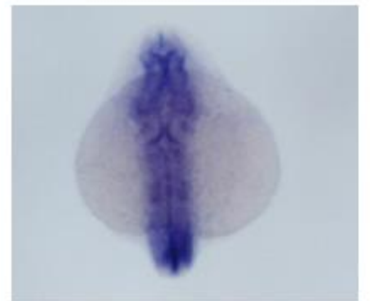

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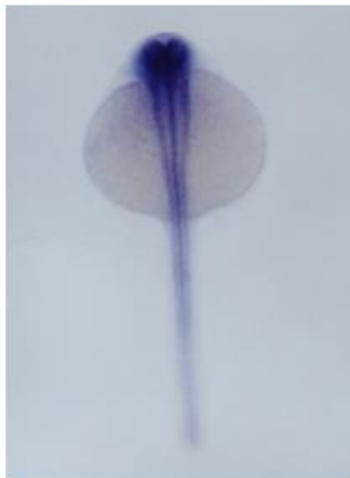
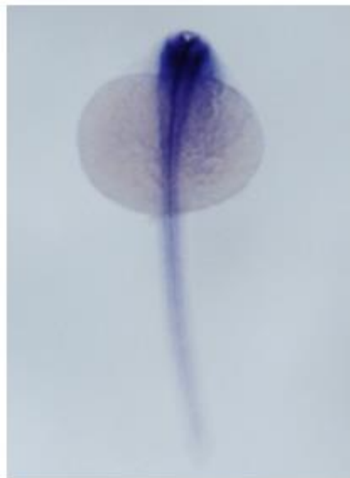
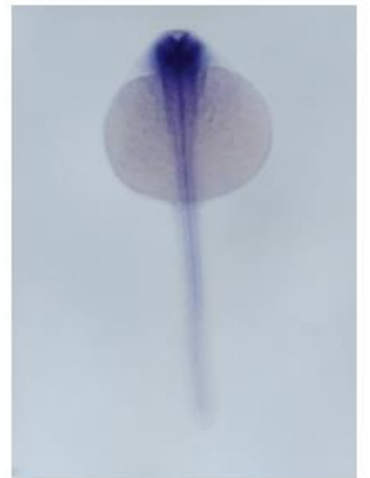

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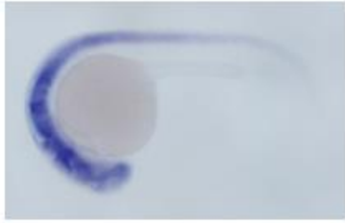
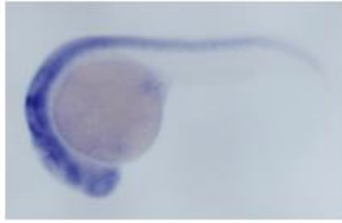
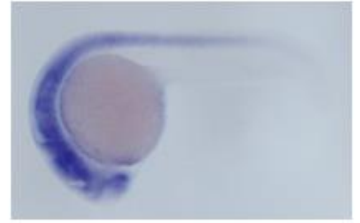

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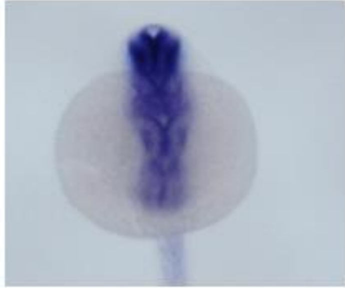
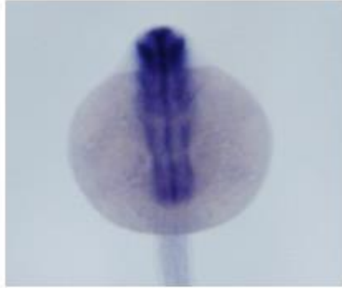
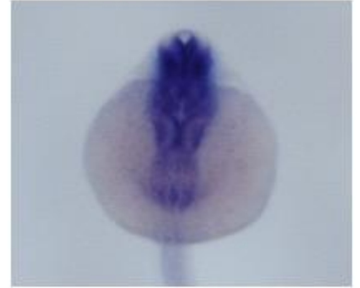

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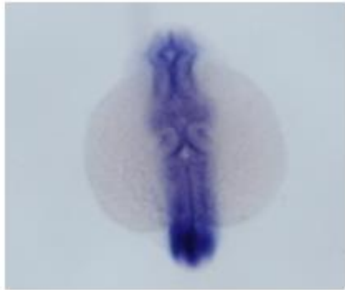
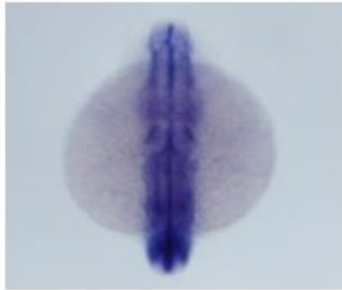
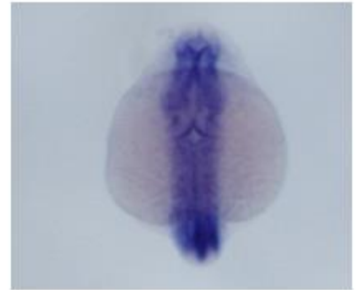

