ID4 KNOCKDOWN DURING ZEBRAFISH DEVELOPMENT REVEALED ITS FUNCTIONAL ROLE IN NEURAL STEM CELL SURVIVAL

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Abstract

Id4 (Inhibitor of DNA binding 4 / Inhibitor of Differentiation 4) is one of the four members of Id protein family that antagonise the function of basic helix-loop-helix (bHLH) transcriptional regulators. In the mouse it has been shown that Id4 plays an important role in the timing of neural stem and progenitor cell differentiation and knockout mice exhibited premature neural stem cell differentiation resulting in significantly smaller brains. To further establish the molecular mechanism underlying Id4 function in neural stem cells during the development of the brain using zebrafish as the model organism. Antisense morpholinos that specifically block translation of the Id4 transcripts were employed to knockdown the expression of Id4 during early zebrafish development. Embryos injected with increasing amounts of Id4 morpholinos exhibited a dose-dependent phenotype at 24 hours post fertilisation (hpf) showing severely damaged and malformed brains with no distinct boundaries. Co-injection of Haemagglutinin (HA)-tagged zebrafish Id4 cDNA (containing the morpholino target sequence) did not rescue the Id4 morphants, but co-injection of HA-tagged mouse Id4 mRNA (not containing the morpholino target sequence) did result in a partial rescue of the phenotype indicating specificity of the knockdown. Western-blot analysis revealed that over expressed HA-tagged Id4 protein was abundant at 8 hpf but levels decreased at 18 hpf and at 24 hpf HA-tagged Id4 protein was undetectable suggesting a rapid turnover of the protein. Nevertheless, injection of high amounts of Id4 cDNA also gave rise to a phenotype with embryos exhibiting malformed head, brain and tail. Preliminary Terminal

deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) assays indicated that Id4 knockdown resulted in an increase in apoptosis in the developing nervous system suggesting a role of Id4 in neural stem cell survival.

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Common Abbreviations

A-P – Antero-Posterior.

APS – Ammonium PerSulphate.

bHLH – basic Helix-Loop-Helix.

BMP – Bone Morphogenic Protein.

bp – base pairs.

CNS – Central Nervous System.

DMSO – DiMethylSulfOxide.

DNA – Deoxyribose Nucleic Acid.

DTT – DiThioThreitol.

dUTP – 2'-Deoxyuridine 5'-phosphate.

D-V – Dorso-Ventral.

GRPs – Glial Restricted Progenators.

HA – Haemagglutinin.

hpf- hours post fertilisation.

Id – Inhibitor of DNA binding/ Inhibitor of Differentiation.

Id4 – Inhibitor of DNA binding 4/ Inhibitor of Differentiation 4.

LB medium - Luria Bertani Medium

MOs – Morpholino Oligos.

mRNA- messenger Ribose Nucleic Acid.

MS222 - Ethyl-3-amino benzoate methane sulphonate salt.

$NaCH_3COOAC$ – Sodium Acetate.

NPCs – Neural Precursor Cells.

NRPs – Neural Restricted Progenators.

NSCs – Neural Stem Cells.

PBS - Phasphate Buffered Saline.

PBST – Phasphate Buffered Saline Tween.

PCR – Polymerase Chain Reaction.

PFA - Paraformaldehyde.

RA – Retinoic Acid.

Rb – Retino blastoma.

SDS – Sodium Dodecyl Sulphate.

SDS-PAGE - Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis.

Shh – Sonic Hedgehog.

STAT – Signal Transducers and Activation of Transcription.

TBE – Tris Borate EDTA.

TdT – Terminal deoxynucleotidyl Transferase.

TEMED – N, N, N', N'-Tetra Methyl Ethylenediamine.

TGFβ – Transforming Growth Factor beta.

Tm – melting temperature.

TUNEL –Terminal deoxynucleotidyl Transferse dUTP Nick End Labelling.

Wnt – Wint (wing)

XbaI – Xanthomonas badrii.

XhoI – Xanthomonas holcicola

YSC – Yolk Syncytial Layer.

1. Introduction

1.1. Id proteins: Inhibitor of DNA-binding / Inhibitor of Differentiation

The first Id protein (now referred to as Id1) was isolated by (Benezra *et al.,* (1990). Subsequently, Id2, Id3 and Id4 have been identified in mammals (Benezra *et al.,* 1990; Riechmann *et al.,* 1994; Christy *et al.,* 1991; Sun *et al.,* 1991). Id proteins contain a helix-loop-helix (HLH) domain and as such are members of the large HLH protein family (Massari and Murre. C, 2000). The HLH domain facilitates dimer formation among family members that form either homo- or heterodimers. In addition, most members of the HLH family also contain a basic (b) DNA-binding domain that is absent in Id proteins. Consequently homo- or heterodimers of bHLH proteins can bind DNA and function as transcriptional regulators activating or repressing target genes but Id proteins cannot bind DNA. In addition heterodimers between Id proteins and bHLH proteins are also unable to bind DNA and as such Id proteins antagonise bHLH function. Given that bHLH proteins often promote cell differentiation (Massari and Murre. C, 2000). Id proteins have been referred to as *Inhibitors of DNA binding* or *Inhibitors of differentiation* (Benezra. R *et al.,* 1990).

1.2. Structure of Id genes and proteins

The four different Id proteins are encoded by genes that in humans are located on chromosomes 20q11 (Id1) (Mathaw, S *et al.,* 1995; Nehlin, J. O *et al.,* 1997), 2p25 (Id2) (Mathaw, S *et al.,* 1995), Ip36.1 (Id3) (Ellmeier. W *et al.,* 1992; Deed. R. W *et al.,* 1994) and on chromosome 6p21-22 (Id4) (Pagliuca. A *et al.,* 1995). The genomic organisation of the different Id genes exhibits a similar pattern of exon-intron boundaries and together with their conserved coding regions suggest an evolutionary relationship from a common ancestral Id gene. In the Id1 and Id3 genes, an alternative open reading frame has been identified which generates variant Id proteins with different C-termini by reading the lines of small intron proximal to the 3' end of the coding region resulting in dramatically attenuated heterodimerization properties in variant Id1 and Id3 proteins (Norton *et al.,* 1998).

The Id proteins are small ranging from 13 to 20 KDa. They share the highly conserved HLH domain (Fig. 1.1 - homology box 3; Norton *et al.,* 1998) and except for four smaller homology domains that might be an indication of additional conserved functional determinant (Fig. 1.1 boxes 1, 2, 4 and 5) are largely divergent. The HLH domain consists of two amphipathic α-helices that are separated by an intervening loop. In the heterodimer of predicted Id3-bHLH monomers form four α-helix bundle structures that are stabilized through a combination of electrostatic and polar interactions at the interface of the monomer.

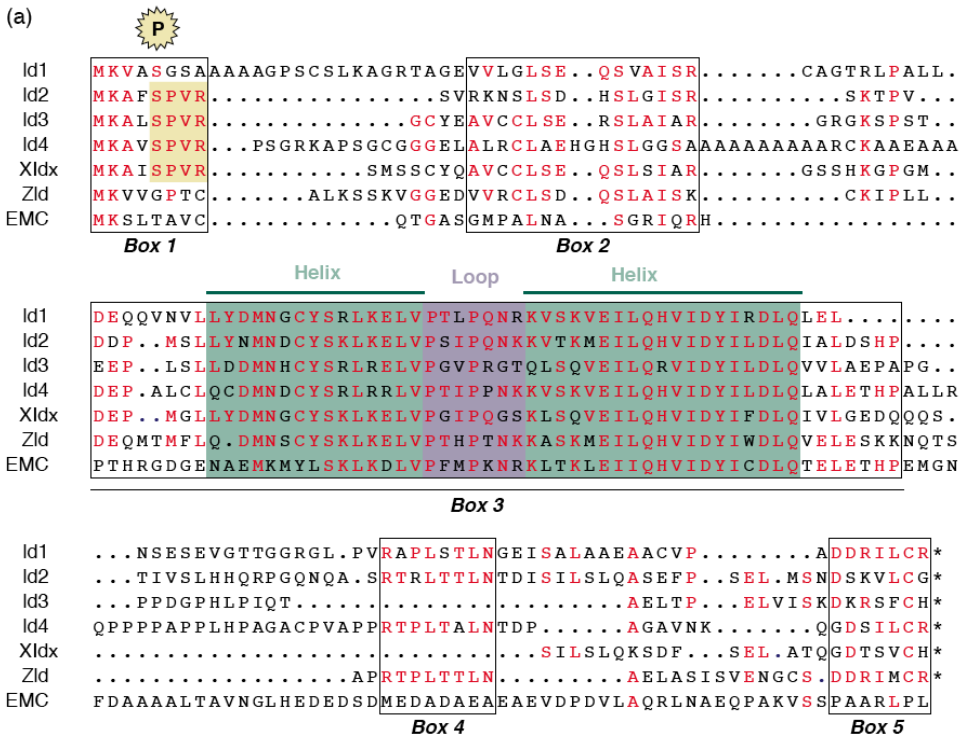


Figure 1.1. Amino acid sequences alignment of Id proteins. The predicted amino acid sequences of mouse Id1, Id2, Id3 and Id4, Xenopus (X1dX), Zebrafish (ZId) and Drosophila (EMC). The Identical amino acids are more than two sequences shown in red and homology region are shown in boxes (1-5). The consensus rite (SPVR) for phosphorylation (P) by cyclin-E/A –CDk2 was shown in yellow within the box1. Stop codons are indicated by *. Taken from Norton *et al.*, (1998).

1.3. Id - bHLH protein interactions

In a variety of cell lineages, the functions of basic helix-loop-helix (bHLH) transcriptional regulators which drive differentiation linked gene expression are opposed by Id proteins. In the absence of Id protein, activation of E-box dependent gene expression and differentiation occurs when bHLH proteins bind to consensus E-box [CANNTG] (Ephrussi *et al.,* 1985) regulatory sequences in a heterodimeric configuration. Typically, heterodimers constitute of class A bHLH proteins that are ubiquitously expressed and class B bHLH proteins that are expressed in a tissue-specific fashion (Table1.1; Norton *et al.,* 1998). Id proteins have been shown to form heterodimers with class A as well as B bHLH proteins preventing bHLH dimmer formation and thus antagonising bHLH function (Littlewood *et al.,* 1995; Benezra, R *et al.,* 1990; Sun *et al.,* 1991).

Class A proteins	E12, E47, E2-5, E2-2, HEB, Daughterless
Class B proteins	Myogenic-MyoD, Myf-5, MRF-4, Myogenin Neurogenic-Neurogenin; NeuroD1, NeuroD2 Neuro D3, NSCL-1, NSCL-2 Mash-1, mash-2 Hes-1, Hes-2, Hes-3, Hes-5 Cardiogenic- eHand, dHand Haematopoietic-SCL, Lyl-1.

Table 2.1. Examples of class-A bHLH proteins and class-B bHLH proteins bHLH proteins of the class A group such as E47 etc. are ubiquitously expressed whereas members of the class B group such as myoD are express in a tissue-specific fashion. (Norton *et al.,* 1998)

1.4. Id gene expression during development

Expression pattern of the Id genes has been extensively studied in the mouse using *in situ* hybridisation (Evans and O' Brien, 1993; Riechmann and Sablitzky, 1995; Zhu *et al.*, 1995; Jen *et al*. 1996, 1997). In general, Id genes are expressed in a variety of organs and tissues during development and their expression patterns are widely overlapping. However, developmental stage-dependent and region-specific expression is also apparent for each of the four Id genes. Id1, Id2 and Id3 are expressed in multiple tissues including the nervous system whereas Id4 appeared to be mainly expressed in the developing nervous system (Riechmann & Sablitzky 1995). In addition Id1, Id2 and Id3 expression in the developing nervous system often overlaps whereas Id4 expression is generally different. For example, in the developing brain, Id3 and Id4 gene expression is reciprocally exclusive (Riechmann and Sablitzky 1995). In the telencephalic vesicle, the transcripts of Id4 are present in the frontal parietal cortex, but Id3 is present only in the hind wall of the telencephalon-which develops into hippocampus.

Also Id genes are expressed in developing neuroblasts representing neural stem cells, where Id1, Id2 and Id3 have similar patterns but Id4 expression is distinct. Remarkably, Id genes are not only expressed in neural stem and progenitor cells but Id2 and Id4 are also expressed in migrating post mitotic neurons in the intermediate zone. In the adult cerebellum, a strong expression of Id2 and Id4 is seen in purkinje cells and the expression of Id2 is observed in neurons of the adult cerebral cortex suggesting a functional

role of these Id proteins other than inhibition of cell differentiation (Riechmann and Sablitzky 1995).

1.4.1. Expression of Id1 in the development of central in nervous system

In the mouse, Id1 is expressed in undifferentiated neural precursors of ventricular zone and in the proliferative neuroepithelial layer of the central nervous system. Id1 mRNA is detected in the neural folds before the closure of neural tube at the embryonic day 8.5 of mouse development (Wang *et al.*, 1992, Jen *et al.,* 1997). Expression of Id1 is seen in the forebrain at the embryonic day 12.5 (E12.5) and is detectable in the ventricular zones of the midbrain, hindbrain and spinal cord (Duncan *et al.,* 1992), but the Id1 mRNA expression is unseen when cells of the ventricular zone stop dividing and undergo migration and differentiation at the embryonic stage of 14.5. The expression is seen along the walls of telencephalon, choroid plexus later at E16.5 in the hippocampus and in some periventricular areas.

1.4.2. Expression of Id2 in the development of central nervous system

Id2 is also expressed in the ventricular zone at the early stages of mouse nervous system development. As the development of nervous system proceeds the expression of Id2 in the presumptive neurons is decreased but is high in the presumptive neurons undergoing maturation (Neuman *et al.*, 1993). Like Id1, the expression of Id2 at E8.5 can be detected in the neural folds (Jen *et al.,* 1997). Id2 shows weak expression in the mantle regions

including the pretectal area, dorsal and ventral thalamus of the diencephalon. In the mid brain, the expression is seen in the alar plate and roof plate. In the hindbrain, expression of Id2 is identifiable in the ventricular zone and the mantle. In the spinal cord, the expression of Id2 is strong in the roof plate and is all over the ventricular zone and the mantle in a dorso-ventral gradient (Zhu *et al.,* 1995). In the cerebellum, the expression of Id2 is seen only in the midline areas of the ventricular zone and is not expressed in the rhombic lip-derived external granule cells (Zhu *et al.,* 1995).

1.4.3. Expression of Id3 in the development of central nervous system

At the embryonic day 8.5 (E8.5), mRNA of Id3 is detectable in the neural folds and in the neural groove (Jen *et al.,* 1997). Id3 is the only Id gene expressed in the floor plate of spinal cord. At the E11.5, the expression of Id3 is seen on the medial slides of the telencephalic vesicles which are located laterally to the 3rd ventricle, where the future hippocampus is derived. The expression pattern of Id3 in the telencephalon is one and the same in both E12.5 and E11.5. Id3 is expressed in the ventricular zone, in the pons and in posterior choroid plexus. The expression of Id3 is also seen in the cells of the tectum and dorsal thalamus (Riechmann and Sablitzky 1995). In the spinal cord, the Id3 continues to express highly at the stage of development and starts decreasing the later stages of development (Jen *et al.,* 1997). At the embryonic day 14.5, the expression of Id3 is seen in

hippocampal region of the forebrain, midbrain and in some areas of the mesencephalon.

1.4.4. Expression of Id4 in the developing nervous system of the mouse

The expression of Id4 is seen in the central nervous system (Riechmann *et al.*, 1994: Riechmann and Sablitzky 1995: Jen *et al.*, 1996. The expression of Id4 is restricted to specific cell types and increases throughout the development of the central nervous system in mouse (Riechmann and Sablitzky 1995). The expression of Id4 is detectable at embryonic day 11.5 all over the telencephalic vesicles (Jen, Manova et al. 1997). Id4 is also expressed in the spinal cord and presumptive motor neurons of the mesencephalon, but a scattered expression is detected in the alar plates of the spinal cord. The expression of Id4 is found in many regions of the brain at E 12.5 including frontal and parietal cortex of the telencephalon, the medulla, cerebellar peduncle, the pyramidal tract, the epithalamus, supra optic, postopic and preoptic areas. The Id4 is also expressed in the sub ventricular zone of the thalamus, post mitotic nuclei in the midbrain and the hindbrain (Riechmann and Sablitzky 1995; Jen, Manova et al. 1997). The expression pattern of Id4 is similar at both E 16.5 and E 14.5 (Jen *et al.*, 1997). At E 17.5, the expression of Id4 is reduced in the telencephalon and the mesencephalon but can be still detectable (Riechmann and Sablitzky 1995). The expression of Id4 is also found during postnatal development of murine brain.

1.4.5. Expression of Id4 in the developing nervous system of zebrafish

The expression of Id4 in the zebrafish during the development of the central nervous system was shown by Thisse *et al.*, (2001) using Id4 markers.

The expression of Id4 in the diencephalon, telencephalon and central nervous system is seen in 14-19 somite stage zebrafish embryos shown in fig. 1.2.1. the expression is also seen in the mid brain, hind brain at 20-25 somite stage the expression is seen in the ventricular zones indicates the importance of Id4 in the neural stem and progenitor cells (fig. 1.2.3).

The expression is limited to the brain indicating the role of Id4 in the development of the central nervous system. The expression pattern is seen in the ganglion cell layer of the retina (fig. 1.2.4).

Expression of Id4 in the Zebrafish during the development of Central Nervous System (Thisse *et al.*, 2001)

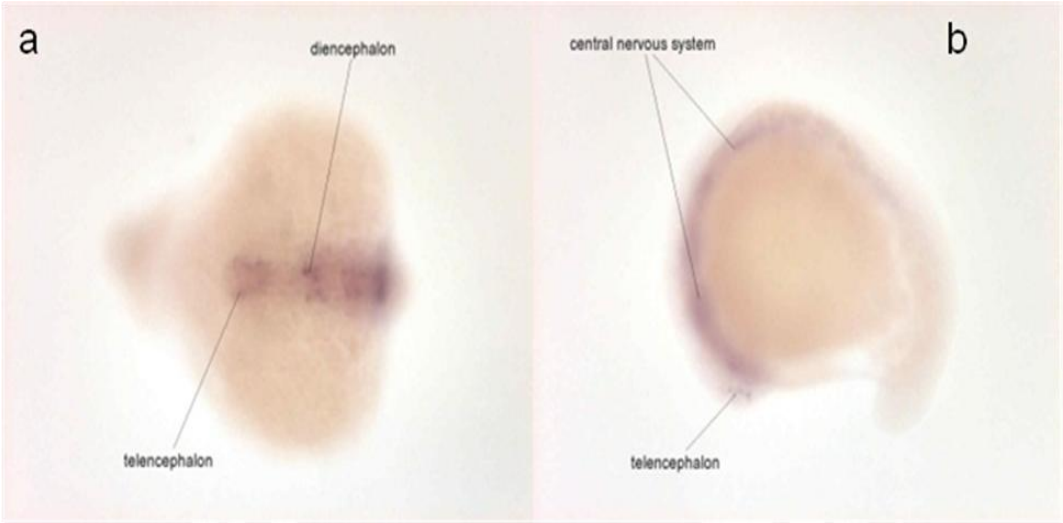


Figure 1.2. *In situ* hybridisation of 14-19 somite staged Zebrafish embryos showing the expression of Id4 in the regions of (a) Diencephalon and Telencephalon (dorsal view). (b) Telencephalon and Central Nervous System (lateral view) (Figures were taken from http://zfin.org) (Thisse *et al.*, 2001).

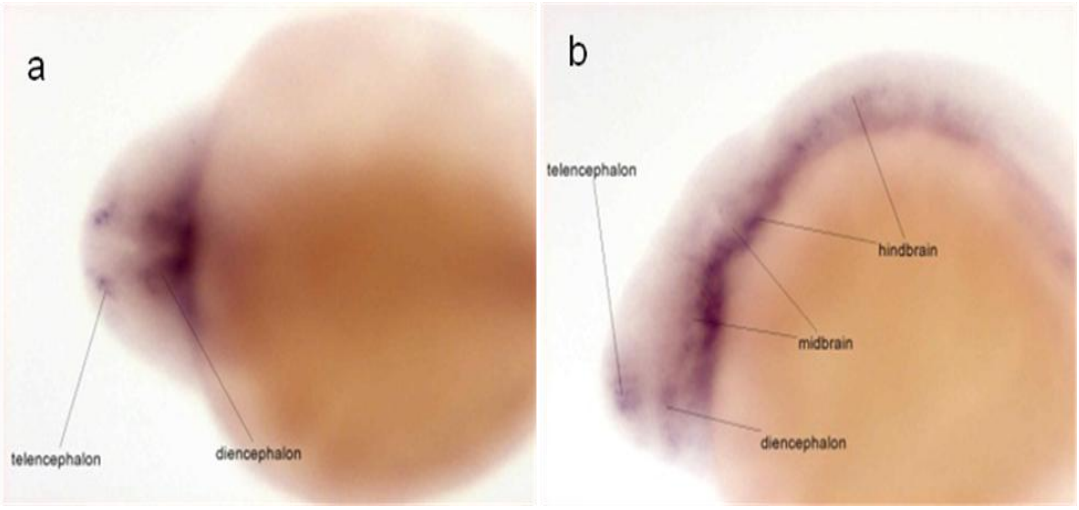


Figure 1.3. *In situ* hybridisation of 20-25 somite staged Zebrafish embryos showing the expression of Id4 in the regions of (a) Diencephalon and Telencephalon (dorsal view). (b) Telencephalon, Diencephalon, Mid brain and Hind brain (lateral view). (Figures were taken from http://zfin.org) (Thisse *et al.,* 2001)

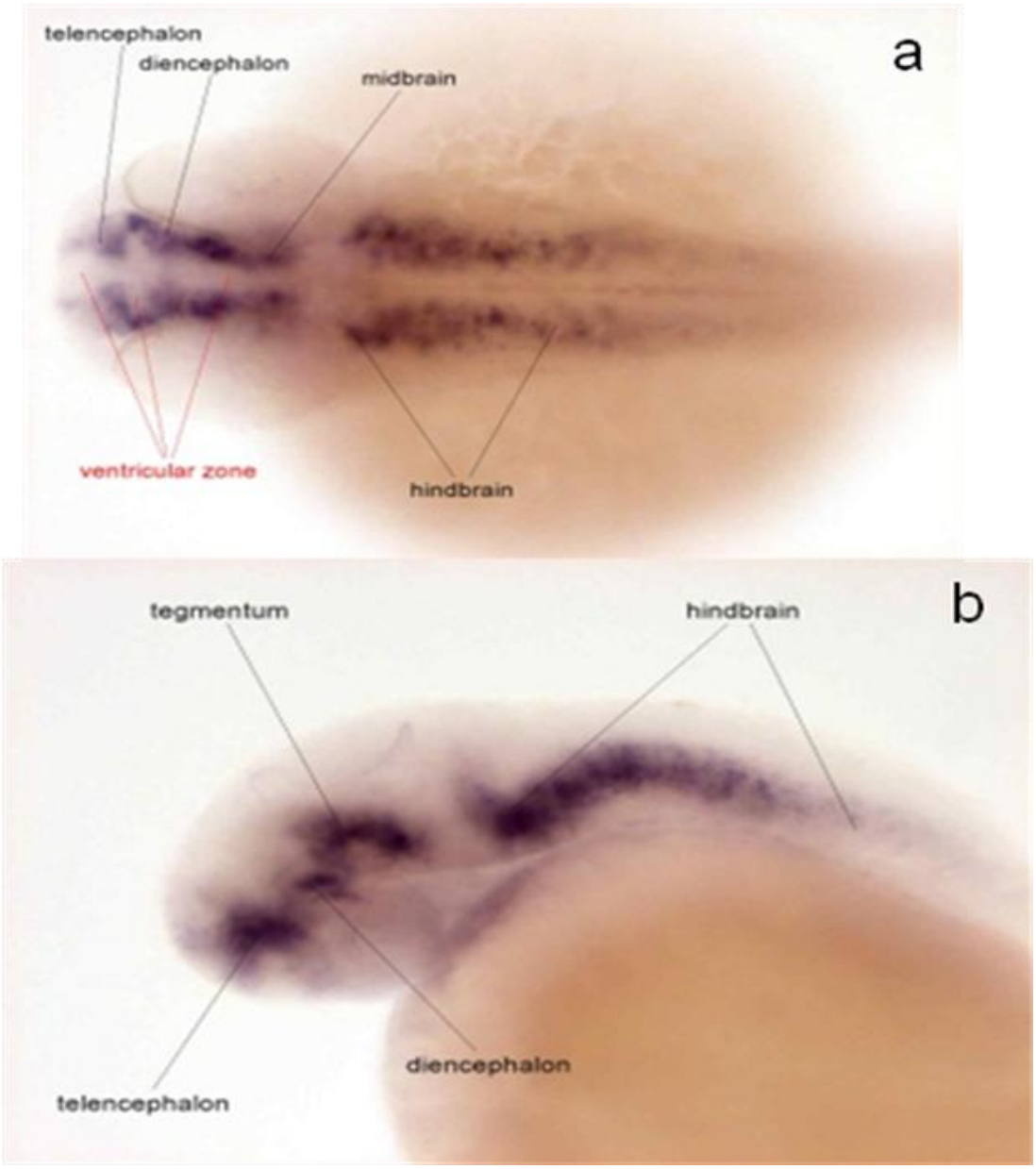


Figure 1.4. ***In situ*** **hybridisation of 15-25 primordial staged Zebrafish embryos** showing the expression of Id4 in the regions of (a) Diencephalon, Telencephalon, Ventricular zones, Mid brain and Hind brain (dorsal view). (b) Tegmentum, Hind brain, Telencephalon and Diencephalon (lateral view) (Figures were taken from http://zfin.org) (Thisse *et al.*, 2001).

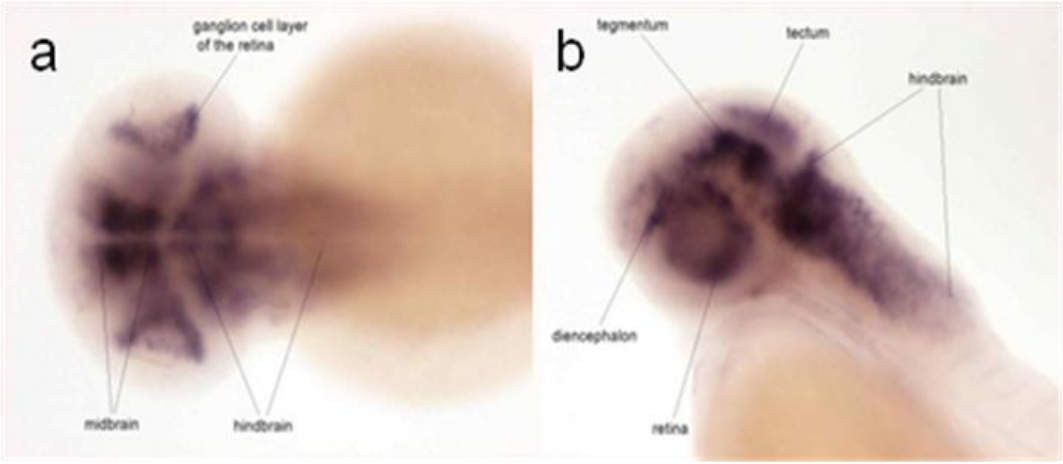


Figure 1.5. *In situ* hybridisation of High pec to Long pec staged Zebrafish embryos showing the expression of Id4 in the regions of (a) Ganglionic cell layer of the Retina, Mid brain and Hind brain (dorsal view). (b) Diencephalon, Retina, tegmentum, tectum and Hind brain (lateral view) (Figures were taken from http://zfin.org) (Thisse *et al.*, 2001).

1.5. Neural Stem Cells (NSCs)

Stem cells are found in most of the multicellular organisms. Stem cells are defined by their properties, the ability to renew themselves through mitotic cell division, and to generate differentiated progeny. The extent of multipotent stem cells existence during the development of the central nervous system is unclear.

During development, neural stem cells divide through mitotic cell division giving rise to all the neurons of the mammalian central nervous system (CNS).

The division of the neural stem cells marks the development of the central nervous system; the neuroepithelial cells undergo symmetric, proliferative divisions generating two daughter stem cells (Rakic, P 1995: McConnell SK 1995). These divisions are followed by asymmetric divisions which generate a daughter stem cell plus a more differentiated cell such as a non-stem cell progenitors or a neuron. The studies suggest that NSCs could give rise to lineage restricted progenitors, neural restricted progenitors (NRPs) and glial restricted progenitors (GRPs). Then neural restricted progenitors differentiated into neuronal cells. Glial restricted progenitors differentiate into glial cells (Rao, 1999). The molecular mechanism of neural cell fate determination is still unclear. It is assumed that intrinsic and external factors may interface to regulate the expression of fate determination genes. When such signaling pathways are altered, the corresponding transcription factors are modified and contribute to NSC fate determination by enhancing the expression of fate determination genes. These signaling pathways and

transcription factors are essential throughtout the course of development. The studies show that the neural associated signaling pathways and transcription factors are closely associated with embryonic development stages (Hirabayashi and Gotoh 2005; Hirsch *et al.*, 2007), with the help of systematic signalling pathways activations and patterns of gene expression, the central nervous system develops into a sophisticated structure with remarkable functions.

During the formation of the central nervous system, neural stem cells undergo many developmental steps, each step is precisely carried out by transcription factors and signalling pathways for the transitions of NSCs to make a distinct cell components in CNS. During this developmental process, the signalling pathways such as Notch, Wnt, Shh (Sonic Hedgehog), TGFβ (Transforming Growth Factor-beta) / BMP (Bone Morphogenic Protein), STAT (Signal Transducers and Activation of Transcription) and RA (Retinoic Acid) act precisely to determine the fates of NSCs through the expression and activity of a panel of transcription factors. Notch signalling plays an important role in NSCs self renewing property because inhibition of Notch signalling pathway stops the self renewal of NSCs. Notch signalling pathways helps in NSCs survival by up regulating the anti apoptotic proteins like Bcl-2 and Mcl-1 (Mason *et al., 2006;* Guentchev and Mc kay, 2006).

1.5.1. Role of basic helix loop helix proteins in Neural Stem Cell maintenance and self renewal

Basic helix loop helix transcription factors play major roles in regulating the maintenance and differentiation of neural stem cells. The bHLH proteins are both activators and repressors of gene transcription (Kageyama *et al.,* 2005). For example, Hairy and enhancer of split genes (Hes) family members such as Hes1, Hes3 and Hes5 are repressor type bHLH genes which are highly expressed in neural stem cells and important for NSC proliferation and maintenance (Hatakeyama *et al*., 2004). Hes1 promotes neural stem cell proliferation by repressing the transcription of a cyclin-dependant kinase inhibitor p27Kip1 (Murata *et al*., 2005). The activator type of bHLH genes helps in promoting the neuronal fate determination and expression of Notch ligands. The Notch ligand such as Delta activates the Notch signalling pathway and up-regulates expression of Hes1 and Hes5 in neighbouring cells, forcing the cell to remain undifferentiated. The activator and repressor type bHLH genes regulate each other to maintain differentiation by allowing only a subset of cells to undergo the process of differentiation. This regulation process is important for central nervous system in maintenance and generation of complex structure, size, shape and arrangement of the cells (Kageyama *et al.,* 2005).

1.6. Functional analysis of Id4

Id4 was first isolated by Riechmann *et al*., (1994). Like the other Id proteins, Id4 forms heterodimers with bHLH proteins by means of HLH dimerisation domain and inhibit their ability to bind DNA (Riechmann & Sablitzky 1995). Id4 has a simple gene structure like other Id proteins. It is located on chomosome 6p21.3-p22 and the gene spans 3.3 kb on plus strand. It has 3 exons of which 2 exons contain the Id4 coding region. The zebrafish Id4 contains 126 amino acids and the molecular weight of protein is 14.44 KDa whereas the molecular weight of mouse Id4 protein is 16.6 KDa and it contains 161 amino acids. The Id4 transcripts were likely to be formed by differential polyadenylation in the 3'-untranslated regions giving rise to four mRNA isoforms. All the transcripts of Id4 contain the same Id4 open reading frame (van Cruchten *et al.*, 1998). The human Id4 promoter contains a functional E box and Sp1 site. The E box is bound by a bHLH zip transcription factor. In band shift, the E box is bound by a bHLH leucine zipper transcription factor called USF1. In reporter assays, bHLH transcription factors such as myoD, E47, E12, E2-2 USF1 (Upstream Stimulating Factor) are efficiently stimulated by the activity of Id4 promoter. Co-expression of Id4 blocks the stimulatory effect of all bHLH transcription factors. It was suggested that the activity of Id4 promoter might be regulated by bHLH transcription factors and such regulation is subject to a negative feedback regulatory loop. (Pagliuca *et al.*, 1998). In band shift assay, downstream of transcription start site a GA motif was bound by the transcription factors Sp1 and Sp3. In reporter assays, the

promoter activity increases when this motif is mutated (Pagliuca *et al.*, 1998). Id4 promoter also contains GA and E box motifs, putative binding sites for SMADs and an Egr1 site and is bound by Egr1 *in vitro*.

1.6.1. Role of Id4 in the central nervous system

The fundamental features of central nervous systems are proliferation, differentiation and programmed cell death of neural cells. Over expression of Id4 leads to apoptosis in astrocytic cells (Jeon *et al., 2008*). Id4 and other Id genes play an important role in control of proliferation and differentiation of neuronal astrocyte and oligodendrocyte progenitors. The ectopic expression of Id4 prevents oligodendrocytic differentiation

In Id4-deficient mice, the histological analysis revealed that the telencephalon was approximately 30% smaller compared to wild type litter mates (Yun *et al.*, 2000); particularly the dorsal, medial pallial regions and the hippocampus appeared to be much smaller approximately 50% than the ventricular regions of cortical and sub cortical tissues (Yun *et al.*, 2000). In adult mice, the brain lacking Id4 was smaller and has enlarged ventricles and a significant decrease in the number of astrocytes. Based on the above observations it was concluded that Id4 is required for the normal development of the brain and for the lateral expansion of the cortical neuro-epithelium (Yun *et al.*, 2000; Bedford *et al.*, 2005).

In the early development of brain investigators found reduced proliferation in the neuroepithelium, but after embryonic day E15.5, the proliferating cells outside the ventricular zone dramatically increased. The dorsomedial

region of the telencephalon is sensitive to both positive and negative activities of HLH protein. *In vivo,* it was shown that bHLH transcription factors function in the telencephalon and suggested a role of Id4 in regulating the development on the telencephalon (Yun *et al.,* 2000). S-phase and M-phase markers show that ectopically positioned cells proliferate and neuroD expression shows that cells differentiate in ectopic positions. In the Id4 deficient cortex, the ectopically positioned proliferating cells are reminiscent of changes as observed in Rb (retinoblastoma) gene lacking animals. Ectopically positioned proliferating cells are found in the cortex of Rb gene lacking animals. Based on the examination of the Rb levels in Id4 animals it was hypothesized that in the cell cycle machinery Id4 may directly interact with other molecules and depending on the context of the cell and interacting partner it either enhances or inhibits the transition of cell cycle (Yun *et al.,* 2000).

Complex molecular interactions that control progenitor proliferation and differentiation are required for the development of central nervous system. It was recognised that Id4 is important for these processes. Id4 plays a role in preventing aberrant cell cycle entry in the neurons undergoing differentiation.

In the mice, the studies using Id4 lacking mice showed that Id4 is required for normal development of the central nervous system and for the correct timing of neural differentiation in the developing forebrain (Bedford *et al.,* 2005). An Id4-deficient mouse has a 20-30% reduction in mitotic neural precursor cells (NPCs) of ventricular zone of the neo cortex, lateral and

medial ganglionic eminences and future hippocampus. Id4 plays an important role in control of Neural Precursor Cells proliferation. Id4 is required for G1-S transition of neuroepithelial cells and such cells lacking Id4 results in reduced brain size due to compromised cell cycle transition of early neural stem cells (Yun *et al., 2004*). *In vitro* analysis of Bedford *et al.,* (2005) showed an impaired proliferation of embryonic and adult Id4 deficient Neural Precursor Cells derived from neurosphere cultures. The above results showed that Id4 is required for normal proliferation of NPCs and expansion in the fore brain development.

In the Id4 deficient mice, there was a significant increase in number of apoptotic cells (Bedford *et al.,* 2005). The failure of self-renewal mechanisms lead to apoptosis during the early expansion phase of Neural Precursor Cells.

1.7. Morpholino-mediated knockdown of gene expression

Morpholinos (MOs) were first introduced in the spring of year 2000 (Heasman *et al.,* 2000) as a means to knockdown gene expression in zebrafish embryos. MOs are single-stranded anti-sense DNA analogues made up of morpholino subunits, each subunit consisting of a nucleic acid base and a morpholino ring. A non-ionic phosphorodiamidate intersubunit substitutes the phospho-diester bond of DNA. These synthetic MOs are very stable allowing gene knockdown in early embryonic development by simple injection of the MOs into 1-8 cell stage embryos. Anti-sense MOs are designed to target either the translational start codon to prevent translation

of the mRNA or to exon-intron boundaries to block pre-mRNA splicing through steric interference (Figure. 1.6).

The translation blocking MOs interfere with the progression of the ribosomal initiation complex, thus inhibiting translation machinery preventing synthesis of protein. Such MO-mediated knockdown of protein expression is very efficient and the target protein is often undetectable in Western blot analysis.

The splice blocking MOs are designed to target intron-exon or exon-intron boundaries of the pre-mRNA sequence thus interfering with splicing of the pre-mRNA through blocking the nucleophilic adenine base and preventing it from forming the splice lariat structure or by interfering with splice regulatory proteins. Steric hindrance of the splicing machinery results in an exon deletion or intron inclusion resulting in protein truncation.

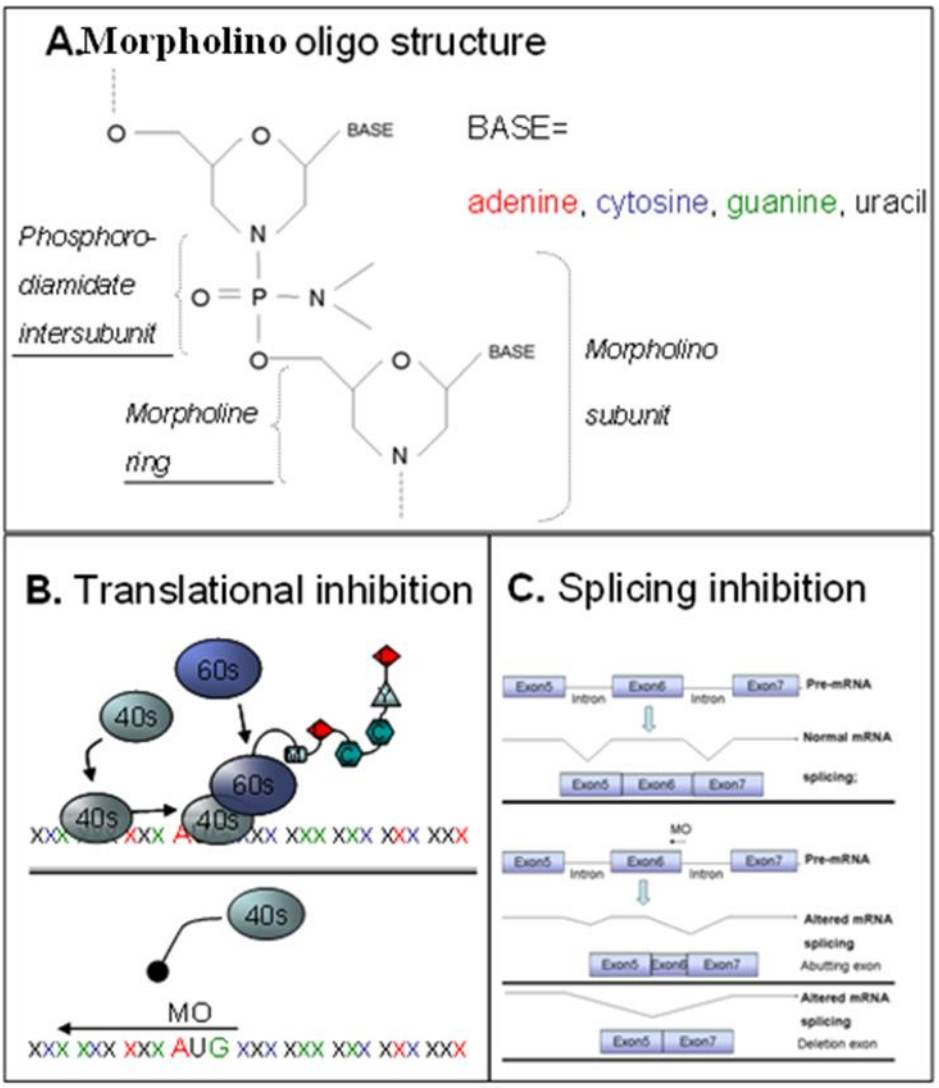


Figure 1.6. Morpholino oligos structure and action mechanism.

(a) Morpholinos oligos are made of a nucleic acid base (either adenine, guanine, cytosine, uracil) and the Morpholine ring is linked by a phosphorodiamidate intersubunit. (b) Translational blocking MOs target the start codon and prevent translation mechanism. (c) Splice blocking MOs interfere with pre-mRNA processing, targeting exon-intron boundaries preventing splicing at the corresponding exon, results in exon deletion or intron insertion, finally resulting in truncated (or) mutated protein. (Figure adopted from Ekker and Larson., 2001; Summerton and Weller., 1997).

1.8. Zebrafish Embryogenesis

1.8.1. Zebrafish as a model organism to study embryogenesis

Zebrafish is commonly used as a model organism to study vertebrate development and gene function. Zebrafish is a teleost fish belongs to cyprinidae family found in the tropical fresh water of southeastern Himalayan region. Zebrafish is scientifically known as *Danio rerio* (Hamilton-Buchanan 1822).

The generation time is short and is 3-4 months. The embryos are transparent and develop fast, are easily manipulated and as such are an ideal model system to study vertebrate development and the molecular mechanisms regulating it (Mayden. R. L *et al.*, 2007).

1.8.2. Zebrafish Development

The following chapters summarises key stages of zebrafish development. The information about zebrafish development was taken from zebrafish: a practical approach Christiane Nüsslein-Volhard, Ralf Dahm, 2000; Kimmel *et al*., 1995; Wilson S.W and Houart C, 2004; Kane and Kimmel, 1989; Kimmel, C.B. 1993).

1.8.2.1. Fertilization

Fertilization is external. The sperms released by the male into the water make their way in order to fuse with the egg through the chorion. The first sperm attaches to microvilli on the egg surface. The microvilli elongate to

create a fertilization cone while the sperms fuses with the cell membrane of the egg and micropyle become plugged, preventing sperm from entering into the cell membrane. In the cortical granule reaction (cortical granules are membrane bound subcellular organelles found in the peripheral cytoplasm of the ripe eggs of many animal species), These granules fuse with the plasma membrane upon activation of the egg rupture and typically releases their contents at the egg surface.

1.8.2.2. Zygotic period (0 hour)

The newly fertilized egg produces the blastodisc at animal pole after undergoing cytoplasmic streaming which separates cytoplasm from yolk.

1.8.2.3. Cleavage period (3/4 -21/4 hours)

The cleavage in the cell begins after 45 minutes. The cells synchronously divide at regular intervals of 15 minutes from one cell stage to two cell stage from two cell stage to four and so on up to 64 cell stage for initial two hours post fertilization. The blastoderm is formed after synchronous process of first six cleavages. In the earlier cleavage period up to 8 cell stage, the cells of the embryo are connected by cytoplasmic bridges so that any substance (ex. morpholinos, mRNA) injected into the yolk will diffuse into all of the cells of the blastoderm. (Fig. 1.7 a).

1.8.2.4. Blastula period (2¼ - 5¼ hours)

The blastula period begins from the 128 cell stage and ends up at 30% epiboly, when gastrulation begins, *i.e.*, from 2¼ - 5¼ hours post fertilisation (hpf, the cells remain synchronously dividing, blastomere is less regular and difficult for staging of embryos, the cell mount giving a greyish appearance. During the blastula period, cell cycle lengthening marks the onset of the mid blastula transition (MBT) (Kane and Kimmel, 1989) at around the 512 cell stage, when the cells lose synchronicity of the division. The zygotic transcription begins at this stage. The yolk syncytial layer (YSL) is formed at the 1000 cell stage, when blastomeres disintegrate and release their cytoplasm and nuclei into the adjacent yolk cell, where the YSL remains throughout embryogenesis. The shape remains spherical but the yolk cell bulges towards the animal pole on the onset of epiboly. The changes in the shape of blastula are the sign of the onset of epiboly appearing a dome like shape characterised by spreading and tuning of blastodisc cell mound and dispersal of the nuclei of the YSL on the yolk cell. Epiboly continues until the end of gastrulation until the entire yolk is covered. (Fig. 1.7 b).

1.8.2.5. Gastrulation Period (5¼– 10 hours)

The process of gastrulation is characterized by the cell movements of involution; it starts at around 50% epiboly, convergence and extension. The thickening of the blastoderm occur giving rise to the germ ring. Gastrulation is visible from the animal pole which is composed of two layers of cells the inner hypoblast and the outer epiblast. The embryonic shield is visible from

animal pole. The antero-posterior (A-P) axis as well as the dorso-ventral (D-V) axis is determined. At this stage, the dorsal blastoderm becomes distinctly thicker and the cells leave from the evacuation zone ventrally by epiboly and dorsally by convergence movements. The visible boundaries in the hypoblast and epiblast indicate the formation of notochord. By 90% epiboly, the brain anlage is formed by the thickening of the dorsal epiblast at the anterior pole. The post mitotic cells are present at this stage forming axial somite-derived muscles, the notochord and specific neurons in the hind brain. At the end of gastrulation stage, i.e., after epiboly is complete, cells in the posterior end forming tail bud contribute to the developing tail. The neural plate is formed by thickening the dorsal ectoderm giving rise to the brain in the anterior part and spinal chord in the posterior part. (Fig. 1.7 c).

1.8.2.6. Segmentation period (10 1/3- 24 hours)

The segmentation period is characterised through formation of somites and the elongation of embryo along the anterior-posterior (A-P) axis. The tail bud develops larger and the development of primary organs is evident. At the end of the segmentation period, the movements in the body of embryos appear for the first time and the cells differentiate. The brain is still unstructured though morphogenesis begins. Somites are mesodermal segments that form every 30 min in bilateral pairs as the embryo extends posteriorly. Somites form the majority of the skeleton, the notochord differentiates and some cells swell to give notochord its structure. At the 5 somite stage, the brain becomes thicker and the optic vessels grow laterally

of the diencephalon. Neural keel forms in the anterior trunk and the appearance of the kupffer's vesicle deep in the tail bud (Fig. 1.7 d). At the 15 somite stage, the four sub divisions of the brain are seen from anterior to posterior. The yolk appears like a kidney bean. At the 20 somite stage, lens placode is visible in the developing eye. In the developing brain all seven rhombomeres, cerebellum and ventricles are recognizable (Fig. 1.7 & 8). The neural tube is formed in the neural rod cavity. The two expansions of diencephalon develop the hypothalamus primordium. The midbrain is horizontally sub divided into a ventral midbrain tegmentum and a dorsal midbrain tectum. The hindbrain neuromeres are prominent and the tail is well extended. The segmentation period ends up with 26 somites stage after 22 hrs of development and the body movements appear for the first time. (Fig. 1.9).