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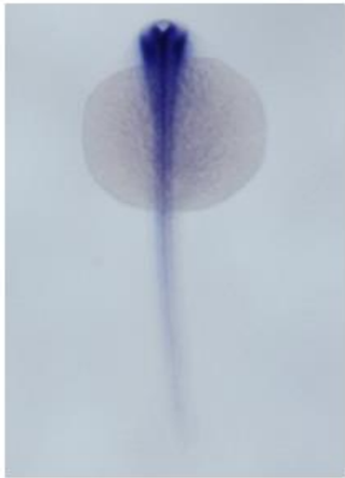
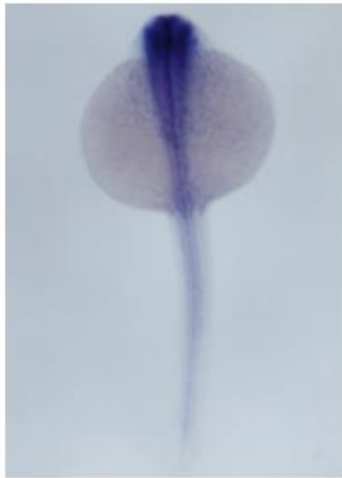
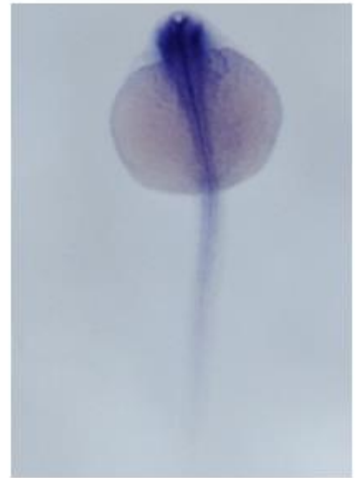

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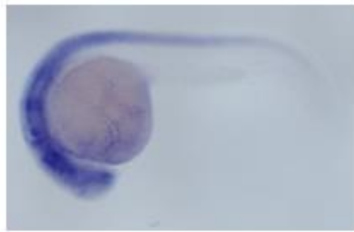
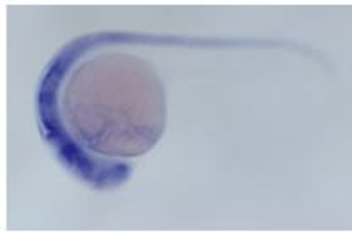
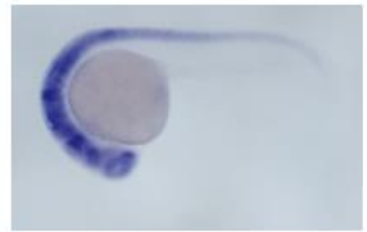

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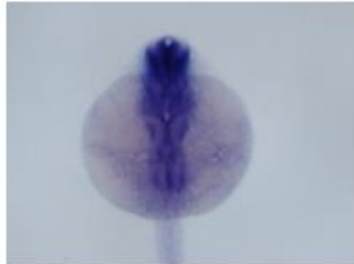
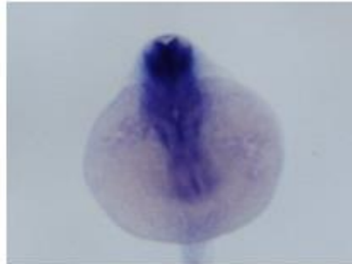
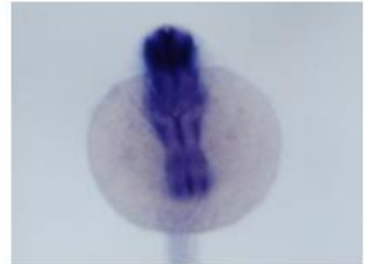

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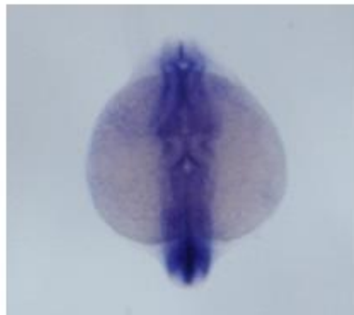
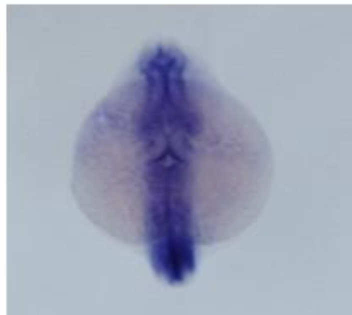
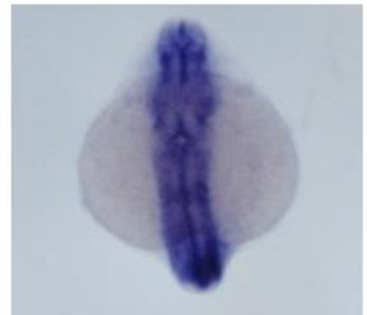