1.8.2.7. Pharyngula period (24-48 hours)

Pharyngula period begins from second day of embryo. The embryo is well developed during his period with central nervous systems shows all the major subdivisions and somitogenesis, another major structure developed during this period is the hatching gland. The Pharyngula period was named after the development of seven pharyngeal arches. The first two arches give rise to the jaw and the other five gives rise to gills. At the prim-5 stage, the pigmentation begins in the cells of the retinal epithelium and the melanophores appear dorsally in the skin. The median fin fold is visible and at 26 hours of period the mesenchyme assemble at the paired locations of

the fin buds. The heart is visible for the first time. Prior to this stage, the heart starts beating with an interrupted rhythm. The functioning of sensors-motor reflexive circuits starts at this period. The red blood cells occupy the yolk ball that indicates the development of cardinal veins and blood circulates soon after the stage. At this prim-15 stage, the yolk ball appears double the size of the head. The pectoral fin buds appears like shallow domes. The lengthening of the tail is slowed down totally. The elimination of the excess cells in the bud after tail somitogenesis by death of cell represents a natural process. (Kimmel *et al.,* 1989). The pigmentation in the retina and skin is darkly visible. Prim-25 is staged at 36 hpf where the head trunk angle is 75 ^{0}C. The heart slightly bends, and the circulating loop connects the caudal artery and moves to end of the tail. Pigmentation in the eye is clear enough to visualize the cell nuclei in the retinal epithelium. Melanophores appear laterally on the trunk and tail. The appearance of reflective pigments cells, iridophores on the eye, although it is difficult to identify at 36 hpf. High-pec stage is named after the appearance of the rudiments of the pectoral fins. The dorsal strips of melanophores are clearly filled to the end of the tail. The xanthophores are appeared for the first time in the head in a pale yellow cast. Iridophores are scattered in an unorganised fashion on the eye. The heart bends and shows clear making between atrium and ventricle. (Fig. 1.7 e).

1.8.2.8. Hatching period (48-72 hours)

Hatching period is an asynchronous process and has no effect on the temporal progression of developing animal, since they hatch at different time points. The morphogenesis of organ rudiments are almost completed and their development is slowed down. The main characteristic features of this period are development of the jaw, the gill arches and the pectoral fins. At the day 3, the head looks straight with the trunk and tail. The protruding month is the most important characteristic feature of this stage. By the end of the hatching period the embryonic period in the developing zebrafish is complete and the fish increase in size entering into the early larval period. Where the larva begins to swim actively and make its independent movements of the pectoral fins, eyes, opercular flaps and jaw. (Fig. 1.7 f).

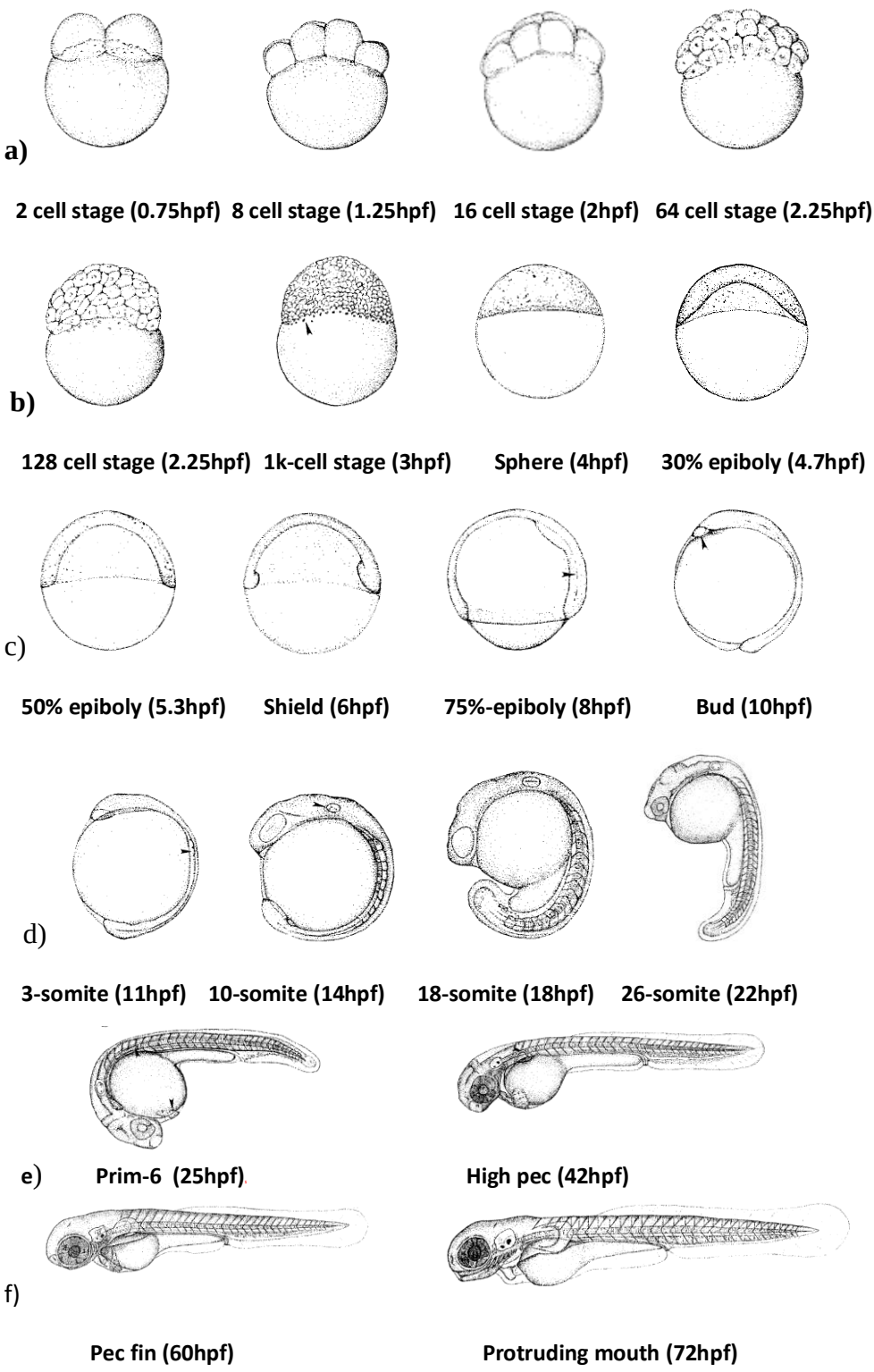


Figure 1.7. Early developmental stages of zebrafish embryo.

The important stages of a) cleavage period, b) blastula period, c) gastrula period, d) segmentation period, e) pharyngula period and f) Hatching period were shown. The arrows indicate the major development of the period. (Adapted from Kimmel *et al*., 1989)

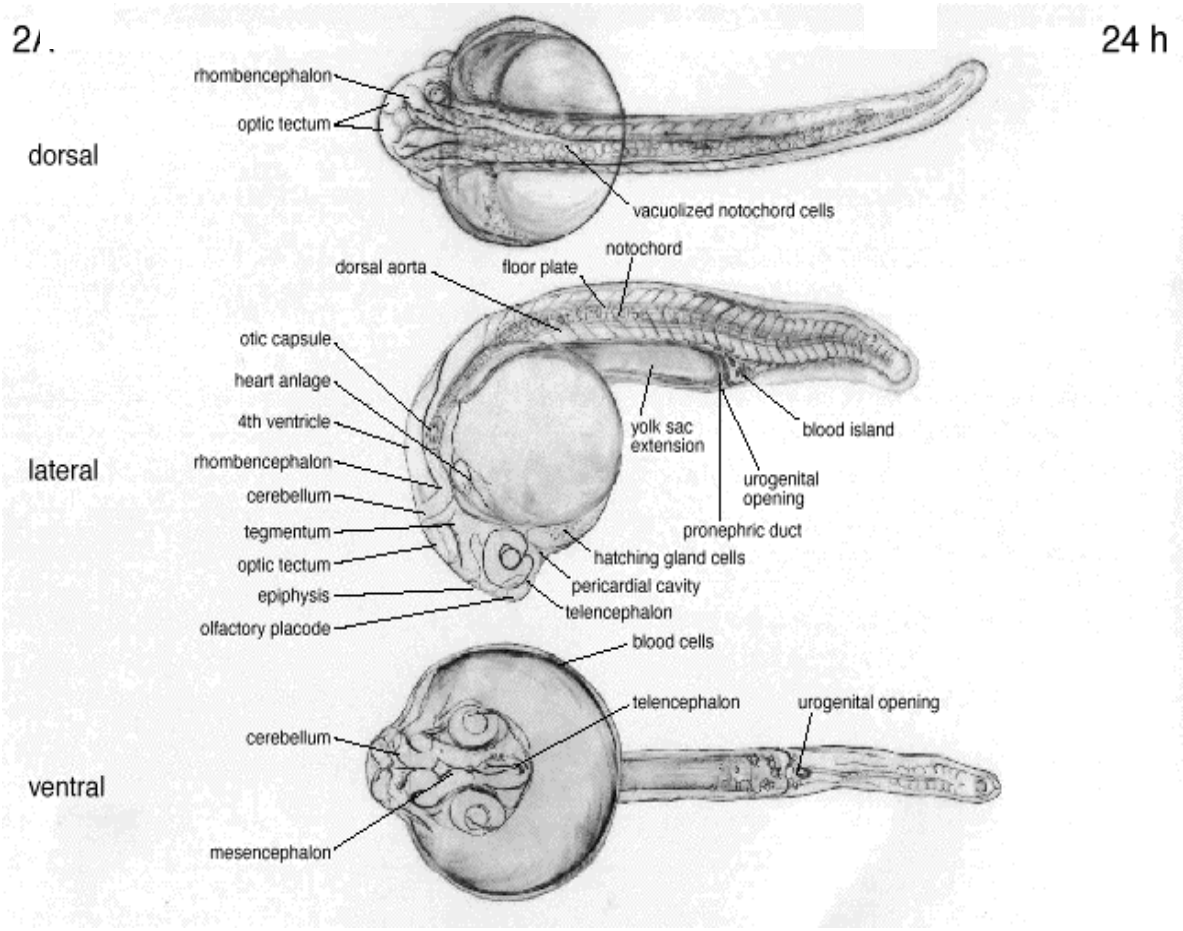


Figure 1.8. The brain morphologies of a zebrafish embryo at 24 hpf.

The dorsal, lateral and ventral view showing all the major rudiments of brain. (Adapted from zebrafish: a practical approach Christiane Nüsslein-Volhard, Ralf Dahm., 2000)

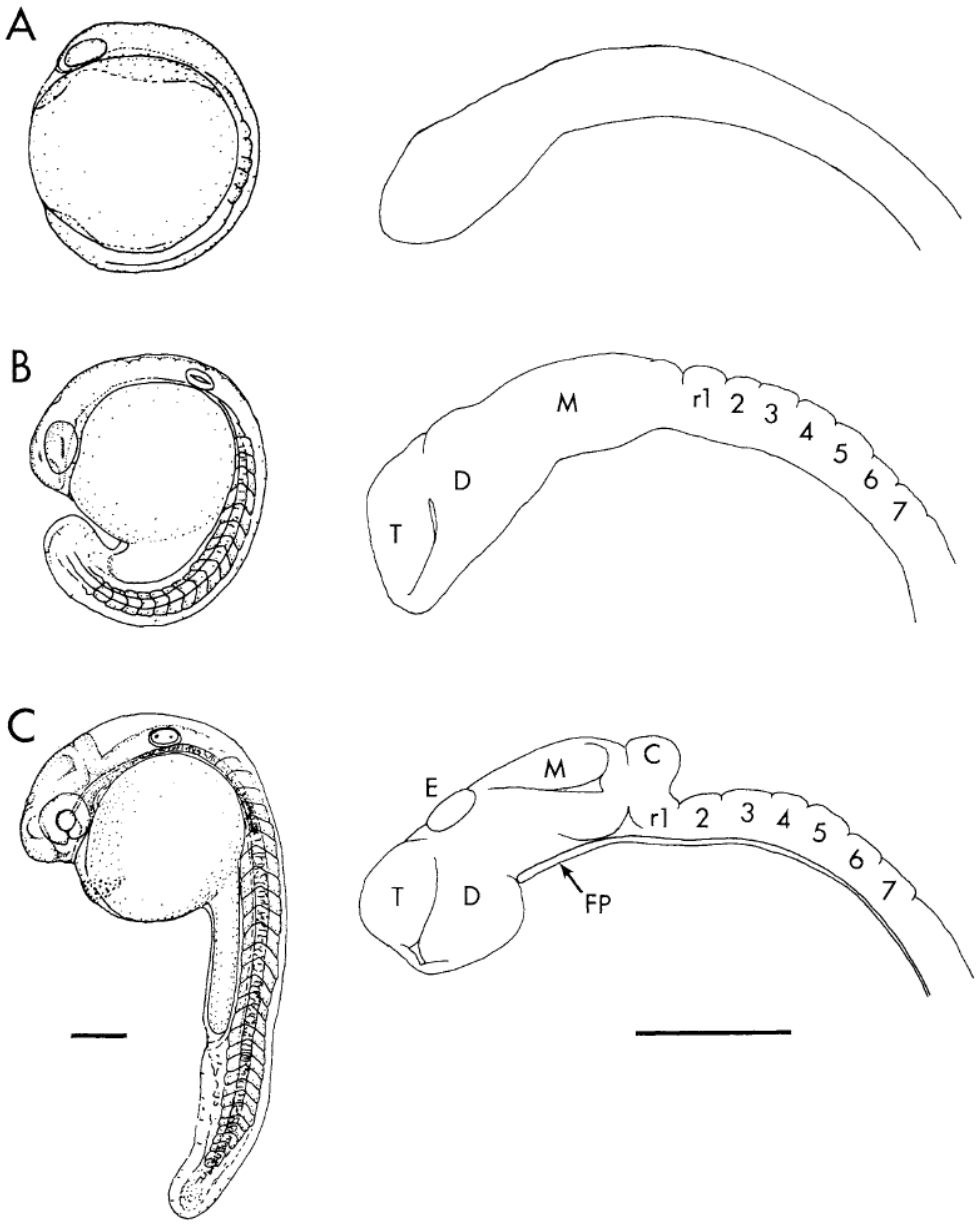


Figure 1.9. Shapes of the brain rudiments during segmentation period.

(a) At the six somite (12 h) stage the morphological subdivisions were not seen. **(b)** At the 18-somite stage (18 h) the telencephalon (T), diencephalon (D), mesencephalon (M) and 7 rhombomeres were seen. **(c)** At the prim-5 (24 h) stage, the brain is marked by clear boundaries, dorsal midbrain, cerebellum (C), floor plate (FP), epiphysis (E) in the midline of diencephalic roof. (Adapted from Kimmel. 1993).

1.9. Aims and Objectives

The aim of the project is to establish the role of Id4 in neural stem cell survival during development using zebrafish as the model organism.

- To study the knockdown effect of Id4 in the zebrafish brain development by employing translation blocking morpholino oligos.
- To validate the knockdown effect of morpholinos, western blotting analysis of Id4 knockdown morphants to be performed by coinjecting with Id4 mRNA and morpholino.
- To study the apoptosis in Id4 knockdown morphants using TUNEL staining assay by *in situ* method establishing the function of Id4 in neural stem and progenitor cells in the developing central nervous system.
- To construct the zebrafish and mouse Id4 clones to allow expression of Id4-HA fusion proteins in zebrafish and to detect the expression of Id4 protein by HA antibody.
- To study the ectopic over expression (gain of function) of zebrafish Id4 taking mouse Id4 as a control using western blotting technique.

2. Material and Methods

2.1. Microbiological techniques

2.1.1. Transformation of competent cells

DH5α competent cells of *Escherichia coli* strain require heat shock to take up exogenous DNA. Competant cells were prepared by growing 5 ml of overnight culture of cells in LB (Luria Bertani) medium and in the morning again diluted into 25-50 ml of fresh LB medium. The diluted culture was grown to an OD_{600} of 0.2-0.5 and the culture tubes were kept on ice. Chilled TSS buffer (10% Polyethylene glycol -5 g, 5% Dimethyl Sulfoxide -2.5 ml, 20mM MgCl2 -1 ml 1M) was added, spun at 3000 rpm for 10 min at 4^0C. The supernatant was discarded and the pellet was resuspended in TSS buffer, then the aliquots of 100 µl were stored at -80 ^{0}C. The competent cells were thawed on ice, and 1 µl (100-500 ng) of plasmid miniprep (or) 5 µl (100-500 ng) of ligation reaction was added to 50 µl of competent cells and mixed gently by tapping without pipetting up and down. The mixture was incubated on ice for approximately 30 min, and then heat shocked at 42 ^{0}C in a water bath for 45 sec and was immediately placed on ice for 2 min. Then 200 µl of LB medium was added to the cells and incubated at 37 ^{0}C shaking at 220 rpm for 60 min. The cells were grown on ampicillin (100 µg/ ml) containing LB agar and incubated at 37 ^{0}C for 12-16 hours.

2.2. Nucleic acid Techniques

2.2.1 Extraction of DNA using Midi prep Kit (Qiagen)

The DNA was extracted using the midi prep kit (Qiagen). The protocols are based on a modified alkaline lysis procedure, binding of plasmid DNA to an anion-exchange resin; impurities are removed by medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and is concentrated and desalted by isopropanol precipitation.

At day I, a single colony was picked and inoculated in 5 ml LB medium containing ampicillin (100 µg/ ml). Then the tubes were kept for incubation at 37 ^{0}C shaking at 300 rpm for 5 hours. Then 50 ml LB medium with ampicillin was taken into conical flask and the culture was added to the flask, incubated at 37 ^{0}C shaking at 300 rpm for 12-16 hrs.

At day II, the culture was transferred into the centrifuge tubes and spun at 6000 rpm for 15 min at 4 ^{0}C, the supernatant was discarded. To the pellet 4 ml buffer P1 (resuspension buffer) (50 mM Tris·Cl, pH 8.0, 10 mM EDTA; 100 µg/ml RNase) was added and vortexed until the pellet was dissolved completely, then 4 ml of buffer P2 (lysis buffer) (200 mM NaOH, 1% SDS (w/v)) was added to lyse the cells, mixed thoroughly by inverting the tube for 4-6 times and incubated for 5 min at room temperature. After incubation 4 ml chilled buffer P3 (neutralization buffer) (3.0 M potassium acetate, pH 5.5) was added and mixed immediately by inverting 4-6 times and incubated on ice for 15 min, spun at 13,000 rpm for 30 min at 4 ^{0}C the Qiagen tip 100 column was equilibrated with 4 ml of buffer QBT (equilibration buffer)

(750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol v/v); 0.15% Triton® X-100 (v/v)), then the supernatant was applied to the column. The Qiagen tip was washed twice with buffer QC (wash buffer) (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)). After washing the eluate was collected into tubes by adding 5 ml of buffer QF (elution buffer) (1.25 M NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol (v/v)), the eluted DNA was precipitated by adding 3.5 ml isopropanol (room temperature), mixed immediately and centrifuged at 6,500 rpm for 1 hour at 4 ^{0}C, the supernatant was discarded carefully. To the pellet 2 ml of 70% ethanol was added and spun at 13,000 rpm for 10 min to remove alcohol-soluble salt. After centrifugation the ethanol was discarded and the pellet was air dried for approximately 10 min. The pellet was resuspended in 200 µl of nuclease free water (from Ambion). The concentration of the DNA was estimated by nano drop in µg/ µl without the use of cuvettes, the sample was placed on the pedestal and closed for analysis. The instrument works on the principle of UV spectrometer, the absorbance was read at 260 nm wavelengths as the DNA absorbs UV light at 260 and 280 nm.

2.2.2 Restriction digestion of DNA

Restriction digestions were performed to excise the desired fragment from the plasmid constructs at the ratio of 1 µg of DNA/ 1 unit of enzyme, as per the manufacturer's instructions using appropriate buffers supplied.

a) Phagemid (gene service) containing the gene ID4 was digested with enzyme XhoI using buffer 4 (NewEngland Biolabs).

b) The pβut-2 plasmid containing HA tag was double digested. Preferably, the plasmid was individually digested with XhoI using buffer 4 and later with XbaI using buffer 4 (NewEngland Biolabs) to ensure complete digestion.

c) The plasmid constructs (Zebrafish and Mouse ID4 gene cloned in pβut-2HA) were initially digested with XhoI using buffer 4 and later with XbaI using buffer 4 (NewEngland Biolabs) to ensure complete digestion.

d) The plasmid constructs (Zebrafish and Mouse ID4 gene cloned in pβut-2HA) were linearized by digesting with an EcoRI enzyme using EcoRI buffer (NewEngland Biolabs).

2.2.3 Agarose Gel Electrophoresis

The agarose gel electrophoresis was run to estimate the size of DNA samples and to validate the restriction digestion. 5X DNA loading dye was added to DNA samples (1 µg) to make the final concentration of 1X. The 1 kb DNA ladder, restriction digested samples and undigested samples were run on a 1% agarose gel. The 1% agarose gel was prepared by dissolving l g of agarose in 100 ml Tris Borate EDTA (TBE) buffer. 5 µl of Ethidium Bromide (0.5 µg/ ml) was added to 1% agarose in order to visualize the bands under UV light using transilluminator and the photos were taken.

2.2.4 Primers

The detailed descriptions of purpose of designing of primers are discussed in section 3.1. The forward primers were designed with an XbaI restriction enzyme site and the reverse primers were designed with an XhoI restriction enzyme site. Both the enzymes produce DNA fragments with cohesive ends. The primers were added with few extra bases at the restriction sites in order to support the excision at the enzyme sites. Primers were designed using NCBI primer designing tool. Primer oligos were ordered from Ocimum Biosolutions Ltd. Tm (melting temperature) values were suggested by the manufacturer.

Detailed primer report of Zebrafish ID4 and Mouse ID4 genes are given in the table below.

Target template	Primer sequences Forward and Reverse in 5'–3' direction	Position of primers in cDNA (in base pairs)	Expected fragment length after PCR amplification (in base pairs)	Length	Melting temperature (Tm in ^{0}C	GC %
(Danio rerio) Zebrafish Id4 (NM-_001039990.1) (ZGC : 123214)	Forward with XbaI site 5' GCTCTAGACCTACTGG ACTGTGCGTTCA-3' Reverse with XhoI site : 5'-CCGCTCGAGAGCGACA CAAAAAAAGTGAG-3'	 4-23 527-546	 562	28 28	68.0 68.1	53.57 51.72
(Mus musculus) Mouse Id4 (NM_031166.2)	Forward with XbaI site 5'-CCTCTAGACGGA GCTCGCTCTACCG-3' Reverse with XhoI site 5'-CGCCTCGAGAGCGGCA GAGAATGCTGTCA-3'	 75-92 536-555	 498	25 29	69.5 72.3	64 62.1

Table 2.1. Detailed primer report of zebrafish Id4 and mouse Id4 genes to subclone ORF into pβUT-2HA.

2.2.5 Polymerase Chain Reactions

The Polymerase Chain Reactions were carried out to amplify the zebrafish Id4 and mouse Id4 on a Thermal Cycler. A 50 µl reaction mixture was prepared by adding the components in an order. The reaction mixture was made with 30 µl of RNase free water, 10 µl of 5X Phusion HF Buffer (1X), 1 µl of 10mM dNTPs (200 µM each), 3 µl of 1.0 µM concentration of forward and reverse primers, 1 µl of template DNA (zebrafish ID4 of concentration 0.5 µg/ µl and mouse ID4 of concentration 0.88 µg/ µl), 1.5 µl of DMSO (3%) (DiMethylSulfOxide) and lastly 0.5 µl of Phusion DNA Polymerase enzyme (0.02 U/ µl) is added. The enzyme possess 5'-3' DNA polymerase activity and 3'-5' exonuclease activity, it generates blunt ends in the amplification product. The amplification parameters are shown below.

Cycle Step	Temperature in ^{0}C	Time	Cycles
Initial Denaturation	98	1 min	1
Denaturation Annealing Extension	98 60 72	30 sec 30 sec 1 min	30
Final Extension	72	7 min	1
Storage	4	hold	-

Table 2.2. The amplification parameters for the polymerase chain reaction.

The amplified DNA was digested with XhoI and XbaI restriction enzymes and run on a 1% agarose gel. The DNA was purified by gel extraction for the ligation reaction.

2.2.6 Gel extraction

Gel extraction technique was used to purify the DNA from the agarose gel. The QIA quick kit (Qiagen) was used for this purpose. The manufacturer's protocol was followed. The DNA portion of the gel was cut under the low intensity UV light. The portion of gel was weighed in a tube and three volumes of buffer QG were added to one volume of gel. The tube was then incubated in a heat block at 50 ^{0}C for 10min until the gel dissolved completely. The colour of the sample should be same as buffer QG. One gel volume of isopropanol was added to the sample to precipitate the DNA present in the sample. The sample was applied to QIA quick spin column placed in a tube and centrifuged at 13000 rpm for 1 min. The flow was discarded and 0.5 ml of buffer QG was added to QIA quick column and centrifuged at 13000 rpm for 1min to remove the remaining buffer. The QIA quick column was placed into a clean 1.5 ml tube to collect the eluted DNA by adding 30 µl of buffer EB (10 mM Tris, pH 8.5) allowed to stand the column for 1 min and centrifuged at 13000 rpm for 1 min. The concentration of the DNA was estimated by nano drop in µg/ µl without the use of cuvettes, the sample was placed on the pedestal and closed for analysis. The instrument works on the principle of UV spectrometer, the absorbance was read at 260 nm wavelengths as the DNA absorbs UV light at 260 and 280 nm.

2.2.7 Ligation Reaction

Joining of linear DNA fragments with covalent bonds is called "Ligation". The DNA fragments with cohesive ends were produced by digestion with restriction enzymes. A 10 µl reaction was set up using 1 µl of 10X concentration ligation buffer, 1 µl of T_4 ligase enzyme (200U) (NewEngland Biolabs) and an insert vector ratio of 1:1-3.5 was used by taking 1 µl of insert and 1 µl of vector and the reaction volume was made up to 10 µl by adding distilled water and the same reaction was set up for control without T_4 DNA ligase enzyme. The reaction mixture was kept for overnight incubation at room temperature. The ligation reaction mixture was used to transform competent cells and the DNA was extracted using a mini prep kit (Qiagen).

2.2.8 Extraction of DNA using mini prep DNA kit (Qiagen)

At day I, a single colony was picked and used to inoculate 5 ml LB medium, and then the tubes were incubated at 37 ^{0}C shaking for overnight at 300 rpm.

At day II, the culture was transferred into the 1.5 ml tube and centrifuged at 7,000 rpm for 5 min at 4 ^{0}C, the supernatant was discarded and the same was repeated for all the culture. The pellet was resuspended in 250 µl buffer P1 and vortexed to dissolve completely. Then 200 µl of buffer P2 was added and mixed thoroughly by inverting the tube, then 350 µl of buffer N3 was added and mixed thoroughly and centrifuged at 13000 rpm for 10 min. The supernatant was applied to the QIA prep spin column and centrifuged for 60

sec and the flow was discarded. The QIA prep spin column was washed with 0.5 ml buffer PB and centrifuged for 60 sec, again it was washed with 0.75 ml buffer PE and centrifuged for 60 sec, after discarding the flow it is centrifuged for an additional 1 min to remove residual wash buffer. The eluted DNA was collected into a clean 1.5 ml centrifuge tube by adding 30 µl buffer EB and centrifuged at 13000 rpm for 1 min. The concentration of the DNA was estimated by nano drop in µg/ µl.

2.2.9 Gene Sequencing

The sequencing results of plasmid constructs (section 3.1) were obtained from Gene Services. Plasmid constructs and HA primers were sent to Gene Services for sequencing to ensure correct fusion of the reading frames.

a. Zebrafish ID4-HA Sequence

NNNNNNGNNNNTCCGCGTATCTGGACNTCGTATGGATACGCGTAATCTGGAACATCGTATGGATAT
CGGGATCCGTCGACGTCTCGAGAGCGACACAAAAAAAGTGAGTCTTCCTTATTGACACCCGTTGTC
CTCTGCTCTGTGTTGATTTGTGTGAGTGGAGTCCGTGTGGAGGCGGGTGGGCTCGTCTGTTTCAG
GAGCGCAGGGTGCGTTTCCAACGCCAGCTGCAGGTCCAGAATATAGTCAATGACATGCTGGAGGA
TTTCCACTTTACTGACTTTCTTATCCTGCGGAATAGTGGGCACCAAGCGTTTGAGTCGGCTGTAGCA
GTCGTTCATGTCGTACTGCAGACAGAAAAGATCCTCATCTTCCATTTTGCATCGGCTGCTCTCCGAC
AAATAACGCAAGGAGAGCTGACTGCAGCTAGAAGGAAGCTTATGAGGGCGAACCGGCACGCTGGC
CTTCATTGTAATAAATAAAACTACAAAAAGAAAAACGTGAAAAAGGAAAAAAAACACGGACGCAACA
ATACGTTTAGTAAAAAAAAACGTAGTATGGTGCACGAAGAAAAAGAGCGCAGATCTATCCTGACCA
CATCTCCTCTCTGAGTGTGAACGCACAGTCCAGTAGGTCTAGAGTCGATCTGCCAAAGTTGAGCGT
TTATTCTGAGCTTCTGCAAAAAGAACAAGCAAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGC
TTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGGTGCCTAATGAGTGAGCTAACTCACATTAATT
GCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGG
CCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGC
TGCGCTCGGTCGNTCGGCTGCGGCGAGCGGTATCANNTCANTCNAAGGCGGTAATACGGTTNTCC
ACAGAATCAGGGGANACGCNNNAAGANATGTGANCANNNNCAGCAAAGNCCAGGANCNTAAAAGN
NNCNTGCTGGNGTTTTNCNNNGGCTCCNNCNCCCNGACNNNCNTNNNANNCGAANNNNTCAAGTC
NAGNGGGNNNNNNNNNNNNANNNNNNNNCNGNNNNNNNNNNANNNNNNNNNNNNNNNNNNNA
NCCNNNNNNNNNNGNANNACNN

Figure 2.1. Sequence of zebrafish ID4-HA clone. The sequencing primer binds downstream of the reading frame. The sequence is reverse and complementary to the coding sequence. *i.e.*, that of the non-coding strand. (a) HA tag is shown in green. (b) Restriction sites are shown in red. (c) zebrafish ID4 sequence is shown in brown and (d) pβut2 sequence is shown in blue. An unedited sequence read is shown which contains unresolved stretches of nucleotide sequence (shown as Ns).

b. Mouse ID4-HA Sequence

NNNNNNCNNNNNNNNNNNCGNNNCTGGACATCGTATGGATACGCGTAATCTGGAACATCGTATGG
ATATCGGGNTCCGTCGACGTCTCGAGAGCGGCAGAGAATGCTGTCACCCTGCTTGTTCACGGCGC
CGGCCGGGTCAGTGTTGAGCGCGGTGAGTGGGGTCCGCGGCGGCGCGACCGGACAAGCCCCGG
CCGGGTGGAGAGGTGGCGCGGGCGGTGGCGGCTGTCTCAGCAAAGCAGGGTGAGTCTCCAGCG
CCAGCTGCAGGTCCAGGATGTAGTCGATAACGTGCTGCAGGATCTCCACTTTGCTGACTTTCTTGT
TGGGCGGGATGGTAGGCACGAGCCTCCGCAGGCGACTGTAGCAGTCGTTCATATCGCACTGCAG
GCACAGCGCCGGCTCATCGGCCGCCGCCTCGGCCGCCTTGCAGCGCGCGGCCGCCGCAGCGGC
GGCGGCGGCTGCCGAGCCACCCAGGCTGTGGCCGTGCTCCGCCAGGCAGCGTAGCGCCAGCTC
CCCGCCGCCGCAGCCCGACGGCGCCTTGCGGCCCGAGGGGCGCACCGGGCTCACCGCCTTCAT
CGCGCGCTTCCTGCGCGAGAGGACCGCGACAAGCGGTAGAGCGAGCTCCGTCTAGAGTCGATCT
GCCAAAGTTGAGCGTTTATTCTGAGCTTCTGCAAAAAGAACAAGCAAGCTTTTGTTCCCTTTAGTGA
GGGTTAATTTCGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCA
CAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCT
AACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGC
ATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCG
CTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGT
AATACGGNNTCCACAGAATCNGGGGANACGCAGGAAANNACATGTGANCAAAAGCNGCAAAAGGC
CAGGANCGTAAAAGCNNCNTGCTGGCNTTTTNCANNGNTCGNCCCCCNGACNANCATNNNNAAAN
NGANNNCTNANNNNNNNGNNNNNNNANNNNNNNNNNNNANNNNNNNNNNNNNCCCNNNNNNNNC
NNNGNNNNGNCNTNNNNCNNNNNNNNNNAACCNNNGNNNN

Figure 2.2. Sequence of mouse ID4-HA clone. The sequencing primer binds downstream of the reading frame. The sequence is reverse and complementary to the coding sequence. *i.e.*, that of the non-coding strand. (a) HA tag is shown in green. (b) Restriction sites are shown in red. (c) mouse ID4 sequence is shown in brown and (d) pβut2 sequence is shown in blue. An unedited sequence read is shown which contains unresolved stretches of nucleotide sequence (shown as Ns),

2.2.10 Production of mRNA

The *in vitro* production of mRNA involves linearization of the vector, phenol-chloroform extraction and transcription reaction. 10 µg of DNA template was used for the reaction. The work area was cleaned with RNase zap (from Ambion) to maintain a RNase free environment.

a) Linearization of vector.

To linearize the vector the template DNA was digested with an enzyme EcoRI (New England Biolabs) using EcoRI buffer and then incubated at 37 ^{0}C overnight. A total reaction mixture of 80 µl volume was made. The digested DNA is analyzed by running 1 µl of mixture using undigested DNA template as control.

b) Phenol-chloroform extraction

Phenol chloroform extraction was performed to clean the DNA template. In the first step, 40 µl of phenol chloroform was added to the 80 µl reaction mixture and vortexed for 30 sec. The mixture was centrifuged at 13,000 rpm for 3min and the bottom layer was removed using filter tip. The process was repeated to remove the protein left below.

In the second step, 40 µl of chloroform was added to the mixture and vortexed for 30 sec. The mixture was centrifuged at 14,000 rpm for 3 min and the bottom layer containing protein was removed using filter tips and the second step was repeated again. In the third step, 8 µl i.e., 1/10th volume of Sodium Acetate ($NaCH_3COO$) of 3M concentration wih pH 5.2 and 200 µl i.e., 2 1/2 th volume of ethyl alcohol was added and was kept in -20 ^{0}C freezer for 30 min and the mixture was spun at 13000 rpm for 30 min at 4

^{0}C. The supernatant was discarded and the pellet was rinsed with 200 µl of 70% ice cold ethyl alcohol and spun at 13000 rpm for 5 min at 4 ^{0}C. The ethyl alcohol was carefully removed and the last bit was centrifuged again and the pellet was air dried for approximately 10min then the pellet was resuspended in 10 µl RNase free dH_20 by heating at 65 ^{0}C and was stored at -20 ^{0}C. To validate the DNA 1 µl of sample is run on 1% agarose gel.

c) Transcription Reaction.

The transcription reaction was carried out using Ambion mMessage mMachine, High yield capped RNA Transcription kit.

To carry out the transcription, reaction mixture of 1 µg of linear template DNA, 10 µl of 2X NTP/CAP, 2 µl 10X reaction buffer, 2 µl of enzyme mix made up to 20 µl with RNase free water. The reaction mixture was incubated at 37 ^{0} C for 2 hours. Then 1 µl (10U) of DNase 1 was added and kept at 37 ^{0}C for 20 min, the reaction was temporarily stopped by placing on ice and 1 µl mixture was analyzed by heating at 65 ^{0}C for 5min. Then the reaction was stopped by adding 115 µl of Nuclease free H_20 and 15 µl ammonium acetate stop solution. The reaction mixture was extracted twice with 75 µl (1/2 the volume of mixture) of phenol/chloroform, vortexed for 30 sec and spun at 13000 rpm for 3 min and the bottom layer was removed. The RNA was precipitated with ½ the volume of isopropanol and kept at -20 ^{0}C for 30mins, and centrifuged at 13,000 rpm for 15 min at 4 ^{0}C. The supernatant was discarded and the dried pellet was resuspended with 30 µl of Nuclease free dH20 (from Kit) and the 3 µl of RNA was analyzed on

1.5% agarose gel, the 3 µl of RNA is aliquoted into tubes and stored at -70 ^{0}C. The concentration of the RNA was estimated by nano drop in µg/ µl.

2.3. Zebrafish Techniques

2.3.1. Collection of embryos

The male and female zebrafish of 8 am or 10 am life cycle were setup in a breeding tank with a divider. In the morning of the next day, the dividers were taken out at 8 am or 10 am. The fish are allowed to mate for 15-20 min. then the embryos of one cell stage were collected and transferred to a petridish with fish water containing methylene blue which prevents fungal growth. The fish were maintained at 28.5 ^{0}C. The embryos were incubated at 28.5 ^{0}C for proper development.

2.3.2. Microinjection

The embryos collected were immobilized by arranging them along a glass slide in a petridish lid. Using a plastic pusher, the embryos were turned so that the animal pole of the embryos was faced towards the injection needle. The needle was filled with 3 µl of zebrafish Id4-HA mRNA (concentrations at 250 pg/nl, 350 pg/nl and 500 pg/nl) or mouse Id4 mRNA (250 pg/nl, 350 pg/nl and 500 pg/nl) or Id4 morpholino (10 ng/nl, 15 ng/nl and 20 ng/nl). The RNase free environment was maintained by cleaning the area with RNase zap. The tip of the needle was broken and the drop size was measured against the scale to inject 1000 pl of sample into the embryos. The

samples were injected into the animal pole of 1-8 cell staged embryos. The injected embryos were incubated at 28.5 ^{0}C. The dead embryos were removed and water from the petridish was changed after 6 or 8 hours to avoid the fungal growth. Morphology of embryos was analysed at 24 hpf using a stereo microscope.

2.3.3. Morpholino Oligos (MOs)

The antisense morpholino oligos (MOs) of 25mers were ordered from Gene Tools which were designed to block the translation of zebrafish Id4 mRNA. The morpholino oligos sequence was written from 5'-3' direction and is complementary to Id4 sequence.

5'- GAA CCG GCA CGC TGG CCT TCA TTG T-3'------MOs

3'-CCG (CTT GGC CGT GCG ACC GGA A**GT A**AC A-5'---
(Sense strand of Id4)

Figure 2.3. Morpholino oligo sequence is complementary to Id4 sequence.

ATG codon is shown in bold and underlined.

The 2.53 mg morpholino oligos (MOs) of 300 nanomoles were diluted with 50.6 µl of distilled water to make the stock concentration of 50 ng/ µl. The diluted sample was heated at 65 ^{0}C for 5 min and was subsequently centrifuged for three times at 13,000 rpm for 1 min each. The 8 µl of morpholinos were aliquoted in tubes and stored at -20 ^{0}C. The desired

concentrations of morpholinos were diluted with distilled water when required for injection.

2.4. Protein Techniques

2.4.1. Extraction of Protein From Zebrafish Embryos

15 embryos of 24 hpf (hours post fertilisation) and 25 embryos of 8 hpf and 18 hpf were collected and the embryos were dechorionated manually after adding 2 drop of MS222 solution (125 mg/L) - an anaesthetic. The embryos were washed with cold PBS and the yolk was removed with deyolking buffer (55 mM NaCl; 1.8 mM KCL; 1.25 mM $NaHCO_3$) (Link, *et al.*, 2006). The buffer was added at 1 µl/ embryo. The embryos were gently pipetted up and down with 200 µl pipette to dissolve the yolk completely and centrifuged at 1,100 rpm for 5 min at room temperature. Then the supernatant was removed as much as possible. The pellet containing the soft tissue was collected (can be stored at -80 ^{0}C if wish to stop the experiment).

The pellet containing the embryos was thawed on ice and SDS sample buffer (0.63M - Tris (pH 8.8), 10% glycerol, 2% SDS, 5mM PMSF (Protease Inhibitor), 0.5mM EDTA) was added at 1 µl/ embryo to disrupt the tissue, 100 mM DTT was added at 1.5 µl/ 100 embryos. The sample was boiled in a beaker containing water for 5 min then the tubes were immediately placed on ice for 5 min. After incubation, the sample was centrifuged at 1,100 rpm for 5 min at 4 ^{0}C. The low speed was maintained to dissolve the pellet in the sample buffer rather than pelleting it down. Then the sample was homogenized with a vortexer to dissolve the tissue

completely. Again the centrifuge tube containing the sample was tightly closed and boiled in a beaker for 5 min, placed on ice for 5 min then centrifuged at 1,100 rpm for 5 min at 4 ^{0}C. The above steps were repeated until complete cell lysis. The sample containing the protein was stored at -80 ^{0}C and the protein sample (15 embryos of 24 hpf, 25 embryos of 8 hpf and 18 hpf) was analysed by running on SDS-PAGE.

2.4.2. SDS-PAGE (Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis)

The protein sample was mixed with gel loading dye of 5X concentration and was boiled in a beaker containing water for 5 min to interact proteins with SDS.

	10% Resolving Gel Composition in ml		Stacking Gel Composition in ml
Dist. Water	4.0	Dist. Water	3.4
30% Acrylamide mix	3.3	30% Acrylamide mix	0.83
1.5 M Tris (pH 8.8)	2.5	1.0 M Tris (pH 6.8)	0.63
10% SDS [(a)]	0.1	10% SDS (a)	0.05
10% APS [(b)]	0.1	10% APS (b)	0.05
TEMED [(c)]	0.004	TEMED (c)	0.005

Table 2.3. Tris/Glycine SDS-PAGE gel composition.

[a]Sodium Dodecyl Sulphate, [b]Ammonium PerSulphate [c]N, N, N', N'- Tetra Methyl Ethylenediamine.

The gel was cast and the samples (15 embryos for 24 hpf , 25 embryos for 8 hpf and 18 hpf) were loaded into the wells along with a prestained standard see blue (R) plus 2 marker (Invitrogen) (Cat. no. LC 5925) to allow visualization of protein molecular weights and evaluation of western transfer efficiency. The wells and the inner compartment of electrophoretic unit were filled with electrode buffer. The appropriate electrodes were plugged in and the gel was run at 35 mA, 100V and the gel was stained with coomassie brilliant blue – protein staining solution (details of composition shown in stock solution table) to ensure the sample contain sufficient amount of protein to be detected by western blot and for the second time the gel was processed further for western blotting without staining.

2.4.3. Western blot

In the first step, the transfer tank was filled with transfer buffer. The nitrocellulose membrane (Amersham) was cut into piece of the size of gel, the two pieces of Whatmann filter paper were also cut to gel size and wet with transfer buffer. The protein in the gel was transferred on to the membrane. The gel was placed between the gel cassettes and inserted into the transfer tank facing the membrane towards anode (+) i.e., facing towards front and the unit was run at 250 mA, 100V for 1 hr 12 min.

In the second step, the membrane was removed and blocked for 2hrs at room temperature or overnight at 4 ^{0}C in a blocking agent [5 ml 5% non fat dry milk (0.1% Tween)]. After blocking the membrane was incubated with appropriate (anti-HA or alpha-tubulin) primary antibody diluted in 5 ml 3% non fat dry milk in PBS for one hour at room temperature or overnight at 4 ^{0}C. The membrane was then washed thrice in PBST (0.1% Tween) for 5 min each on a shaker and incubated with appropriate secondary antibody conjugated with horse radish peroxidase diluted in 5 ml 3% non fat dry milk in PBS for 40 min at room temperature, membrane was then washed thrice in PBST (0.1% Tween) for 5 min each on a shaker. Signals were detected using ECL reagent (Applied Biosystems) was mixed at 1:1 ratio as per manufacturer's instructions. Blots were exposed to x-ray films for 2-5 minutes, depending on signal strength. The technique was used to analyse the knockdown morphants to ensure the knockdown of ID4-HA fusion protein with morpholino oligos.

Primary Antibody	Secondary Antibody
1) Anti-HA (Rat monoclonal antibody) from Calibiochem. Dilution used at 1:3000 1) Anti α-Tubulin (Abcam) Dilution used at 1:750 Polyclonal, produced in Rabbit, concentration 0.200mg/ml, stored at 4 ^{0}C. Catalogue no. ab15246.	1) Anti-Rat (Goat anti-Rat IgG peroxidase conjugate) from (Calibiochem) Dilution used at 1:5000 1) Anti-Rabbit (Dako) Dilution used at 1:10,000 Polyclonal, produced in swine Catalogue no. P0217.

Table 2.4. Details of antibodies used in western blots.

2.5. Apoptosis Assay (TUNEL Staining by *In situ* Method)

Terminal deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) is a common method to detect DNA fragmentation that results from apoptotic signal cascades by labelling the terminal end of nucleic acids. The assay depends on the nicks present in the DNA which can be identified by an enzyme terminal deoxynucleotidyl transferase (TdT) which catalyzes the addition of dUTPs that are secondarily labelled with a tag, here fluorescein, and can also label the cells whose DNA is severely damaged.

During apoptosis the enzyme DNase not only generates double stranded low molecular weight DNA fragments but also introduces strand breaks into the

high molecular weight DNA, these DNA nicks were identified by labelling the free 3'-OH termini with the enzyme TdT ensuing a synthesis of a polydeoxynucleotide polymer. The apoptosis cell appearance is limited to only a few minutes (Russell et.al., 1972; Sanderson, 1976; Matter, 1979; Kerr et. al., 1987).

25 embryos of 24 hpf were dechorionated manually after adding two drops of MS222 solution and dechorionated embryos were washed twice with PBST (0.1% Tween) for 5 min each, and then the embryos were fixed with 4% PFA in PBS for one hour at room temperature. After fixation the embryos were washed twice with PBST (0.1% Tween) for 5 min each and the embryos dehydrated with series washes in MeOH (25% MeOH/ 75% PBST, 50% MeOH/ 50% PBST, 75% MeOH/ 25% PBST and 100% MeOH) and stored at -20 ^{0}C for at least a night (longer storage can actually improve *in situ*).

Embryos were rehydrated with series washes to PBST (0.1 % Tween) (25% PBST/ 75% MeOH, 50% PBST/ 50% MeOH, 75% PBST/ 25% MeOH and 100% PBST), then the embryos were treated with proteinase k (10 µg/nl) diluted in PBS and kept for 20 minutes at room temperature (1 in 1000 dilutions of stock). Half of the embryos were separated and the remaining half were fixed in PFA for 20 min then washed 3 times with PBS 5min each, the PBS was removed as much liquid as possible at the last wash. Half of the wild type embryos were treated with DNase I (1 µl enzyme/ 50µl buffer) at room temperature for 10 min then the embryos were washed 3 times with PBS for 5min each. Then 5 µl of enzyme (mixture containing

TdT and fluorescein-dUTP (Roche) and 45 µl of buffer (Roche) is added to the embryos in dark and kept on ice, incubated for one hour in water bath maintained at 37 ^{0}C while the tubes were covered with an aluminium foil. After incubation the embryos were washed three times with PBS and the care was taken while washing as the embryos bust. After washes the embryos were viewed under Leica fluorescent microscope coupled with a digital camera and photos were taken by adjusting the exposure time to minimum as the stained embryos were tend to lose their fluorescence.

2.6. Stock Solutions

MS222	4 g of MS222 [Ethyl-3-amino benzoate methane sulfonate salt] made up to 1 litre with distilled water; pH 7.0 (stored at 4 ^{o}C)
Paraformaldehyde (PFA) (4%)	4g of paraformaldehyde made up to 100 ml with PBS (stored at 4 ^{o}C)
Phosphate Buffered Saline (PBS)	150 mM phosphate buffer, pH 7.2, 0.85% NaCl
SDS (Sodium Dodecyl Sulphate) 10%	10 g of SDS in 100ml distilled water.
1.5 M Tris (pH 8.8)	18.165 g of Tris in 100 ml dist. H_20; pH 8.8; autoclaved
1.0 M Tris (pH 6.8)	12.11 g of Tris in 100 ml; pH 6.8; autoclaved
APS [Ammonium PerSulphate] 10%	10 g of APS in 100ml of dist. H_20.
Electrode Buffer	0.05 M Tris; 0.192 Glycine; 0.1% SDS; dist. H_20.
Gel loading buffer	5 ml Tris HCL buffer (pH 6.8); 0.5 g sucrose, 0.25 ml Mercaptoethanol; 1 ml Bromophenol Blue (0.5% w/v solution in water); water to 10ml).
Transfer Buffer	7.57 g Tris, 36.02g glycine made up to 1500 ml with dist. water; 500 ml methanol.
PBST (0.1% Tween)	1 ml of Tween20 per litre of PBS.