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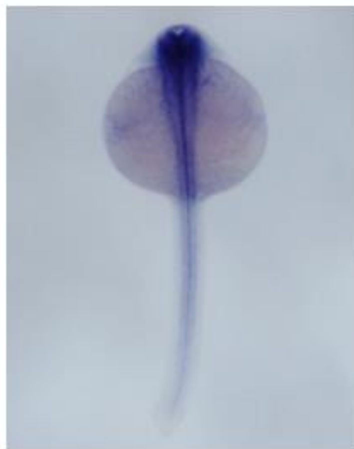

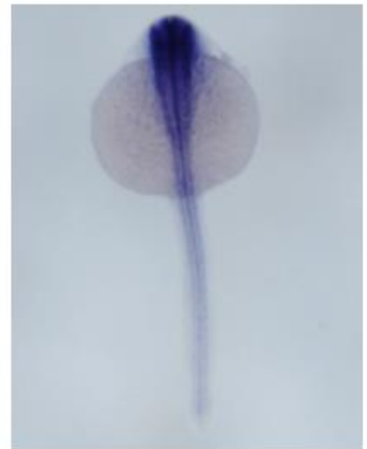

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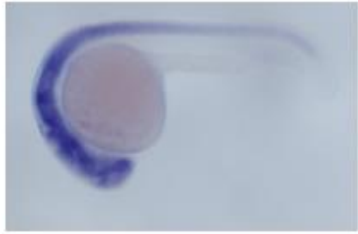
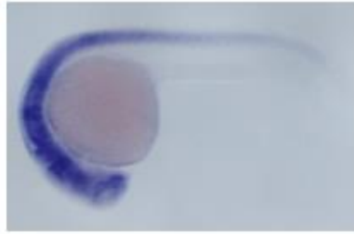
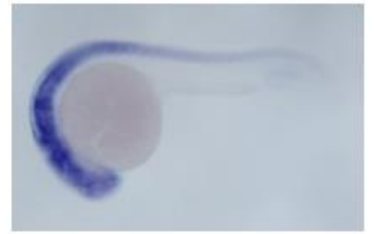

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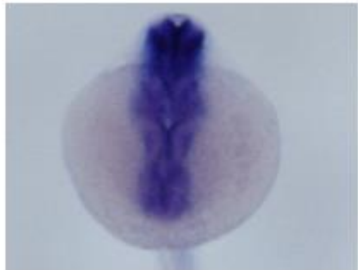
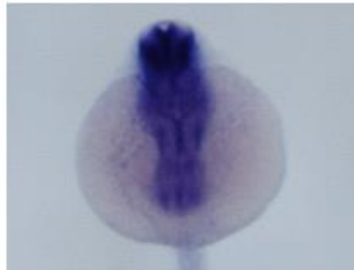
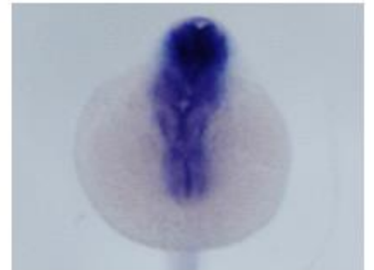

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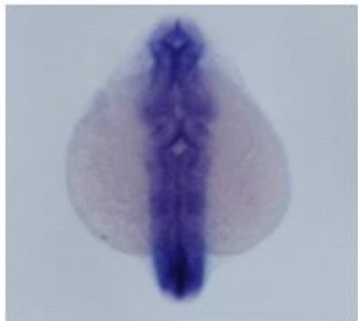
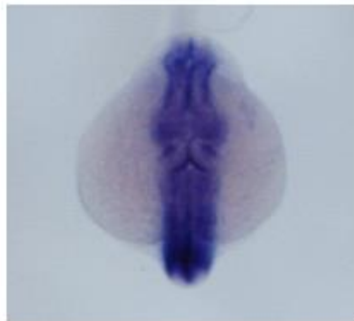
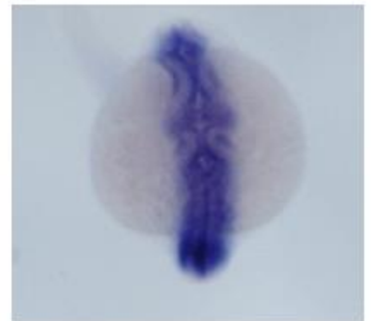