Agarose (1%)	1 g of agarose in 100 ml TBE buffer; 5 µl Ethidium Bromide.
Agarose (1.5%)	1.5 g of agarose in 100 ml TBE buffer; 5 µl of Ethidium bromide.
Blocking agent for Western blotting	5% non fat dry milk in PBST (0.1% Tween)
Acrylamide solution 30% [Severn Biotech Ltd]	29.2% Acrylamide and 0.8% N, N'–methylene-bis-acrylamide.
Deyolking buffer	55 mM NaCl; 1.8 mM KCL; 1.25 mM $NaHCO_3$.
Protein Stain Solution	0.1 g Coomassie Brilliant Blue R250; 40 ml Methanol; 10 ml acetic acid; 50 ml distilled water
Destainer	40 ml Methanol; 10 ml Acetic acid; 50 ml distilled water
SDS sample buffer	0.63M - Tris (pH 8.8), 10% glycerol, 2% SDS, 5mM PMSF (Protease Inhibitor), 0.5mM EDTA
TBE (Tris Borate EDTA)	53 g of Tris base, 27.5 g of boric acid, 20 ml of 0.5 M EDTA (pH 8.0). TBE was diluted to 0.5X prior to use in electrophoresis.
LB medium - Luria Bertani Medium	Tryptone 10 g, Yeast extract 5 g, NaCl 10 g

Buffer P1 (resuspension buffer) (Qiagen)	50 mM Tris·Cl, pH 8.0, 10 mM EDTA; 100 μg/ml RNase A. storage: 2–8°C, after addition of RNase A
Buffer P2 (lysis buffer) (Qiagen)	200 mM NaOH, 1% SDS (w/v) Storage: 15–25°C
Buffer P3 (neutralization buffer) (Qiagen)	3.0 M potassium acetate, pH 5.5 Storage: 15–25°C or 2–8°C
Buffer QBT (equilibration buffer) (Qiagen)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol v/v); 0.15% Triton® X-100 (v/v); storage: 15–25°C
Buffer QC (wash buffer) (Qiagen)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v), Storage: 15–25°C
Buffer QF (elution buffer) (Qiagen)	1.25 M NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol (v/v) Storage: 15–25°C.
TSS Buffer	10% Polyethylene glycol -5 g, 5% Dimethyl Sulfoxide -2.5 ml, 20mM MgCl2 -1 ml 1M

3. Results

3.1. Tagging of zebrafish and mouse Id4 cDNA clones to allow the expression of Id4-HA fusion proteins in zebrafish.

Given that there are no antibodies available that interact with zebrafish Id4 protein, it was not possible to analyse endogenous Id4 protein expression to ensure that morpholino-mediated knockdown of Id4 was actually happening in the injected zebrafish embryos. In order to verify Id4 knockdown two alternative approaches were chosen: (i) co-injection of zebrafish Id4 cDNA that contained the target sequence of the morpholino and (ii) rescue of the morpholino-mediated phenotype through over expression of mouse Id4 cDNA that did not contain the target sequence due to sufficient nucleotide variation between mouse and zebrafish Id4 (see figure. 3.5). In addition, both cDNAs were tagged at the 3' end with a short sequence encoding a peptide from the haemagglutinin protein (HA-tag) to allow the detection of the fusion proteins after injection of zebrafish or mouse Id4 mRNA using HA-tag-specific antibody and Western-blotting.

3.1.1. Construction of Zebrafish Id4-HA plasmid.

The zebrafish Id4 (*Danio rerio* zgc:123214, http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg) cDNA was obtained from Gene Service (image clone 7403544). The cDNA (~1.2 kb XhoI fragment) had been cloned into the plasmid vector pME18S-FL3 (Figure. 3.1). The plasmid DNA containing the Id4 cDNA was used to amplify the Id4 coding

region excluding the translational stop codon using a forward primer (nucleotides 4 – 23 of the cDNA) containing an XbaI (CTCGAG) restriction site and a reverse primer (nucleotides 527 – 546) containing an XhoI (CTCGAG) restriction site (details of primers in Table. 2.1). The amplified cDNA as well as the target vector pβut-2 HA (provided by Dr M. Gering) was digested with XbaI and XhoI, ligated and recombinant clones isolated (see M&M section. 2.2.2). The nucleotide sequence of the Id4-HA clones was determined (Gene services) to verify in-frame fusion of the cDNA and the HA-tag (see figure. 2.2)

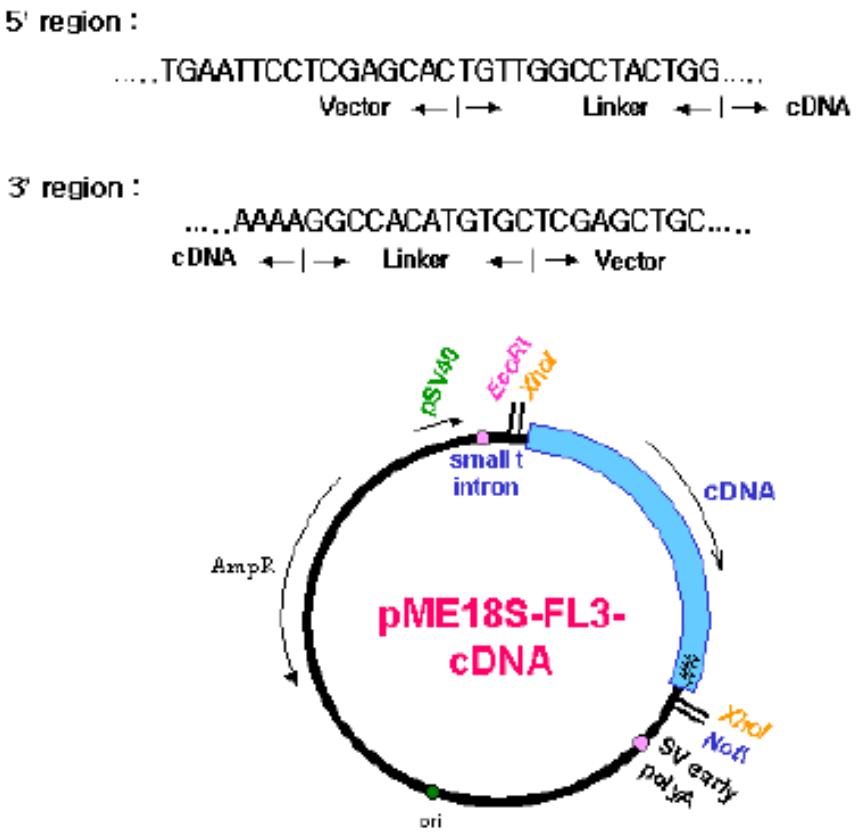


Figure 3.1. Vector map of pME 18S-FL3 with Zebrafish Id4 cDNA. The 5' and 3' regions showing the linker sequences. A ~1.2 kb XhoI fragment containing the zebrafish Id4 cDNA is shown in blue. SV40-derived

promoter, small t intron and poly A signal are also shown. Amp^R: ampicillin resistance gene, ori: origin of réplication. (information provided by Gene Service).

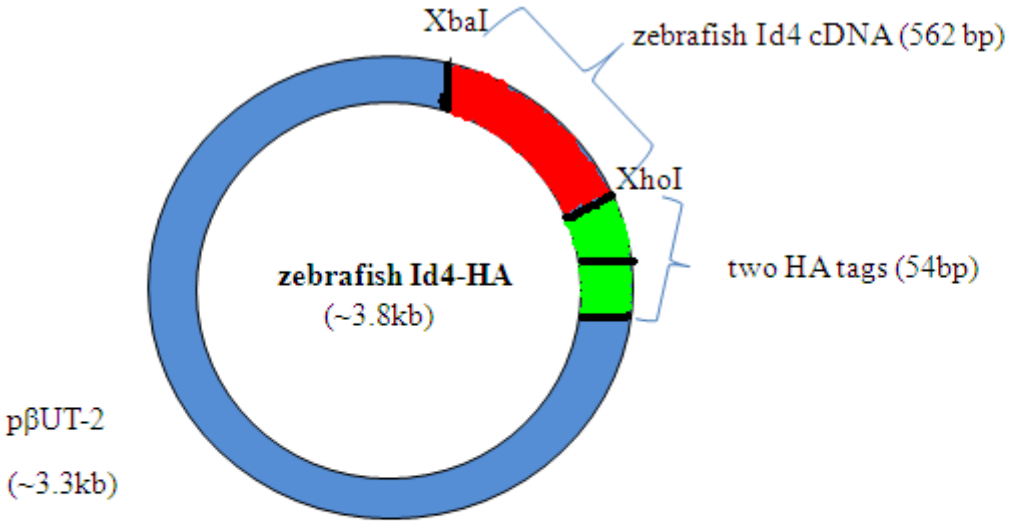


Figure 3.2. A rough vector map of zebrafish Id4-HA. The zebrafish Id4 gene (shown in red) of length 562 bp was cloned in between the XbaI and XhoI restriction sites with two HA (`YPYDVPDYA`) tag sequences (shown in green).

3.1.2. Construction of mouse Id4-HA plasmid.

The mouse Id4 cDNA was amplified from pCI-neo Vector containing a 686 bp XbaI fragment (Figure. 3.2).

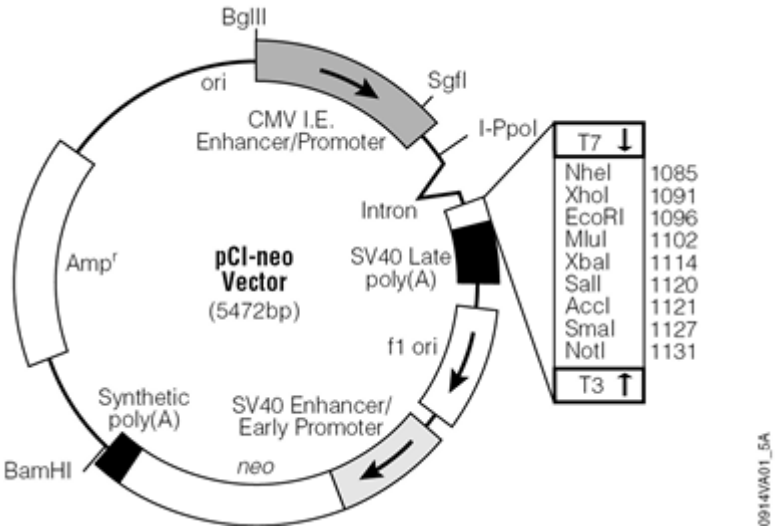


Figure 3.3. Vector map of pCL-neo containing the mouse Id4 cDNA. The mouse Id4 cDNA (686 bp XbaI fragment) had been inserted in the mammalian expression vector pCI-neo (Promega). Neo: neomycin phosphotransferase; CMV I.E: cytomegalovirus immediate-early enhancer promoter, SV40 enhancer/promoter, small t intron and poly A signal are also shown.

The forward (nucleotide sequence 22 – 38 bp of the mouse cDNA) and reverse primers (536 - 555 bp position) were designed with XbaI (TCTAGA) or XhoI (CTCGAG) restriction sites (details of primers in Table 2.1). The Id4 cDNA (22-555 bp region) was amplified by PCR and cloned into pβUT-2 HA vector in the same way as described for the zebrafish Id4 cDNA. Sequence analysis of recombinant clones confirmed in-frame cloning of the Id4 cDNA and the HA-tag (see figure. 2.3)

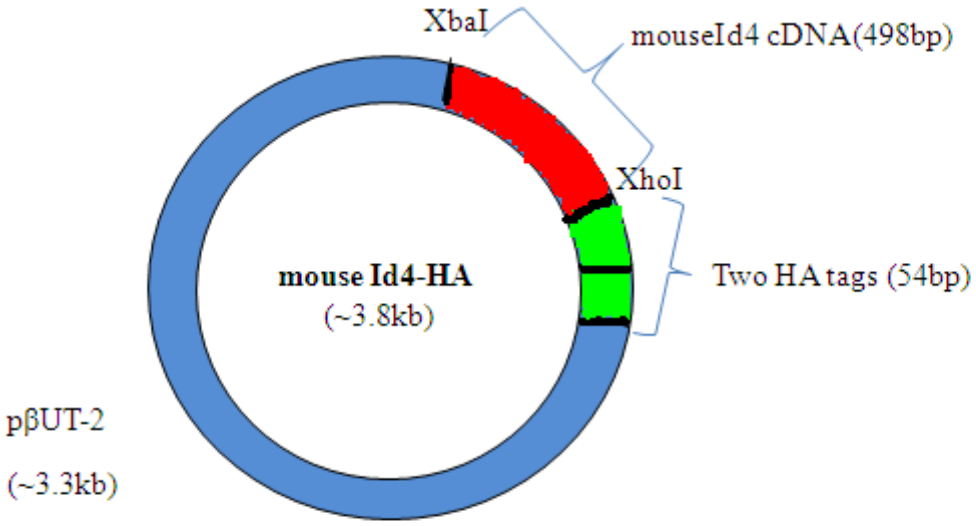


Figure 3.4. An overview vector map of mouse Id4-HA. The mouse Id4 gene (shown in red) of length 498 bp was cloned in between the XbaI and XhoI restriction sites with two HA (YPYDVPDYA) tag sequences (shown in green).

3.2. Morpholino design to knockdown Id4 expression during zebrafish development

To establish the role of Id4 in neural stem cell fate during the zebrafish development antisense morpholinos (MOs) were designed to target the zebrafish Id4 mRNA. Given that the Id4 gene contains only 2 coding exons with the majority of the coding sequence in exon 1, it was not suitable to use splice MOs. Therefore only a translation blocking (AUG) MO was employed.

3.2.1 Morpholino oligos design

A translation blocking MO was designed (Gene Tools) that targeted the AUG start codon of the zebrafish Id4 mRNA (zgc:123214). The antisense MO is specific to zebrafish Id4 but will not interfere with the translation of mouse Id4 mRNA due to sequence variation (Figure 3.5).

```
5′-GAA CCG GCA CGC TGG CCT TCA TTG T-3′—MOs
3′-CTT GGC CGT GCG ACC GGA AGT AAC A-5′-Zf Id4
3′-CGT GGC CCG AGT GGC GGA AGT AGC G-5′-mouse Id4
```

Figure 3.5. Sequence comparison of the AUG MO and the 5' end of zebrafish and mouse Id4 mRNA. Morpholino sequences (25mer) are complementary to zebrafish Id4 but not to mouse Id4. Ten nucleotide bases are common in zebrafish (zf) and mouse Id4. Sequence of the anti-sense MO is shown in 5'-3' direction and zebrafish and mouse sequences are shown in 3'-5' direction.

3.3. MO-mediated knockdown of Id4 expression

The translational blocking MO was injected into the animal pole of the 1-8 cell stage zebrafish embryos. 1 nl of MO solution at concentrations of 2.5 ng/ nl, 5 ng/ nl, 7.5 ng/ nl, 10 ng/ nl, 15 ng/ nl and 20 ng/ nl was injected and the embryos were analysed after 24 hpf. Phenotypic analysis was performed between 24 hpf to 28 hpf and images were taken using bright field stereo microscopy. Initial test experiments revealed that injection of 2.5, 5 or 7.5 ng MO did not result in any obvious morphological phenotype (not shown). In contrast, embryos injected with 10, 15 or 20 ng exhibited a specific phenotype in the developing brain of various severities. To quantify the observed outcome, the severity of the phenotypes was arbitrarily divided into 'normal' (no difference from wild type), 'mild' (little effect in brain morphology), 'moderate' (obvious effect in brain morphology lacking clear brain boundaries) and 'severe' (brain morphology severely disrupted, no distinct boundaries of rhombomeres and malformed or no definite eyes). The latter embryos were not showing proper movement and development was delayed compared to wild type embryos. Representative examples of each category are shown in Figures. 3.7.

Embryos injected with 10 ng MOs exhibited only a mild (~12%) or moderate (~2%) phenotype and more than 80% of the embryos looked normal with distinct boundaries of the brain (Table 3.1. & Figure.3.7). Embryos injected with 15 ng MOs were more severely affected showing all categories of phenotypes from normal (~11%), mild (~16%), moderate (~58%) and severe (~16%) (Table. 3.2 & Figure 3.7). Embryos injected

with 20 ng MOs were most affected the majority of embryos (~63%) exhibiting a severe phenotype with completely malformed brains lacking distinct boundaries (Table. 3.3 & Figure 3.7.) As in the case of 10 and 15 ng, the observed phenotype was restricted to the developing central nervous system most notably the brain and other tissues such as the somites and the tail appeared unaffected. It was noted that with increasing concentration of MO the mortality rate of the injected embryos increased. However, at the time when these experiments were performed the mortality rate of un-injected wild type embryos was also unusually high and therefore dead embryos were excluded from the analysis.

Taken together MO-mediated knockdown of Id4 resulted in a specific, dose-dependent phenotype disrupting neurogenesis in the developing embryos.

10 ng/ nl Morpholino	**No. of Embryos**	**Percentage**
Normal	84	85.71
Mild	12	12.25
Moderate	2	2.05
Severe	0	0

Table 3.1. Percentages of phenotypic morphants injected with 10 ng/ nl Id4 morpholinos

15 ng/ nl Morpholino	**No. of Embryos**	**Percentage**
Normal	10	10.5
Mild	15	15.7
Moderate	55	57.9
Severe	15	15.7

Table 3.2. Percentages of phenotypic morphants injected with 15 ng/ nl Id4 morpholinos

20 ng/ nl Morpholino	No. of Embryos	Percentage
Normal	1	1.2
Mild	6	7.23
Moderate	24	28.9
Severe	52	62.65

Table 3.3. Percentages of phenotypic morphants injected with 20 ng/ nl Id4 morpholinos

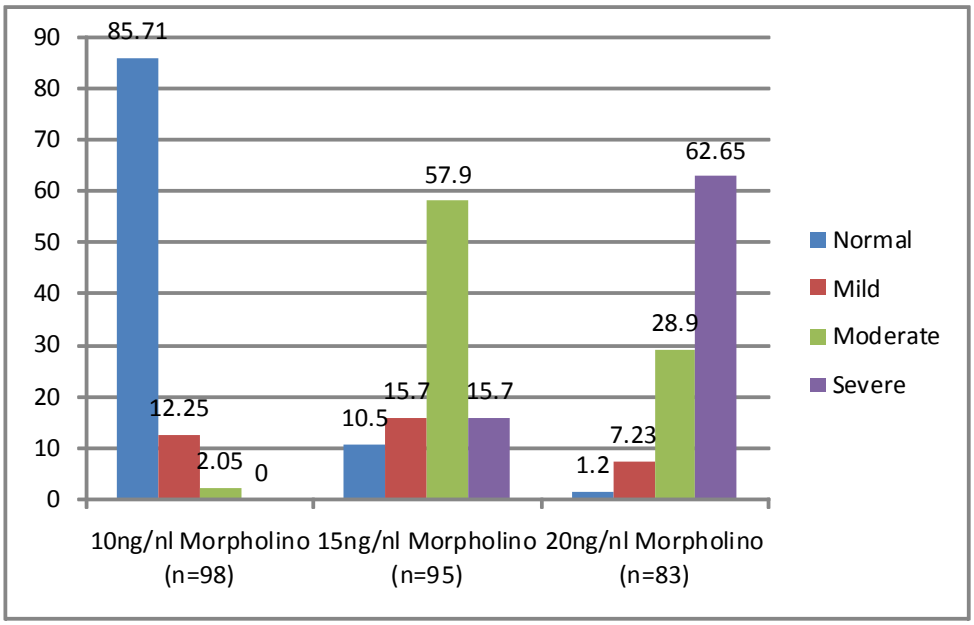
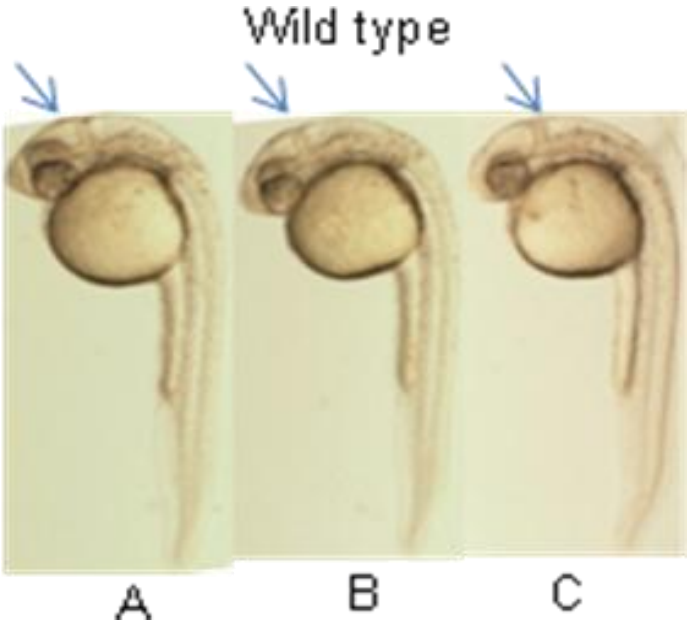


Figure 3.6. Id4 morphants exhibit a dose-dependent increase in the severity of the observed phenotype. Percentage of injected embryos that looked normal (blue), or exhibited a mild (red), moderate (green) or severe (purple) phenotype are shown. The number of embryos analysed for each concentration is given in brackets.


Wild type
A
B
C


10 ng/ nl Morpholinos
D
E
F

Figure 3.7. Effect of Id4 morpholinos in brain of zebrafish embryos observed at 24-28 hpf.

A-C showing wild type embryos with no brain phenotypes, D-F showing mild phenotypes when injected with 10 ng/ nl morpholinos, G-I embryos have moderate phenotypes injected with 15 ng/ nl morpholinos and embryos injected with 20 ng/ nl morpholino have severe phenotypes (J-L). The brain defect is indicated by arrows.

3.4. Ectopic over expression of zebrafish Id4-HA results in severely malformed embryos

1- 8 cell stage embryos were injected with various amounts of zebrafish Id4-HA mRNA (produced by *in vitro* transcription as described in Material & Methods section 2.2.10) and analysed after 24 hpf as described above.

Embryos were injected with 1 nl of zebrafish Id4-HA mRNA at a concentration of either 250, 350 or 500 pg/ nl. As shown in Figure 3.9 over expression of zebrafish Id4 resulted in severely malformed embryos exhibiting phenotypic alterations in head and tail as well as shortened body axis (Figure 3.9). As for the MO-mediated knockdown the severity of the phenotype obtained was quantified in four categories normal, mild (with partial rhombomeric boundaries), moderate (with no distinct rhombomeric boundaries) and severe (complete damage of brain). Injection of 250 pg zebrafish Id4-HA mRNA gave rise to mainly normal (~49%) embryos but ~38% showed a mild, ~9% a moderate and ~5% a severe phenotype (Table 3.4). As expected, increasing amounts of mRNA resulted in a higher percentage of affected embryos as well as an increase in severity (Tables. 3.5). At 500 pg only 10% of the injected embryos looked normal and about 30% exhibited a mild, moderate or severe phenotype (Table. 3.6).

250 pg/ nl zebrafish Id4-HA	No. of Embryos	Percentage
Normal	51	49.04
Mild	39	37.5
Moderate	9	8.65
Severe	5	4.8

Table 3.4. Percentage of phenotypic morphants injected with 250 pg/nl of zebrafish Id4-HA mRNA.