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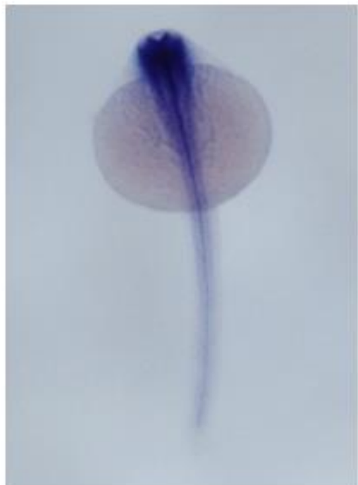
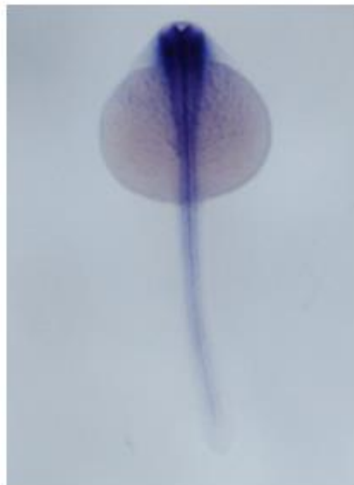
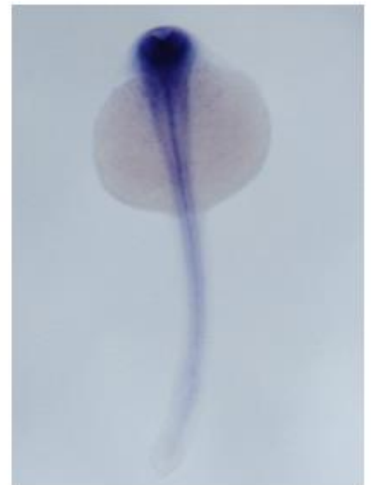

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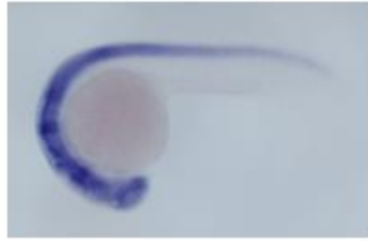
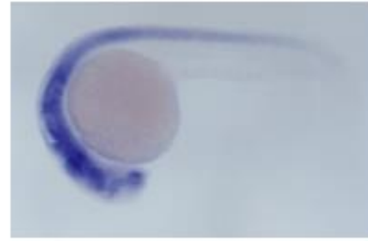

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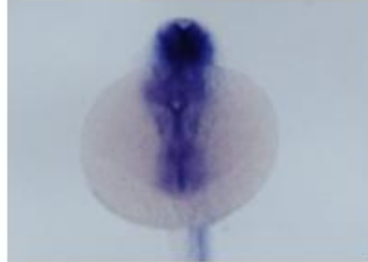
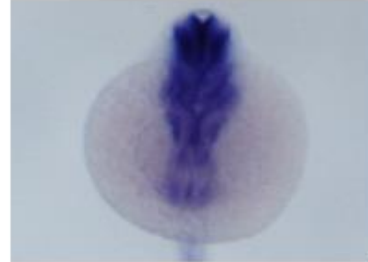

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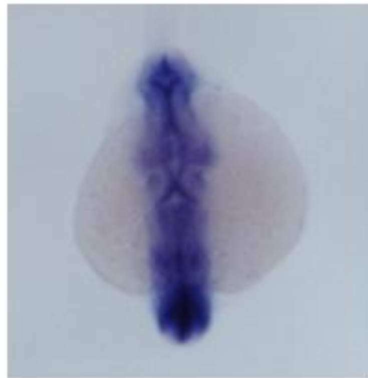
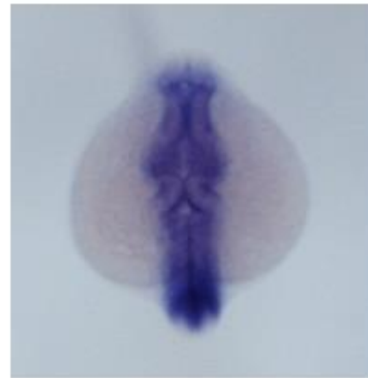

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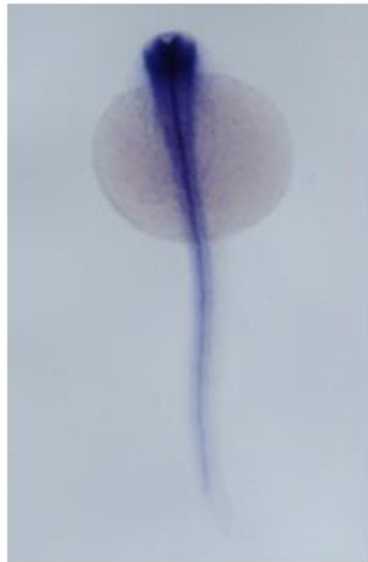
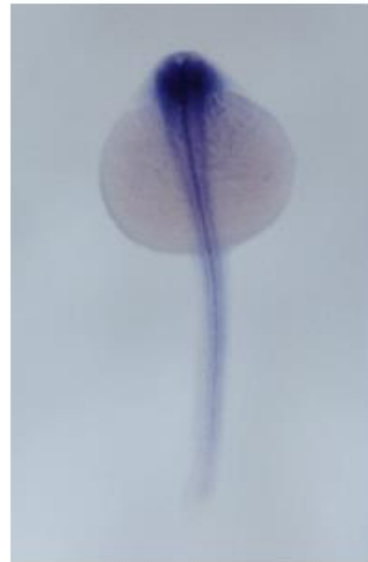
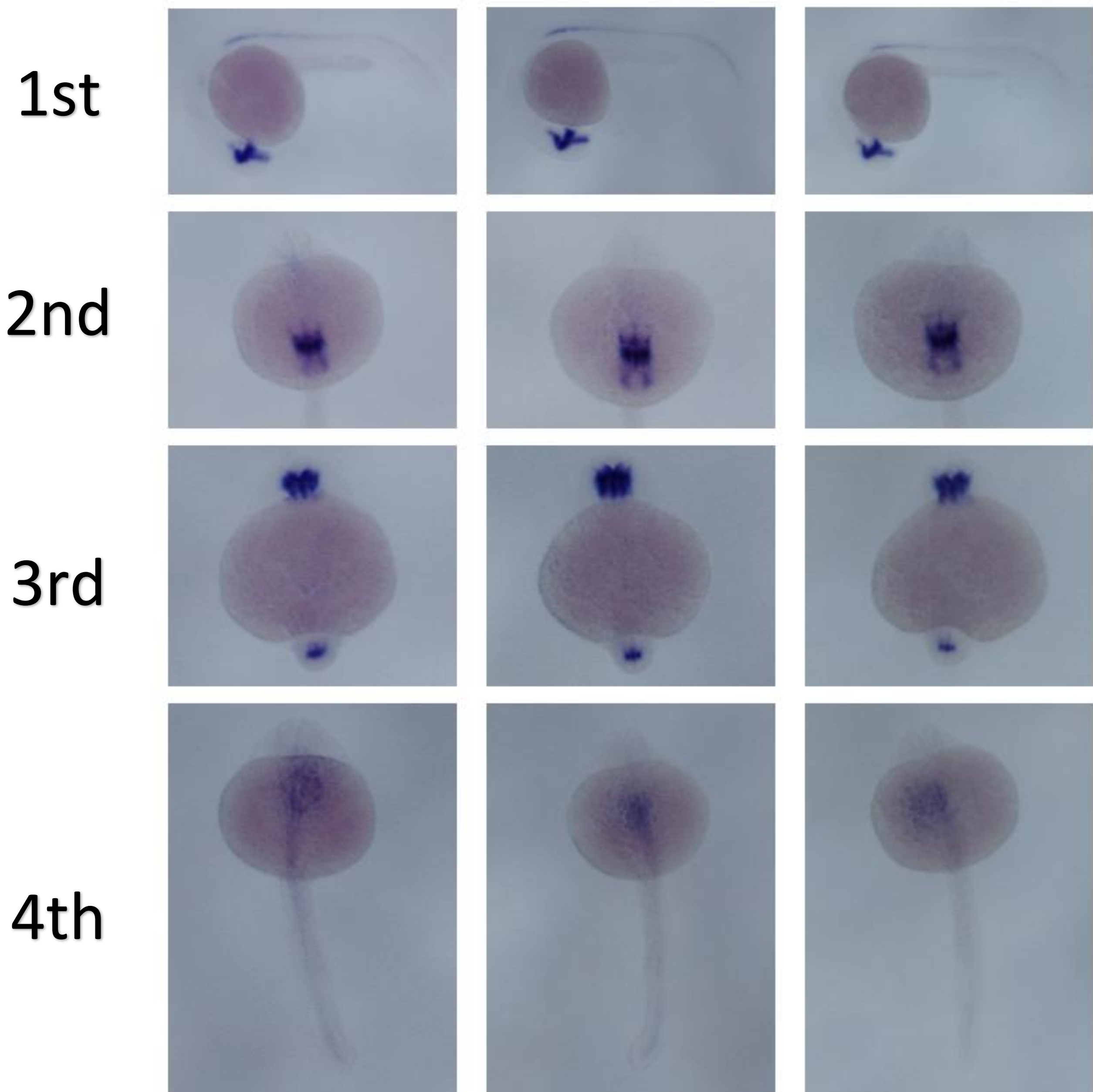



4) Olig2 Hybridisation Results at 24 hpf

First Lane: Lateral View, Second Lane: Frontal View, Third Lane: Anterior View, Fourth Lane: Dorsal View. 3/19 embryos obtained from *in situ* hybridisation were genotyped by sequencing, chosen as possible candidates for three different genotypes. Here, images of the remaining not-sequenced 6 embryos presented. Remaining 10 embryos not shown since their expression were indistinguishable from the ones shown in the Appendix.



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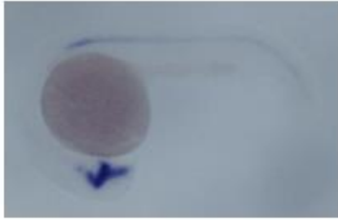
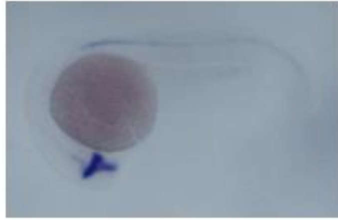
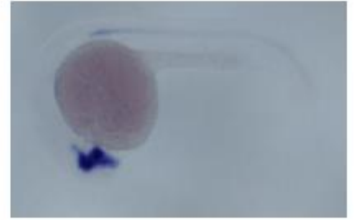