350 pg/ nl zebrafish Id4-HA	No. of Embryos	Percentage
Normal	5	6.41
Mild	45	57.7
Moderate	18	23.07
Severe	5	6.41

Table 3.5. Percentage of phenotypic morphants injected with 350 pg/nl of zebrafish Id4-HA mRNA.

500 pg/ nl zebrafish Id4-HA	No. of Embryos	Percentage
Normal	7	10
Mild	22	31.43
Moderate	19	27.14
Severe	22	31.43

Table 3.6. Percentage of phenotypic morphants injected with 500 pg/nl of zebrafish Id4-HA mRNA.

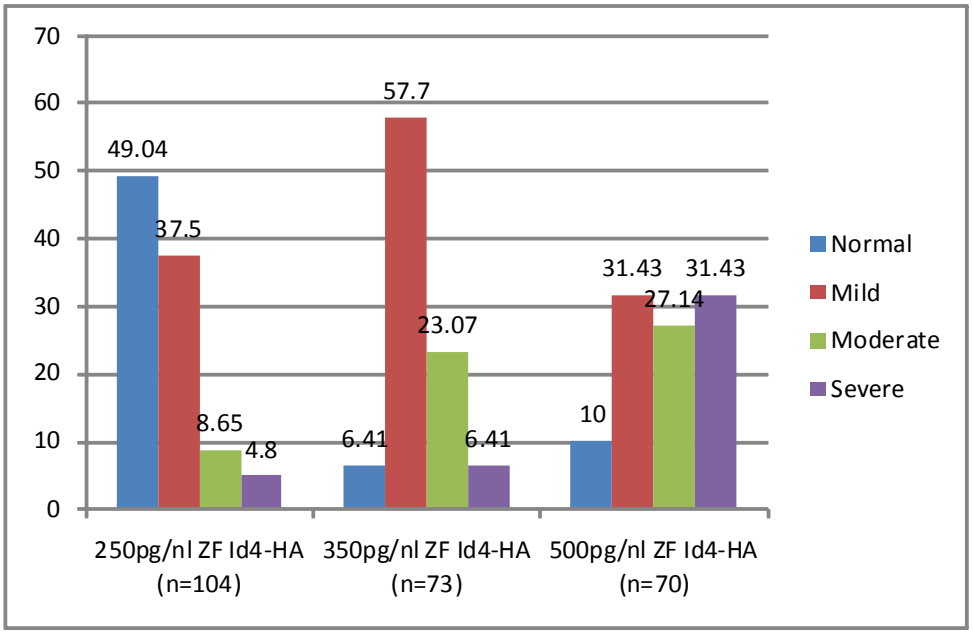
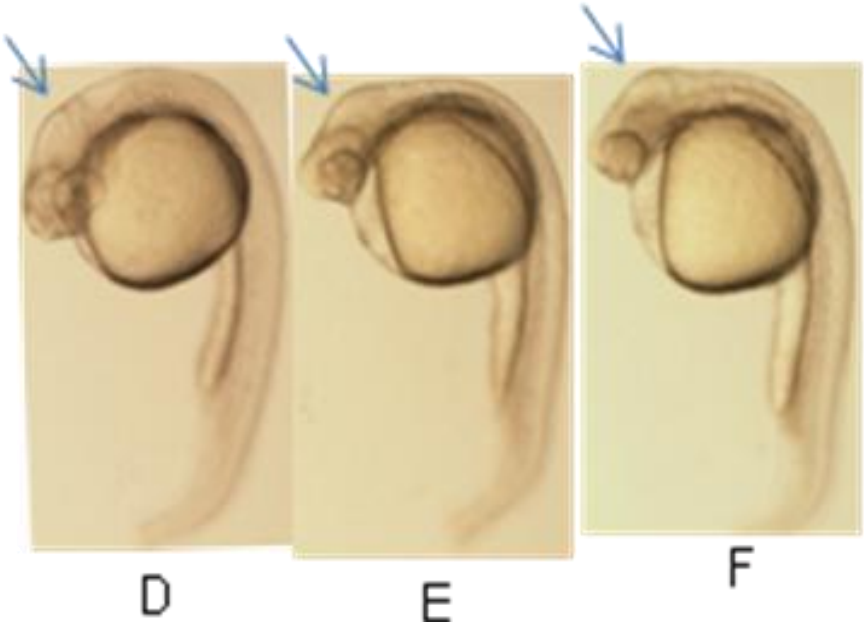


Figure 3.8. Embryos with ectopic over expression of zebrafish Id4 exhibit a dose-dependent increase in the severity of the observed phenotype. Percentage of injected embryos that looked normal (blue), or exhibited a mild (red), moderate (green) or severe (purple) phenotype are shown. The number of embryos analysed for each concentration is given in brackets.

250 pg/ nl Zebrafish Id4-HA mRNA



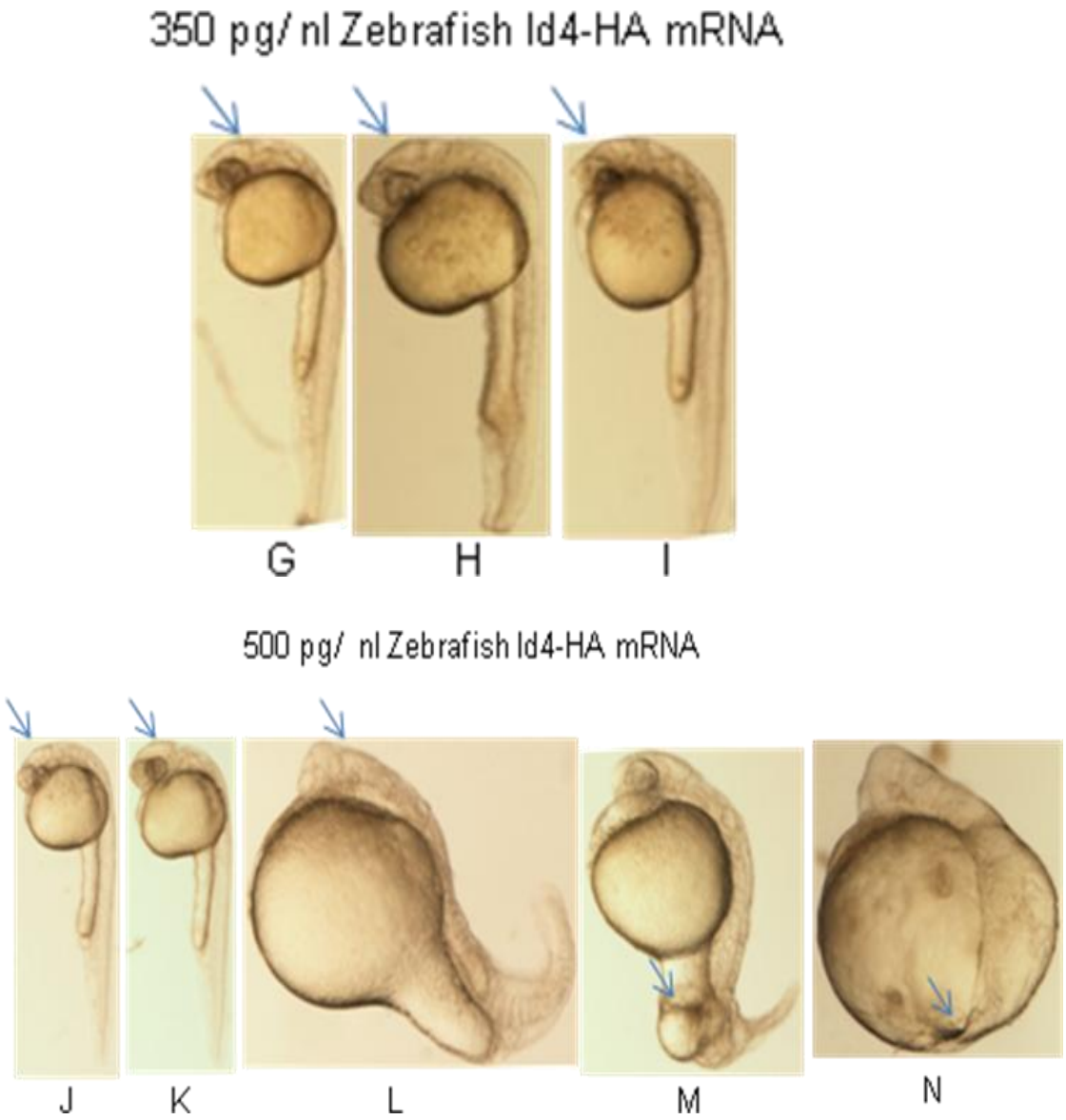


Figure 3.9. Over expression of zebrafish Id4-HA mRNA in embryos observed at 24-28 hpf. A-C wild type embryos showing normal phenotypes. Embryos injected with 250 pg/ nl Zf Id4-HA mRNA showing normal (D) and mild (E-F) phenotypes. Embryos injected with 350 pg/ nl Zf Id4-HA mRNA showing mild (G) and moderate (H-I) phenotypes, where as embryos injected with 500 pg/ nl Id4-HA mRNA has given a wide range of phenotypes with mild (J), moderate (K), severe (L), yolk extension with a kink in tail (M) and a tail is completely absent with severe brain defect (N).

3.5. Ectopic over expression of mouse Id4-HA results in mild phenotype

1- 8 cell stage embryos were injected with various amounts of mouse Id4-HA mRNA (produced by *in vitro* transcription as described in Material & Methods section 2.2.10) and analysed after 24 hpf as described above.

Embryos were injected with 1 nl of zebrafish Id4-HA mRNA at a concentration of either 250, 350 or 500 pg/ nl. As shown in Figure 3.10 ectopic over expression of mouse Id4-HA resulted in a much less severe phenotype compared to ectopic over expression of zebrafish Id4-HA.

Injection of 250 or 350 pg mouse Id4-HA mRNA did not give rise to abnormal development and most (~99%) injected embryos were indistinguishable from wild type embryos (Table 3.7 & 3.8). However, about 30% of embryos injected with 500 pg mouse Id4-HA mRNA did exhibit a phenotype similar to those injected with zebrafish Id4-HA mRNA (Figure 3.9. & Table. 3.6).

250 pg/ nl mouse Id4-HA	**No. of Embryos**	**Percentage**
Normal	94	98.95
Mild	1	1.05
Moderate	0	0
Severe	0	0

Table 3.7. Percentage of phenotypic morphants injected with 250 pg/ nl of mouse Id4-HA mRNA.

350 pg/nl mouse Id4-HA	No. of Embryos	Percentage
Normal	77	98.72
Mild	1	1.28
Moderate	0	0
Severe	0	0

Table 3.8. Percentage of phenotypic morphants injected with 350 pg/ nl of mouse Id4-HA mRNA

500 pg/nl mouse Id4-HA	No. of Embryos	Percentage
Normal	68	68.69
Mild	10	10.1
Moderate	9	9.09
Severe	12	12.12

Table 3.9. Percentage of phenotypic morphants injected with 250 pg/ nl of mouse Id4-HA mRNA.

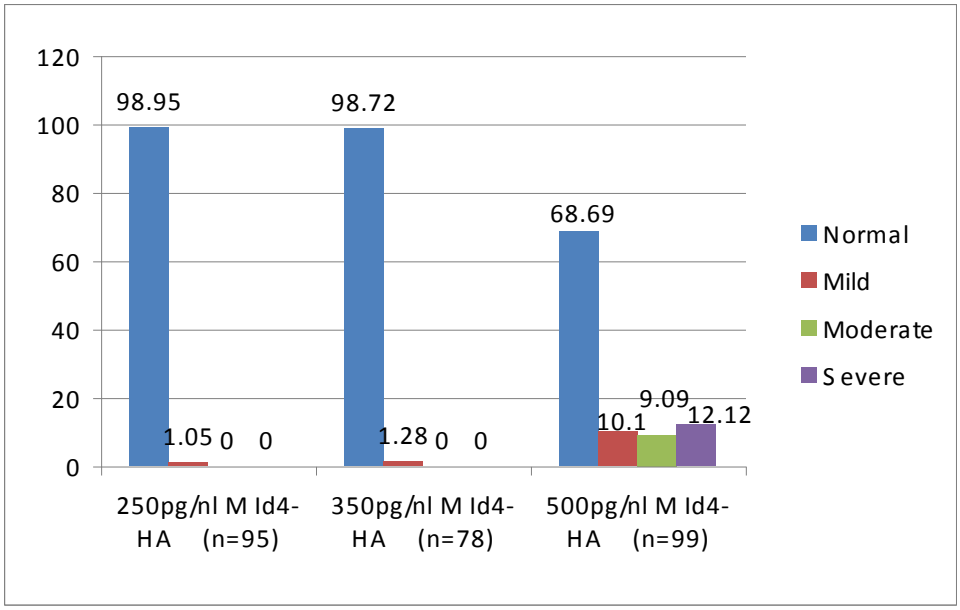
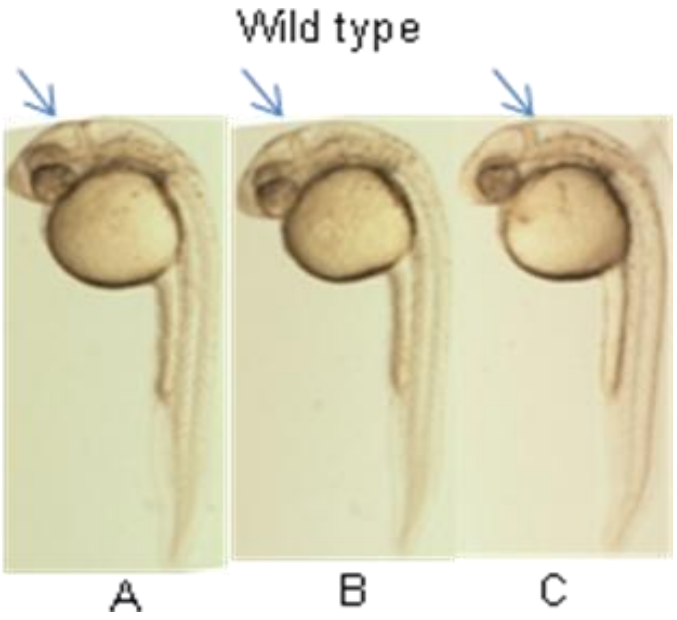


Figure 3.10. Comparison of morphants injected with Mouse Id4-HA mRNA. Percentage of injected embryos that looked normal (blue), or exhibited a mild (red), moderate (green) or severe (purple) phenotype are shown. The number of embryos analysed for each concentration is given in brackets.


Wild type
A
B
C


250 pg/nl Mouse Id4-HA mRNA
D
E

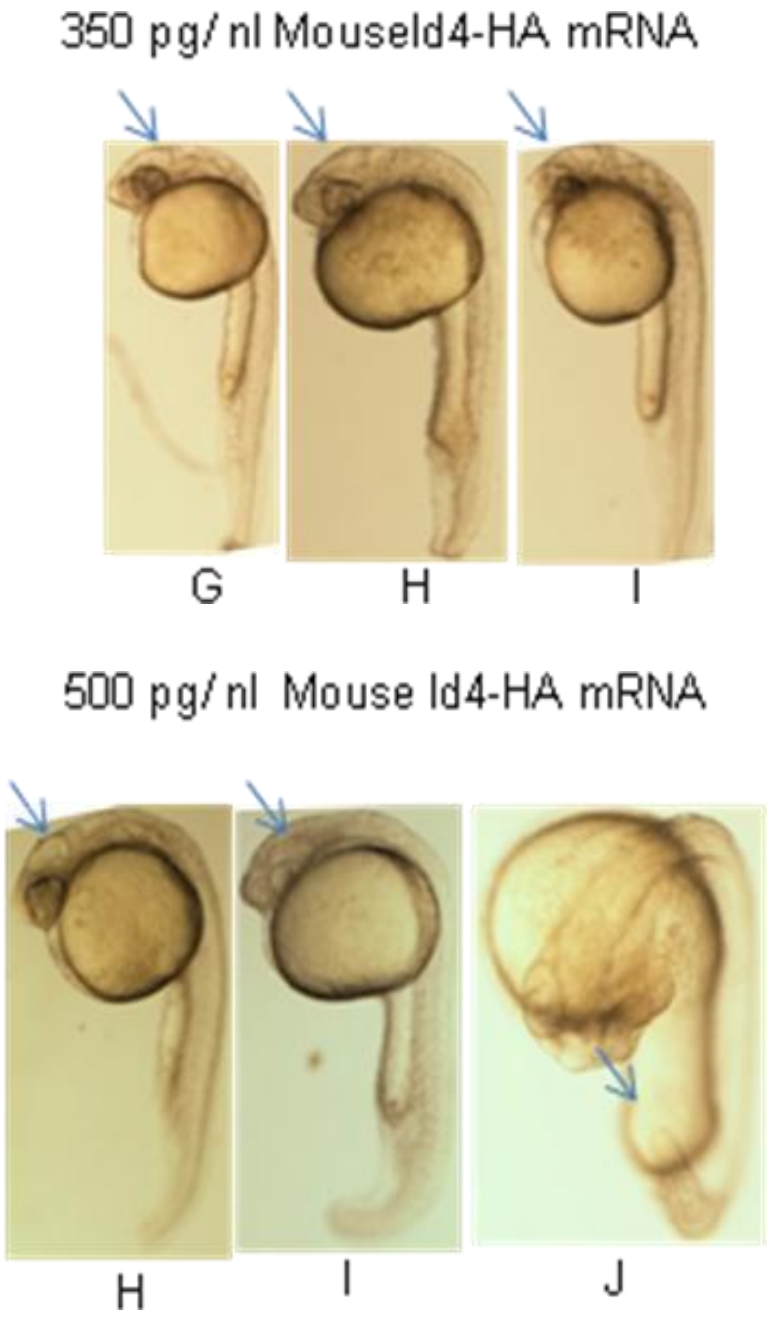


Figure 3.11. Ectopic over expression of mouse Id4-HA results in mild phenotype. A-C are wild type embryos taken as controls. Embryos injected with 250 and 350 pg/ nl Mouse Id4-HA mRNA were not showing any phenotypes (D-I) and embryos showed mild (H), severe (I) and yolk extension (J) phenotypes when concentration of mRNA is increased.

3.6. Over expression of mouse Id4-HA partially rescues Id4 morphants

MO-mediated knockdown of Id4 described in section 3.3 suggested a functional role for Id4 in neurogenesis. However, to exclude off-target effects and to ensure that the observed phenotype is specific, embryos were co-injected with MO and Id4-HA mRNA. As described in section 3.4, the zebrafish Id4-HA mRNA contains the target sequence of the MO whereas the mouse Id4-HA mRNA is sufficiently different and cannot be targeted by the MO. Accordingly, only co-injection of mouse Id4-HA with Id4 MO should result in a rescue of the Id4 morphants.

Initially embryos were co-injected with 15 ng MO and 250 pg zebrafish Id4-HA mRNA. As shown in Table 3.10 co-injected embryos exhibited a phenotype similar to embryos injected with zebrafish Id4-HA mRNA alone. Similarly, co-injection of 20 pg MO and 500 pg zebrafish Id4-HA mRNA resulted in a severe phenotype in the majority of embryos (~76%, Table 3.11). These results suggest that ectopic expression of zebrafish Id4-HA mRNA was not sufficiently blocked by the MO. Indeed, Western blot analysis confirmed that similar amount of Id4-HA protein was present in embryos either injected with zebrafish Id4-HA mRNA alone or in combination with the MO (see section 3.7, Figure 3.18 & 3.20). This was unexpected since the Id4-HA mRNA does contain the MO target sequence and it was anticipated that co-injection of both would result in a reduction of the Id4-HA protein. Presumably, the amount of mRNA injected was too high relative to the amount of co-injected MO. Given that injection of

zebrafish Id4-HA mRNA on its own resulted in a severe phenotype it was not possible to determine whether or not ectopic expression of zebrafish Id4-HA protein rescued the MO-mediated phenotype.

15 ng/ nl MO + 250 pg/ nl zebrafish Id4-HA	**No. of Embryos**	**Percentage**
Normal	2	4.44
Mild	8	17.78
Moderate	10	22.2
Severe	24	53.3

Table 3.10. Percentage of phenotypic morphants coinjected with 15 ng/nl Id4 MO + 250 pg/nl of zebrafish Id4-HA mRNA.

20 ng/ nl MO + 500 pg/ nl zebrafish Id4-HA	**No. of Embryos**	**Percentage**
Normal	3	3.45
Mild	3	3.35
Moderate	15	17.24
Severe	66	75.86

Table 3.11. Percentage of phenotypic morphants co-injected with 20 ng/nl Id4 MO + 500 pg/nl of zebrafish Id4-HA mRNA.

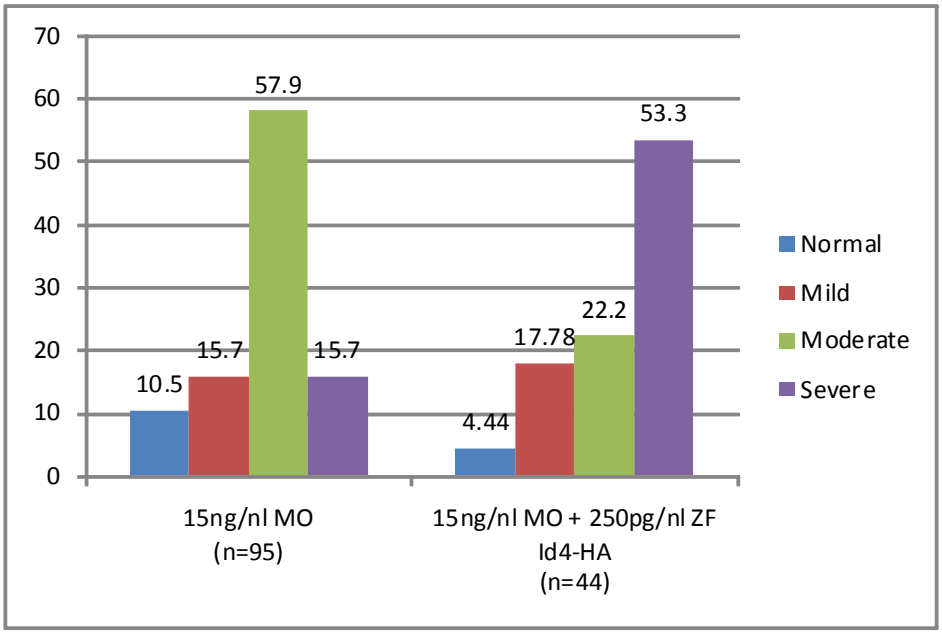


Figure 3.12. Comparison of phenotypes observed after injected of 15 ng MO alone or in combination with 250 pg zebrafish Id4-HA mRNA. Percentage of injected embryos that looked normal (blue), or exhibited a mild (red), moderate (green) or severe (purple) phenotype are shown. The number of embryos analysed for each concentration is given in brackets.