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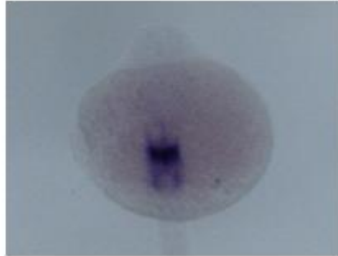
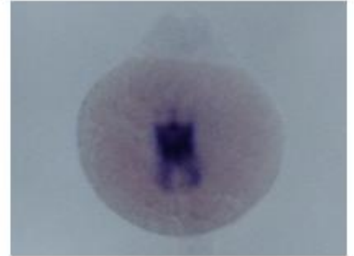

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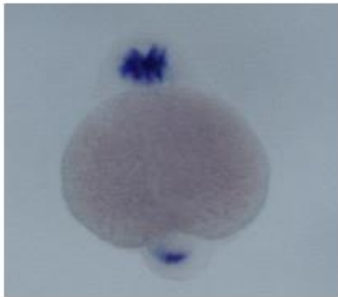
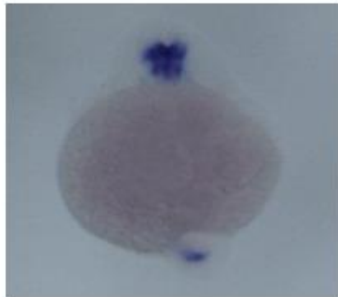
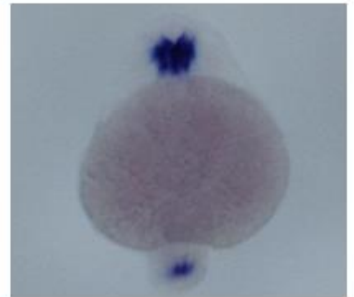
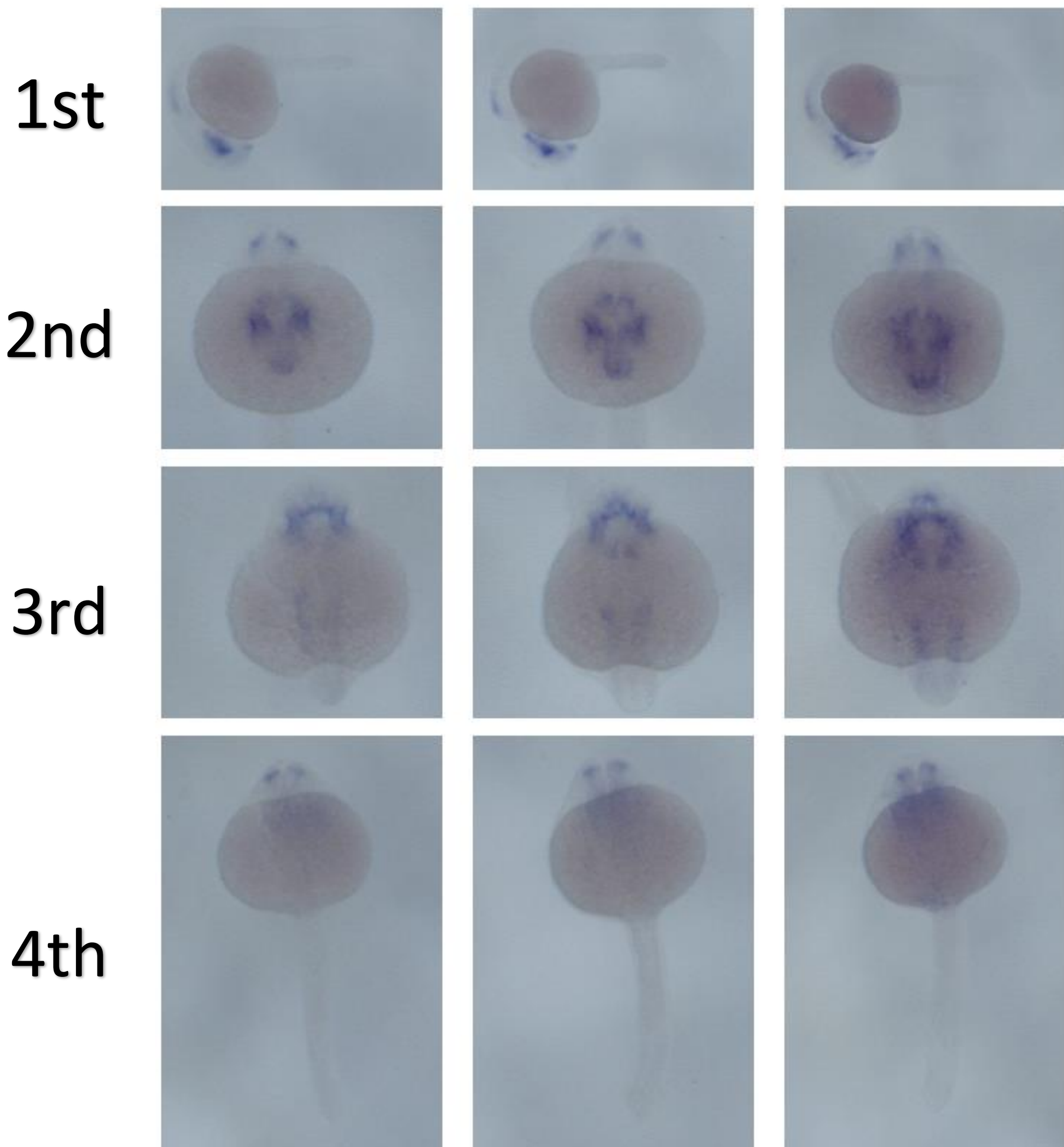




4th

5) Ascl1a Hybridisation Results at 24 hpf

First Lane: Lateral View, Second Lane: Frontal View, Third Lane: Anterior View, Fourth Lane: Dorsal View. 6/18 embryos obtained from *in situ* hybridisation were genotyped by sequencing, chosen as possible candidates for three different genotypes. Here, images of the remaining not-sequenced 12 embryos presented.



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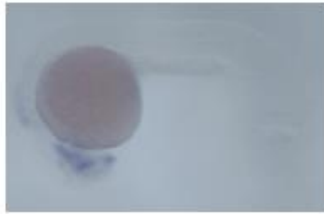
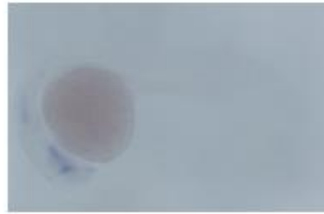
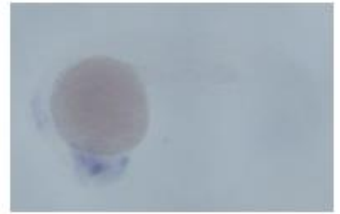

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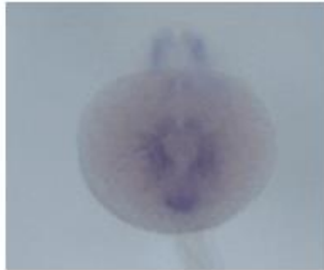
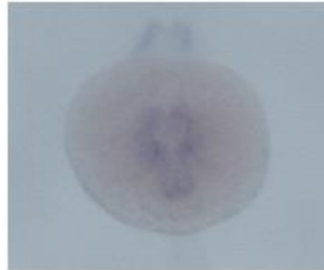
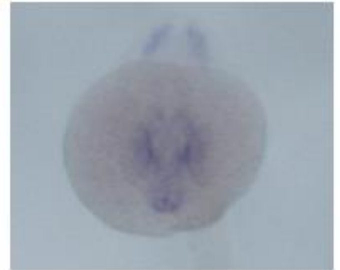

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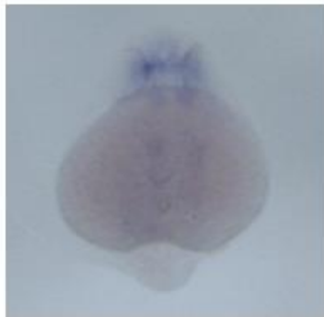
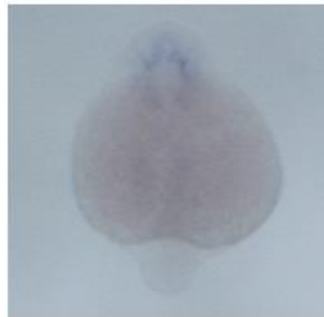
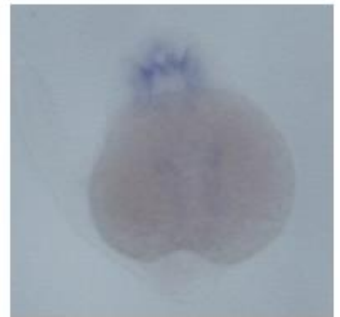

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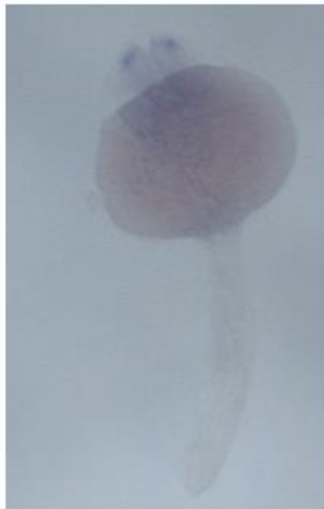

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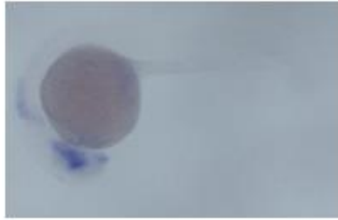
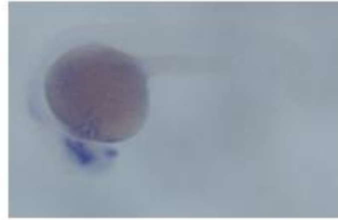
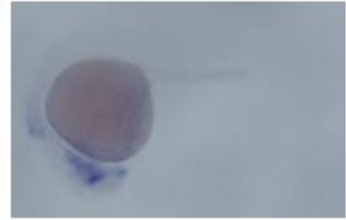