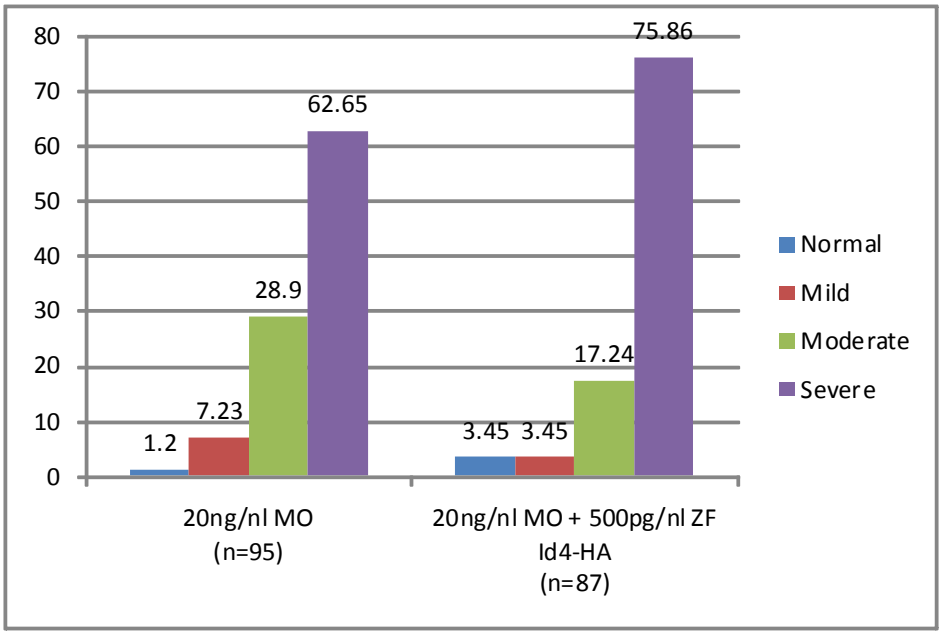
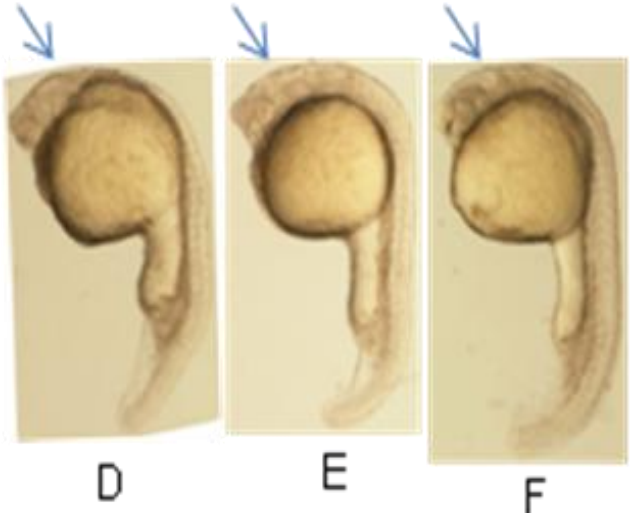


Figure 3.13. Comparison of phenotypes observed after injected of 20 ng MO alone or in combination with 500 pg zebrafish Id4-HA mRNA. Percentage of injected embryos that looked normal (blue), or exhibited a mild (red), moderate (green) or severe (purple) phenotype are shown. The number of embryos analysed for each concentration is given in brackets.

250pg/ nl Zf Id4-HA mRNA + 15ng/ nl MO



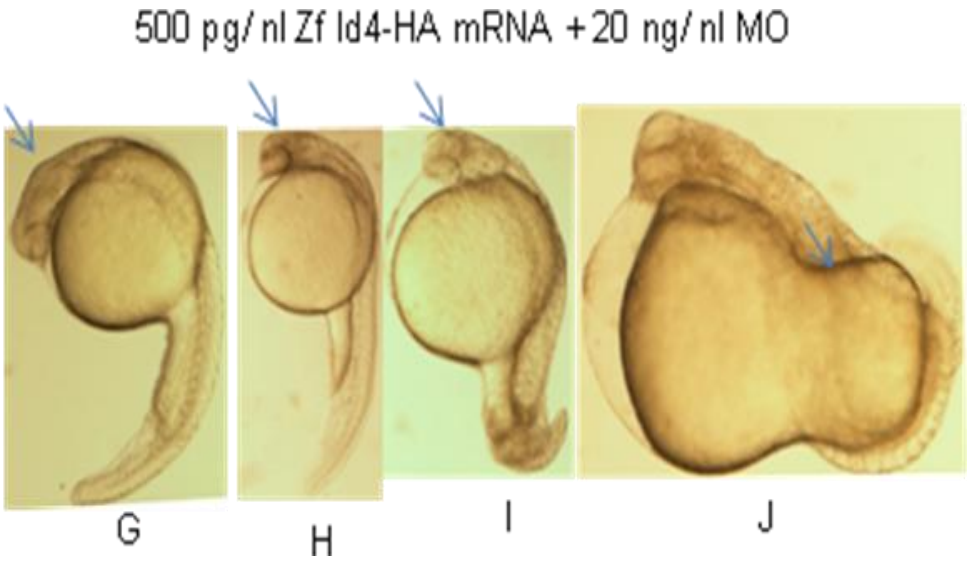


Figure 3.14. Ectopic expression of zebrafish Id4-HA mRNA was not sufficiently blocked by the MO. Effect of morpholino was observed when injected with 250 pg/ nl Zf Id4-HA mRNA. D-F showing severe brain phenotypes indicating the knockdown of protein Id4 expression. G-H embryos with severe brain phenotypes and an extended yolk in J indicated by arrow. A-C are wild type embryos showing normal growth.

Co-injection of mouse Id4-HA mRNA on the other hand resulted in a partial rescue of the Id4 morphants. Using 15 ng MO and 250 pg mouse Id4-HA mRNA, (fig. 3.15) less than 10 % of the embryos showed a severe phenotypes and the majority (~60%) of embryos exhibited a mild phenotype (table 3.12). Comparison of phenotypes observed after injected of 15 ng MO alone or in combination with 250 pg mouse Id4-HA mRNA clearly indicated a partial rescue (Figure 3.15). In addition, the phenotypic rescue was reversed when higher a concentration of MO was co-injected with the same amount of mRNA (Table 3.13). However, comparison of phenotypes observed after injected of 20 ng MO alone or in combination with 250 pg mouse Id4-HA mRNA still indicated a partial rescue (Figure 3.16).

15 ng/ nl MO + 250 pg/ nl mouse Id4-HA	**No. of Embryos**	**Percentage**
Normal	4	4.39
Mild	55	60.44
Moderate	24	26.37
Severe	9	9.9

Table 3.12. Percentage of phenotypic morphants co-injected with 15 ng/ nl Id4 MO + 250 pg/ nl of mouse Id4-HA mRNA.

20 ng/ nl MO + 250 pg/ nl mouse Id4-HA	**No. of Embryos**	**Percentage**
Normal	7	6.86
Mild	23	22.54
Moderate	24	23.53
Severe	48	47.06

Table 3.13. Percentage of phenotypic morphants co-injected with 20 ng/ nl Id4 MO + 250 pg/ nl of mouse Id4-HA mRNA.

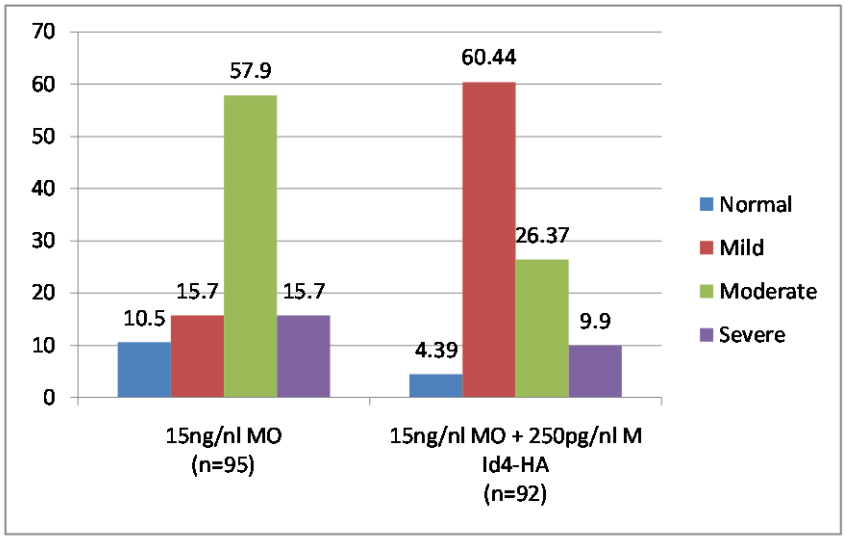


Figure 3.15. Comparison of phenotypes observed after injected of 15 ng MO alone or in combination with 250 pg mouse Id4-HA mRNA. Percentage of injected embryos that looked normal (blue), or exhibited a mild (red), moderate (green) or severe (purple) phenotype are shown. The number of embryos analysed for each concentration is given in brackets.

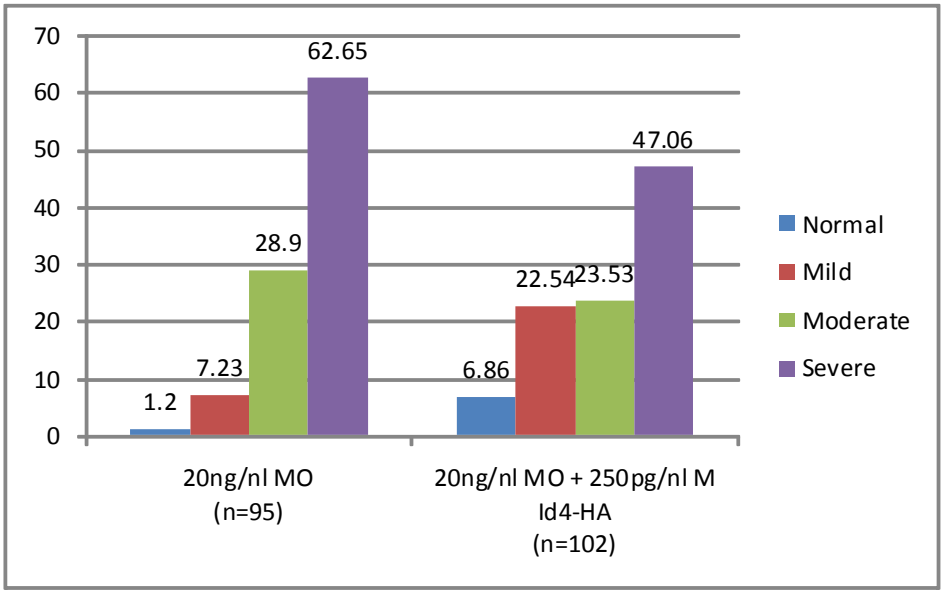
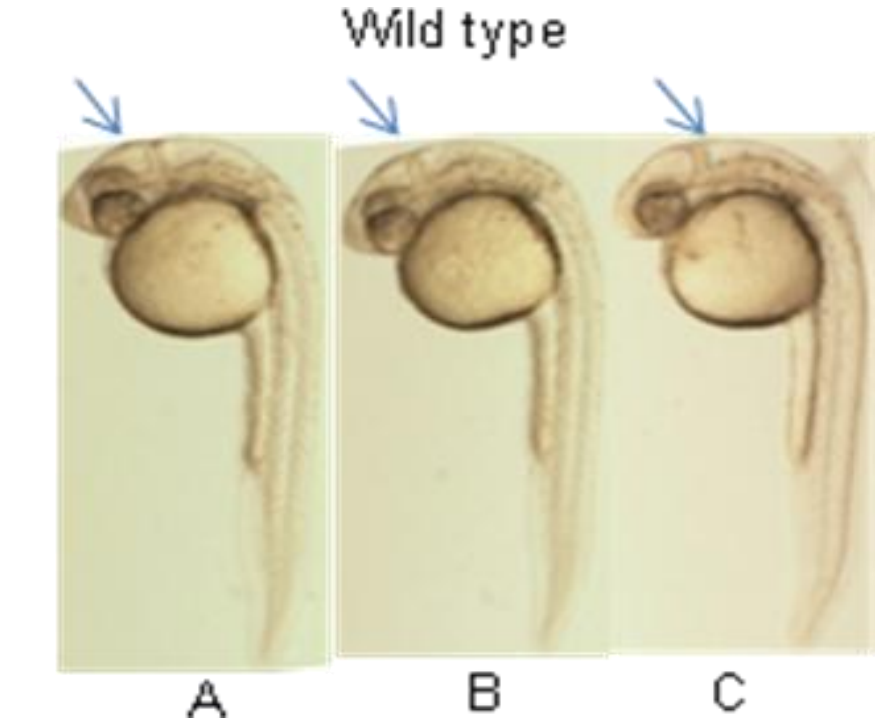
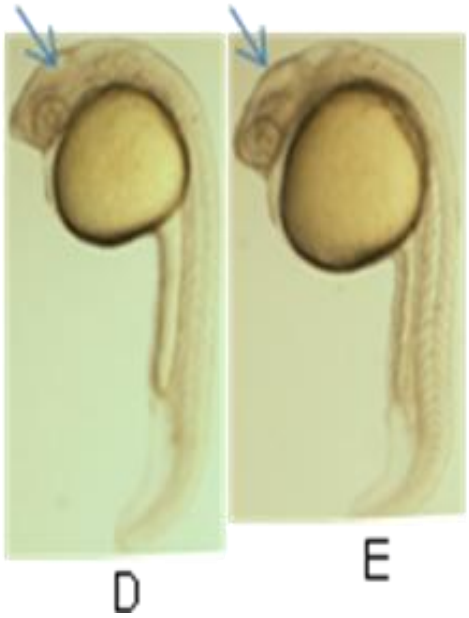


Figure 3.16. Comparison of phenotypes observed after injected of 20 ng MO alone or in combination with 250 pg mouse Id4-HA mRNA. Percentage of injected embryos that looked normal (blue), or exhibited a mild (red), moderate (green) or severe (purple) phenotype are shown. The number of embryos analysed for each concentration is given in brackets.



250pg/ nl Mouse Id4-HA mRNA + 15ng/ nl MO



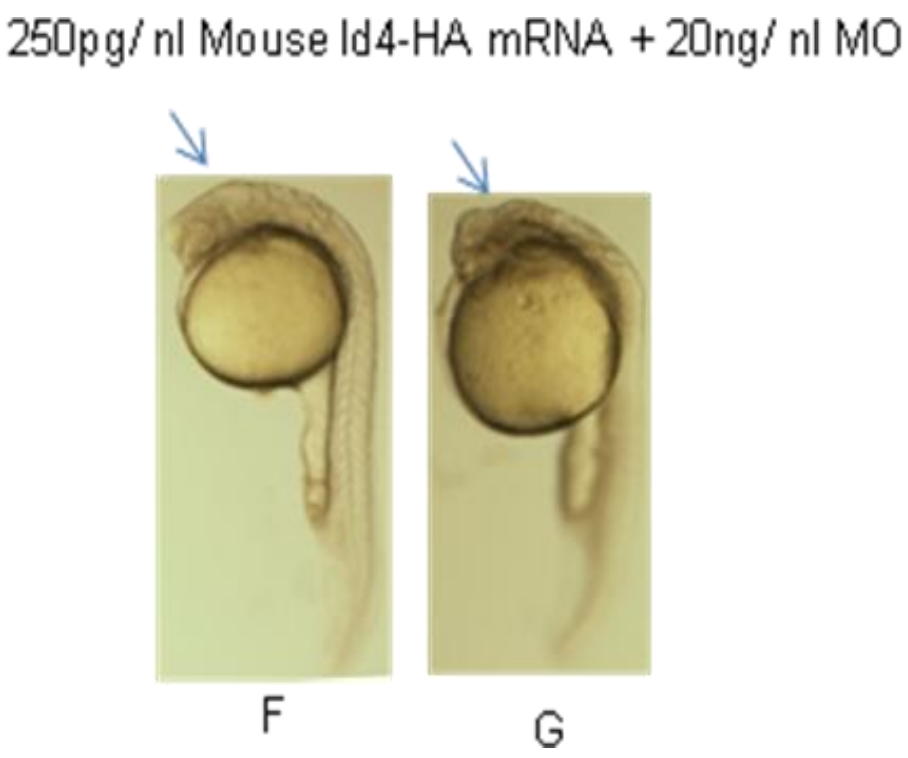


Figure 3.17. Over expression of mouse Id4-HA partially rescues Id4 morphants observed at 24 hpf. Embryos co-injected with 15 ng/ nl morpholino + 250 pg/ nl Mouse Id4-HA mRNA did not give any phenotypes (D-E) and looked similar to wild type embryos (A-C), indicating a rescue of morpholino injected embryos. Embryos giving moderate (F) and severe phenotypes (G) with 250 pg/ nl Mouse Id4-HA mRNA+20 ng/ nl indicated by arrows.

From the above results it can be stated that morpholino mediated knockdown of protein Id4 can be rescued with Mouse Id4-HA mRNA but not with Zebrafish Id4-HA mRNA, as the morpholinos are specific to ZebrafishId4 protein whereas Mouse Id4 has a different amino acid sequence lacking the sequence complementary to the morpholinos.

3.7. Western blot analysis indicated a high turnover of ectopically over expressed Id4-HA protein.

Protein extracts were prepared from embryos injected with zebrafish or mouse Id4-HA mRNA as well as Sox3-HA mRNA (kindly provided by Shih Yu-Huan). Sox3-HA was included as a positive control for HA antibodies used in the Western blot analysis. The protein from zebrafish Id4-HA mRNA injected embryos was extracted at different developmental stages of 8 hpf, 18 hpf and 24 hpf. The protein from mouse Id4-HA mRNA injected embryos was extracted at 8 hpf and 24hpf. The protein from Sox3-HA mRNA injected embryos was extracted at 8 hpf. Protein extracts were separated using SDS-PAGE gel electrophoresis (chapter 2.4.2) and blots were exposed to anti-HA antibody (fig 3.18, 19, 20, and 21). The same blots were used to detect alpha–tubulin as the loading control. The molecular weights of zebrafish Id4-HA, mouse Id4-HA and Sox3-HA proteins are approximately 15, 18 and 35 KDa, respectively.

Zebrafish Id4-HA protein was readily detectable at 8 hpf (fig 3.18) but the amount of protein was reduced at 18 hpf (fig. 3.20) and was undetectable by 24 hpf (fig 3.21). Similarly, mouse Id4-HA protein was present at 8hpf (fig 3.19) but undetectable at 24 hpf.

Western blot analysis using protein extracts from embryos co-injected with MO and either zebrafish or mouse Id4-HA mRNA revealed that both Id4-HA proteins were readily detectable at 8 hpf (fig 3.20) indicating that the MO did not sufficiently target the zebrafish Id4-HA mRNA to prevent translation.

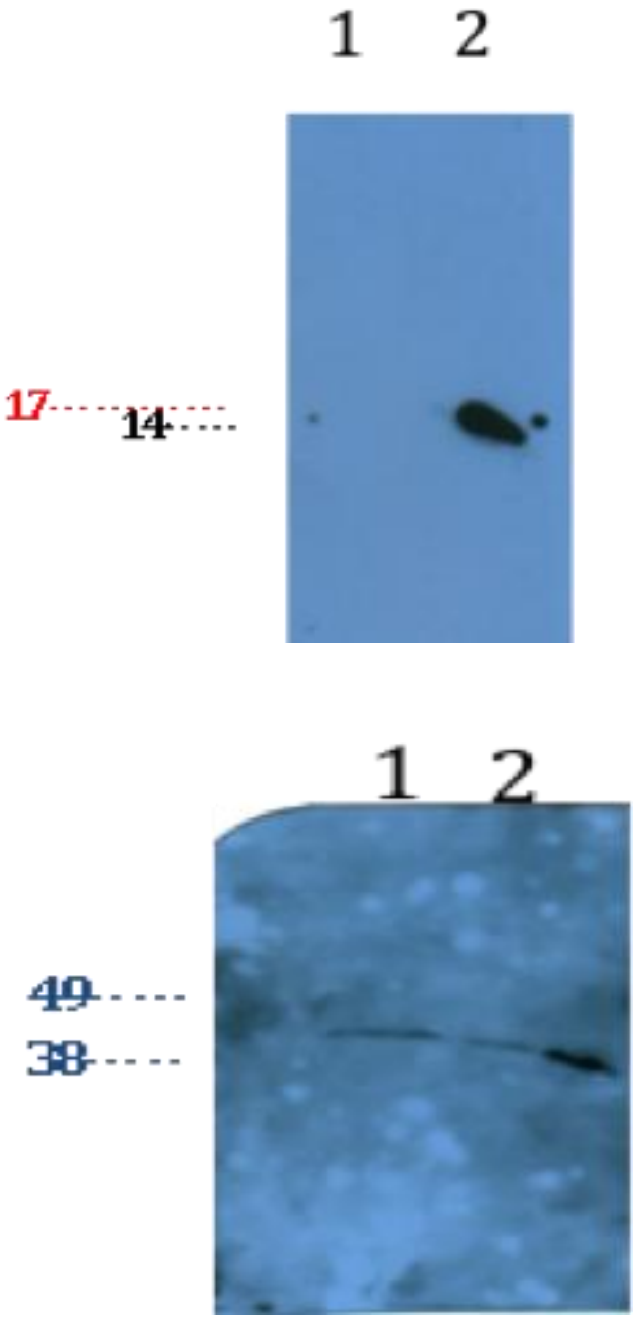


Figure 3.18. Western blot analysis of protein extracts isolated from embryos injected with zebrafish Id4-HA mRNA.

Upper panel: over expressed zebrafish Id4-HA protein is readily detectable at 8 hpf using HA antibodies. Lane 1: wild type, lane 2: zebrafish Id4-HA mRNA injected.

Lower panel: the same blot was re-used with the alpha-tubulin antibody

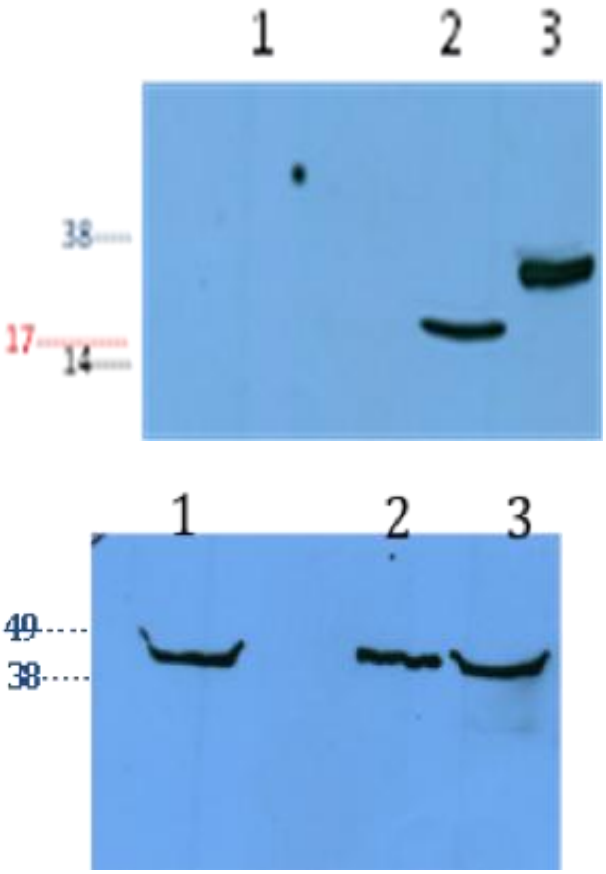


Figure 3.19. Western blot analysis of protein extracts isolated from embryos injected with mouse Id4-HA mRNA.

Upper panel: over expressed mouse Id4-HA protein is readily detectable at 8 hpf using HA antibodies. Lane 1: wild type, lane 2: mouse Id4-HA mRNA injected, lane 3: Sox3-HA mRNA injected.

Lower panel: the same blot was re-used with the alpha-tubulin antibody

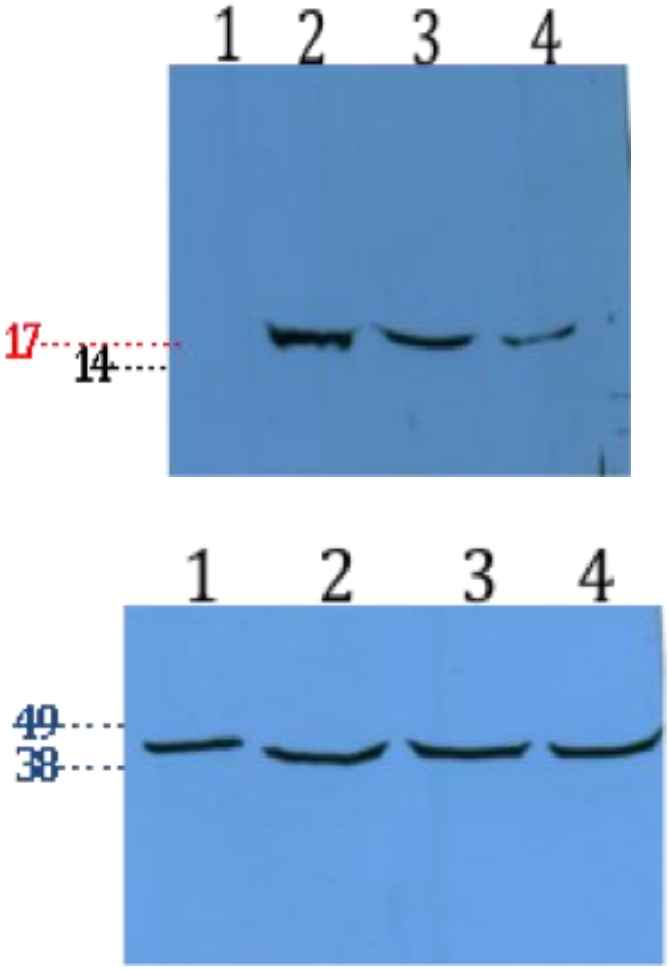


Figure 3.20. Western blot analysis of protein extracts isolated from embryos co-injected with MO and either zebrafish or mouse Id4-HA mRNA.