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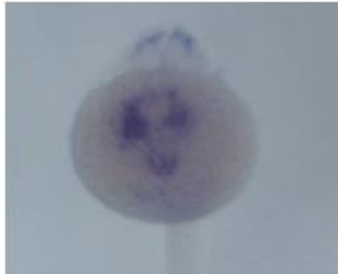
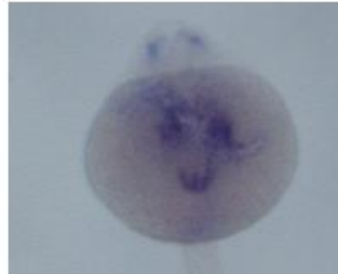
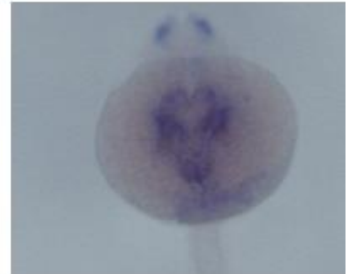

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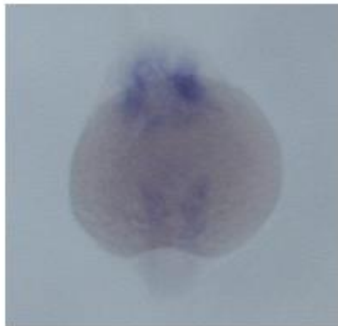
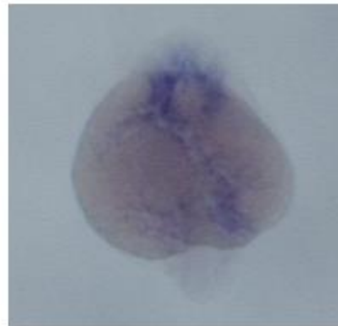
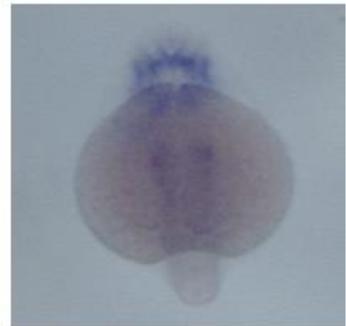
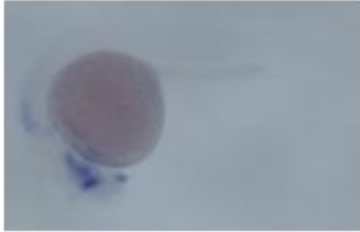
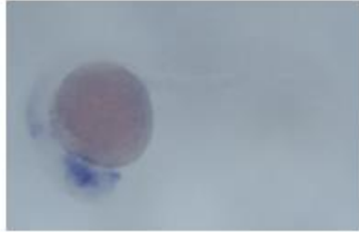
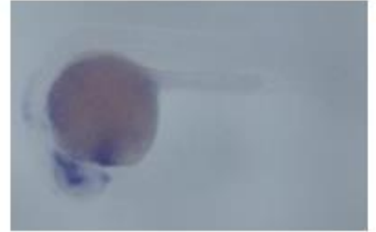
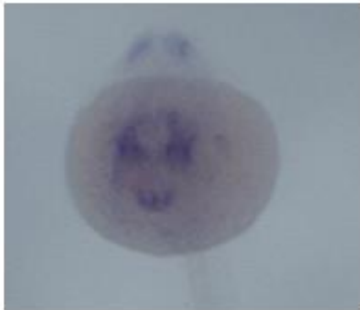
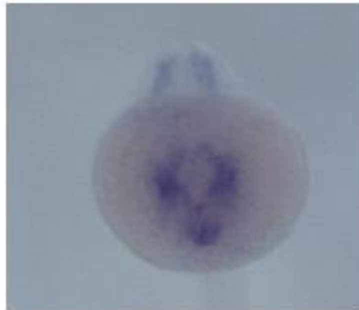




4th

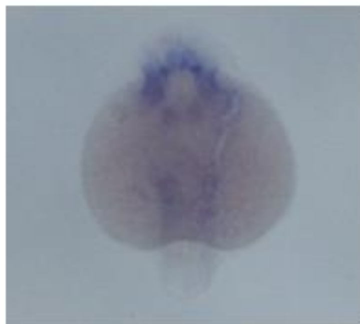
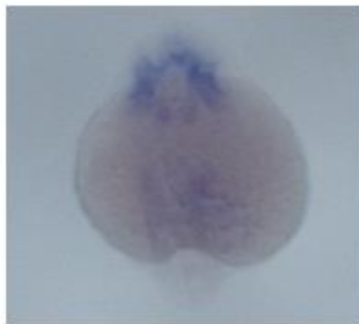
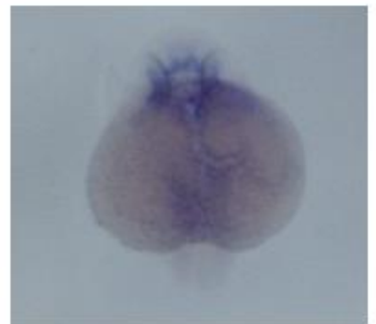
1st

2nd

3rd

4th

Sequencing Data from wild type, heterozygous mutant and homozygous mutant fish: The purple frames indicate the sequence difference where TALEN induced 8 bp deletion starts.