Upper panel: over expressed zebrafish and mouse Id4-HA protein is readily detectable at 8 hpf using HA antibodies. Lane 1: wild type, lane 2: 15 ng MO and 250 pg zebrafish Id4-HA mRNA, lane 3: 15 ng MO and 250 pg mouse Id4-HA mRNA analysed at 8hpf, lane 4: 250 pg/ nl zebrafish Id4 analysed at 18 hpf.

Lower panel: the same blot was re-used with the alpha-tubulin antibody

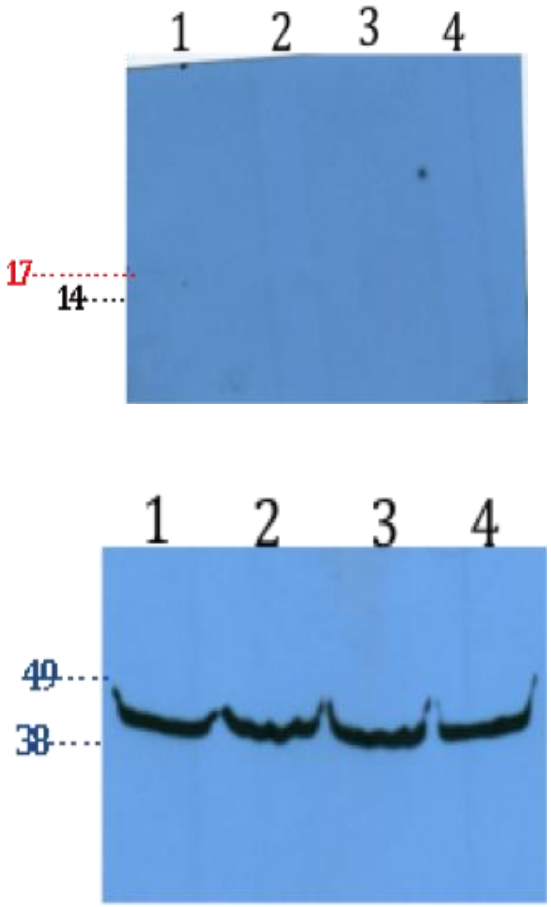


Figure 3.21. Western blot analysis of protein extracts isolated from embryos 24 hpf injected with zebrafish or mouse Id4-HA mRNA.

Upper panel: over expressed zebrafish or mouse Id4-HA protein is undetectable at 24 hpf using HA antibodies. Lane 1: wild type, lane 2: zebrafish Id4-HAmRNA, lanes 3 &4: mouse Id4-HA mRNA injected

Lower panel: the same blot was re-used with the alpha-tubulin antibody

3.8. MO-mediated knockdown of Id4 results in increased apoptosis in the developing nervous system.

Terminal deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) is a common method to detect DNA fragmentation that result from apoptotic cell death. Double-strand breaks in the DNA are substrates for the enzyme Terminal deoxynucleotidyl Transferase (TdT) which catalyze the addition of dUTPs. DNA strand breaks are labelled with modified nucleotide using exogenous terminal transferase (end labelling). End-labeling of DNA with fluorescein-dUTP (Gavrieli *et al.*, 1992). End-labelled DNA can then be detected using Flourescent microscopy is used to detect labelled cells undergoing apoptosis.

The TUNEL assay was performed on whole embryos injected with either 15 or 20 ng MO. Wild type embryos served as negative controls and wild type embryos pre-treated with DNAse were used as positive controls (see M &M section 2.5).

As expected, untreated wild type embryos did not show any apoptotic cells. However, green signal (fluorescein) was seen in the yolk ball and yolk extension but these cells do no contribute to the formation of organs (L.A. D'Amico and M.S. Cooper) (figure 3.23.) but wild type embryos treated with DNase exhibited a high amount of apoptotic cells (average of 29 per embryo) throughout the entire embryos (figure 3.23.). Embryos injected with 15 ng MO showed an average of approximately six apoptotic cells per embryos (figure 3.24.) and embryos injected with 20 ng MO showed an average of 22 apoptotic cells per embryo (figure 3.24.). In contrast to DNase

treated embryos, MO injected embryos exhibited apoptotic cells exclusively in the brain and spinal cord and the amount of apoptotic cells appeared proportional to the severity of the phenotype. These results suggest that the observed phenotype in the Id4 morphants is at least in part due to increased programmed cell death implicating Id4 in the survival of neural stem and progenitor cells. This indicates that the cell death is due to the knockdown of Id4 protein.

Type of embryos	**No. of embryos analysed**	**Total no. of apoptotic cells**	**Average no. of apoptotic cells per embryo**
Wild Type	15	0	0
DNase treated	12	More than 350	29.2
15 ng/ nl MO injected	16	~95	5.93
20 ng/ nl MO injected	9	~200	22.2

Table 3.14. Increased apoptosis in the developing nervous system of Id4 morphants. Apoptotic cells were counted analysing wild type, DNAse treated, wild type and MO injected embryos as indicated.

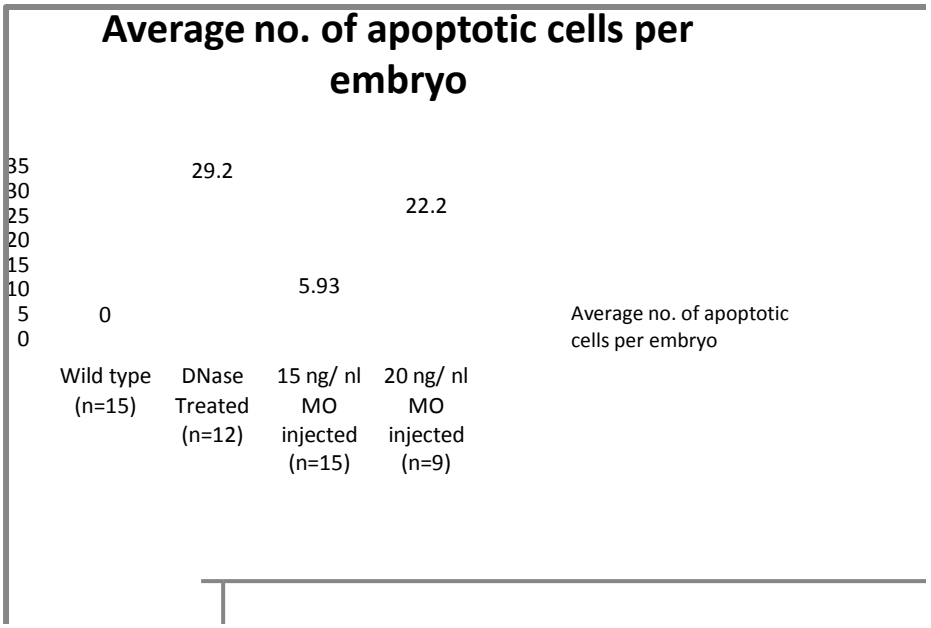


Figure 3.22. Graphical representation of the data shown in Table 3.7.

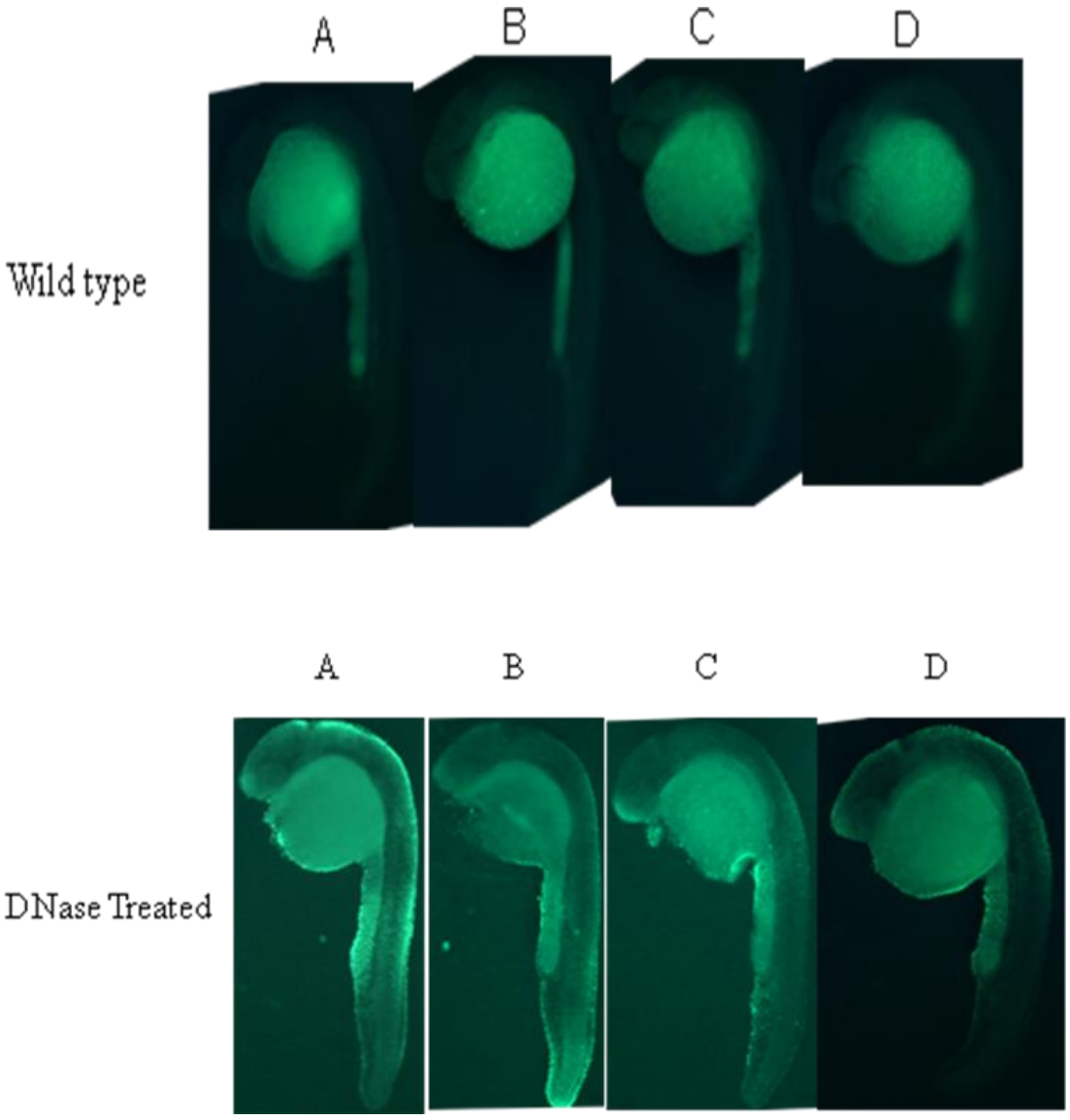


Figure 3.23. TUNEL stained Zebrafish embryos at 24 hpf.

Upper figure: Wild type embryos were taken as negative control for TUNEL; they did not show any apoptosis. **Lower figure:** Embryos treated with DNase were taken as positive controls for TUNEL staining, produced a large number of apoptotic cells.

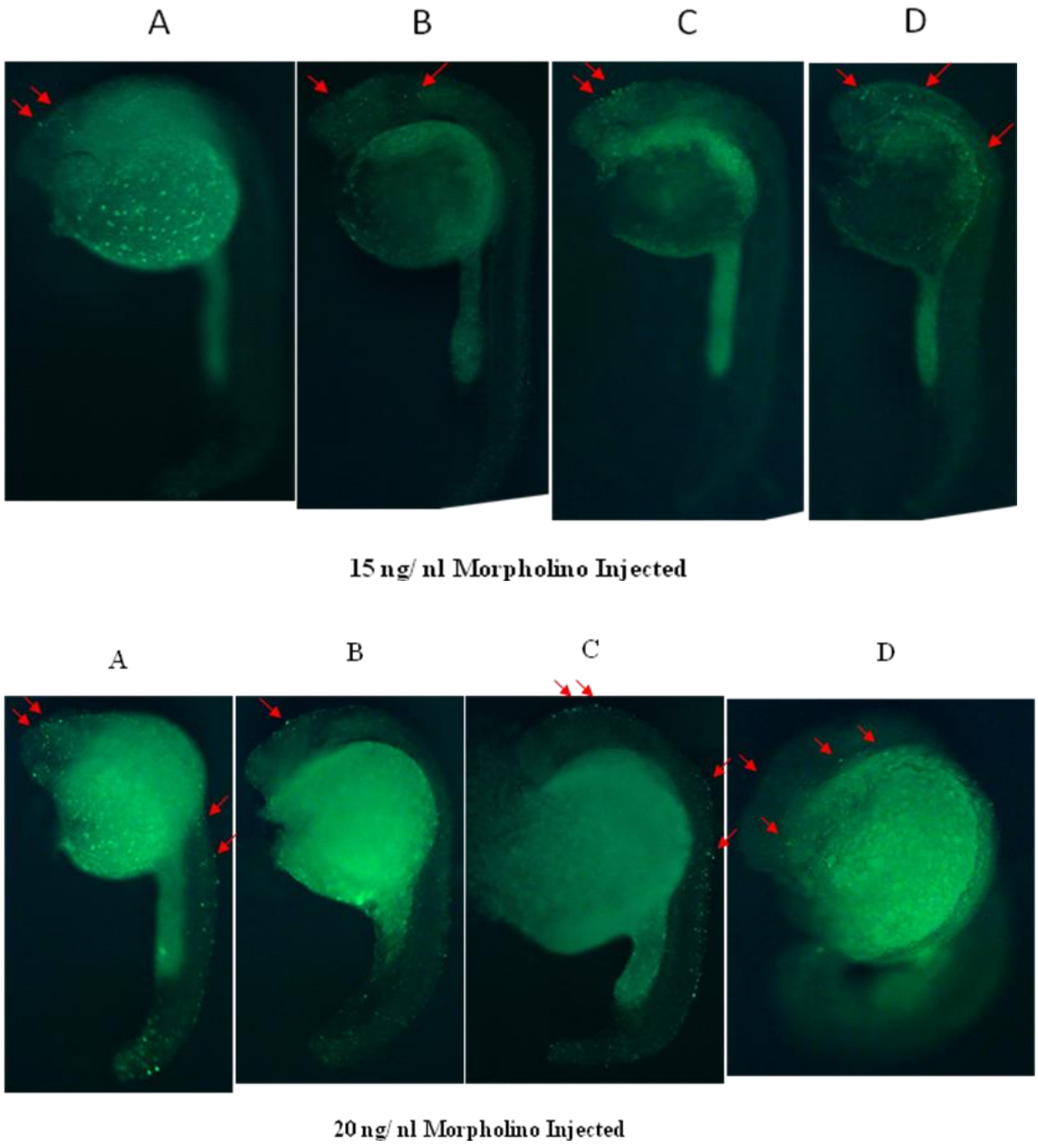


Figure 3.24. TUNEL staining in Zebrafish Id4 knockdown morphants.

TUNEL stained Id4 knockdown morphants show increased apoptosis in the brain. **Upper figure:** Embryos (A, B, C and D) showing fluorescent signals of apoptotic cells in the brain injected with 15 ng/ nl Id4 Morpholinos, **Lower figure:** whereas a significant increase in apoptotic cells in the brain and spinal cord of 20 ng/ nl Id4 morpholino injected morphants (A, B, C and D). The fluorescent signals of some of the apoptotic cells were indicated by red arrows.

4. Discussion

4.1. Id4 function in neural stem and progenitor cell fate

Id4-deficient mice exhibited a 20–30% reduction in mitotic neural progenitor cells during early telencephalon development and *in vitro* analysis of embryonic neurosphere cultures revealed that proliferation of Id4-deficient progenitors was impaired. In addition, cortical progenitor cells exit the cell cycle prematurely and neuronal differentiation was accelerated during embryogenesis resulting in an increase in early-born neurons in the adult Id4-null cortex. Late-born cortical neurons were decreased however, presumably a consequence of precocious depletion of the progenitor cell pool. Consequently, adult Id4-deficient brains were smaller and exhibited enlarged ventricles (Yun *et al.,* 2004; Bedford *et al.,* 2005). In addition, Bedford *et al.,* (2005) showed a significant increase in apoptotic cells in the developing forebrain of Id4-deficient embryos. However, Yun *et al.,* (2004) did not observe any increase in apoptosis in their mutant Id4 mice. To see whether Id4 is indeed required for survival of neural stem and progenitor cells during embryogenesis a MO-mediated knockdown approach was employed using zebrafish as a tractable model system. A translation blocking MO was designed that specifically targeted the AUG start codon of the zebrafish Id4 mRNA but would not target the mouse Id4 mRNA due to several nucleotide differences between zebrafish and mouse Id4. Knockdown of Id4 resulted in a specific phenotype in the developing brain. The severity of the phenotype was proportional to the amount of MO injected and in the extreme cases the normal patterning of the brain was

heavily disturbed and no clear boundaries were visible. Given the restricted expression pattern if Id4 in the ventricular zone of the developing brain in zebrafish (Thisse *et al.,* 2001) this result suggested for the first time a critical role of Id4 function during early neurogenesis in zebrafish.

To ensure that the observed phenotype was due to Id4 knockdown and not to 'off-target effects', it was important to show that ectopic over expression of Id4 could rescue the Id4 morphants. Two recombinant vectors were generated that allowed the production of zebrafish or mouse Id4 mRNA. In addition both sequences were fused to an HA-tag at their C-terminal end to allow detection in western blot analysis using anti-HA antibodies.

Morpholino injections have been shown to cause off-target effects, including cell death in the brain. The latter was correlated with an upregulation of p53 and was shown to be eliminated by co-injection of a p53 morpholino (Robu *et al.,* 2007). To further validate the Id4 knockdown, the knockdown should be carried out in combination with p53 knockdown morpholino oligos. The developmental defects and increased cellular apoptosis induced by the loss of Id4 could be rescued by knockdown of p53 protein along with Id4-MO. The injection of 4 ng/embryo of a p53-specific MO specifically blocks the translation of the zebrafish p53 protein and inhibits p53-induced apoptosis (Langheinrich *et al.,* 2002).

4.2. Ectopic over expression of Id4 protein results in wide spread disruption of zebrafish development

Ectopic over expression of zebrafish Id4-HA protein disrupted normal development of the injected embryos. The observed phenotype however was not restricted to the developing brain but also disrupted the formation of the tail and in severe cases the antero-posterior body axis was shortened. In addition, very large yolk extensions were observed in some of the more severely affected embryos. Ectopic over expression of the mouse Id4-HA was less effective but embryos injected with a high amount of mRNA exhibited a similar phenotype to that observed with zebrafish Id4-HA.

Western blot analysis confirmed over expression of the Id4-HA proteins in 8 hpf and 18 hpf embryos. However, no exogenous protein could be detected at 24 hpf suggesting a rapid turnover of the Id4-HA protein. A similar rapid loss of exogenous protein expression was observed when Sox3-HA mRNA was injected (Dee *et al.*, 2008).

Given that bHLH transcription factors play pivotal roles during embryogenesis ectopic expression of Id4-HA is likely to interfere and antagonise bHLH protein function and thus disrupting development of many different cell types and tissues resulting in a more wide spread phenotype as observed.

4.3. Id4 morphants can be partially rescued through over expression of mouse Id4-HA protein

To ensure that the observed phenotype in Id4 morphants was actually due to a specific knockdown of Id4, embryos were co-injected with the MO and zebrafish or mouse Id4-HA mRNA. Given that the zebrafish Id4-HA mRNA sequence is identical it the endogenous Id4 RNA it was possible that the MO would not only block translation of the endogenous Id4 mRNA but also the exogenous Id4-HA mRNA. It was anticipated therefore that co-injection of zebrafish Id4-HA mRNA would not result in the rescue of Id4 morphants. Co-injected embryos exhibited a phenotype that was similar. In addition, the severe and wide spread phenotype caused by over expression of zebrafish Id4 HA protein alone would possibly complicate the interpretation. This was indeed the case. Co-injected embryos exhibited a similar phenotype to those injected with only zebrafish Id4-HA mRNA suggesting that the co-injected MO did not block efficiently the translation of the Id4-HA mRNA. Indeed, Western blot analysis showed that the zebrafish Id4-HA protein was readily detectable after 8 hpf. Presumably, the amount of mRNA injected was too high and the experiments should be repeated co-injecting more MO and/or less mRNA. Due to time constraint and limitation of availability of embryos it was not possible to do theses experiments.

Co-injection of the mouse Id4-HA mRNA on the other hand resulted in a partial but clear rescue of the Id4 morphants. Co-injecting 15 ng MO with 250 pg mouse Id4-HA mRNA (that on its own did not give rise to a phenotype) resulted in a clear difference in severity with the majority of

embryos exhibiting a mild phenotype. Increasing the amount of the co-injected MO to 20 ng but keeping the amount of mouse Id4-HA mRNA at 250 pg increased the severity. Again as mentioned above, it was not possible to repeat these experiments using 15 ng MO in combination with 350 or 500 pg mouse Id4-HA mRNA but the results presented suggest that higher amounts of mRNA could result in a complete rescue of the Id4 morphants.

The short lived nature of the expression of exogenous Id4 may not have effect on morpholino rescue experiment as the embryosclearly showed the phenotypic characters of a morphant embryo when they were analysed at 24 hpf.

Taken together, the results strongly suggest that Id4 function is required for the normal development of the zebrafish brain.

4.4. Id4 knockdown results in increased apoptosis in the developing nervous system

In zebrafish, the knockdown of Id4 function resulted in significant increase in apoptosis in the developing central nervous system. Id4 knockdown morphants were analysed for apoptosis in the developing brain of zebrafish embryos at 24 hpf. Apoptotic cells were restricted to the fore brain, mid brain and hind brain of embryos injected with 15 ng MO. Embryos injected with 20 ng MO exhibited many more apoptotic cells that were also present in the spinal cord but not signals were present in other tissues of the

embryos as it was evident in DNAse treated embryos. Given that Id4 is expressed in the ventricular zone of the developing brain these results suggest a critical role for Id4 for the survival of neural stem and progenitor cells.

The weak signals detected in the TUNEL assay suggest that the experiments were possibly not sensitive enough to reveal the full extent of apoptosis in the morphant embryos. To improve the sensitivity, the experiments should be repeated and the fluorescein-labelled DNA nicks should be detected with an enzyme-coupled fluorescein antibody to amplify the signal.

Further investigations should be carried out to establish the role of Id4 in proliferation and differentiation of neural stem cells. In the Id4 deficient zebrafish embryos, the CNS was severely affected resulting in severe impairment of the brain. Bedford *et al.,* (2005) showed that the Id4 is required for normal proliferation of the neural precursor cells (NPCs) of the developing brain and correct timing of neural differentiation. The proliferation of the neural stem cells should be detected by prelabeling of cells by injecting BrdU into the yolk of the 18 somite cell stage (18 h) zebrafish embryos, followed by immunodetection of BrdU labeled DNA fragments with anti-BrdU antibody to amplify the signal (Miyake A. *et al.,* 2005, Del Bene F. *et al.,* 2008).

5. Conclusion

MO-mediated knockdown of Id4 revealed a critical role for Id4 in the developing nervous system of the zebrafish likely to be required for the survival of neural stem and progenitor cells.

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