Identification and functional analysis of a novel BTB-domain gene family.

Thesis

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Identification and functional analysis of a novel BTB-domain gene family

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ABSTRACT

In a screen for novel genes involved in chick hindbrain development, clone cV11 was isolated, encoding a gene expressed in the hindbrain. Sequence analysis established that cV11 is a novel gene, now designated *cBTBD6*, which encodes protein containing three functional domains: BTB, BACK and PHR. Human, mouse and zebrafish *cBTBD6* orthologs were cloned, as well as highly related *BTBD3* genes that form a distinct subgroup of the same family of genes. Within each subgroup there is a single copy of human, chick and mouse *BTBD6* and *BTBD3* and two copies of zebrafish orthologs. Spatio-temporal analysis of *BTBD6* and *BTBD3* expression patterns in chick and zebrafish revealed that, while *BTBD3* othologs have divergent expressions, *cBTBD6* shares some aspects of its expression pattern with its counterparts in zebrafish, *zBTBD6a* and *zBTBD6b*. In particular, both *cBTBD6* and *zBTBD6a* are expressed in the developing central nervous system, suggesting a potential involvement in neurogenesis. This potential function was investigated in zebrafish. Comparative analysis of *zBTBD6a* expression with markers of neurogenesis revealed that *zBTBD6a* is expressed in cells during neuronal differentiation, starting from early stages labelled by proneural gene *neurog1* until later phases marked by *isl1*, when *zBTBD6a* expression starts to be downregulated. Knockdown experiments demonstrated that zBTBD6a promotes neurogenesis and acts downstream of *neurog1* and upstream of *neurod* and *neurod4* in the neurogenesis cascade. I found that zBTBD6a interacts with Cul-3, a component of the ubiquitin ligase complex, and the PHR motif and to a lesser extent the BTB domain are required for this interaction. Overexpression experiments showed that the presence of both BTB and PHR is required for zBTBD6a function. Since several BTB-containing proteins have been recently reported to be implicated in targeting proteins for ubiqutination, it is proposed that zBTBD6a positively regulates neurogenesis by mediating ubiquitination of one or more proteins that regulate the formation of neurons.

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This thesis is dedicated to my family: Piotr, Ada and Bartek. Without them, nothing would be possible or make sense. Thanks to them, my projects continue...

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ABBREVIATIONS

AP	Anterior-Posterior
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BMP	Bone Morphogenetic Protein
bp	base pairs
BTB/POZ	broad complex, tramtrack, and bric-a-brac
cDNA	complementary DNA
cV11	chick V11
CNS	Central Nervous System
CTP	Cytidine triphosphate
Da	Dalton
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DV	Dorsal-Ventral
EDTA	Ethylenediaminetetraacetic
Elf1α	Elongation factor-1 alpha
EST	Expressed sequence tag
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
GFP	Green Fluorescent Protein
GTP	Guanosine triphosphate
HH	Hamburger and Hamilton
HRP	Horseradish peroxidase
kb	kilobase
LB	Luria-Bertani
M	Molar
MAPK	Mitogen-activated protein kinase
ml	millilitre
mM	millimolar
mRNA	messenger RNA

mV11	murine V11
NCBI	National Center for Biotechnology Information
nm	nanometer
nmol	nanomol
NP40	Nonidet P-40 (detergent)
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline/0.1% Tween20
Pfam	Protein family searches
pg	picogram
PNS	Peripheral Nervous System
poly(A)	polyadenylic acid or polyadenylate
POZ	poxvirus and zinc finger
r	rhombomere
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	revolutions per minute
RT-PCR	Reverse transcriptase PCR
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TBE	Tris-borate-EDTA buffer
TBS	Tris buffered saline
Tris	2-amino-2-(hydroxylmethyl)-propane-1:3-diol
µg	microgram
µl	microlitre
µM	micromolar
UTP	Uridine triphosphate
UTR	untranslated region
°C	Degree Celsius

INTRODUCTION

The nervous system is an extremely complex organ. It is constituted of neurons and glia that must be produced in the correct numbers and appropriate positions for their function, in for example movement, sensory perception, emotion and memory. Neurons are generated in a multistep process of neurogenesis, which commences with neural induction and leads to the differentiation of functional neurons (Appel and Chitnis, 2002). Understanding the molecular mechanisms promoting neurogenesis in embryos is one of the fundamental tasks in developmental neurobiology.

NEURAL INDUCTION

The default model

Neurogenesis in vertebrates begins with the formation of the neural plate that arises from the embryonic ectoderm during gastrulation. Generation of the neural plate involves an activation (neuralisation) of the ectoderm, and its transformation (regionalisation). In all classes of vertebrate, the prospective neural plate – the rudiment of the adult central nervous system (CNS) and a major contributor to the peripheral nervous system (PNS) – arises in close proximity to the organiser (the embryonic shield in fish, the dorsal lip of the blastopore in amphibians and reptiles, Hensen's node in birds and the node in mammals) (Beddington, 1994; Gimlich and Cooke, 1983; Shih and Fraser, 1996; Waddington and Schmidt, 1933). Until recently, largely based on studies in amphibians, neural induction was explained by the "default model", which proposes that the ectoderm is pre-programmed towards a neural fate (Fig. 1A). This was shown in experiments in *Xenopus*, in which when ectodermal cells are dissociated and thus unable to receive signals from neighbouring cells, they adopt a neural fate (Furthauer et al., 1999; Hemmati-Brivanlou and Melton, 1997; Hemmati-Brivanlou and Melton, 1994; Wilson and Hemmati-Brivanlou, 1997). This autonomous tendency of ectoderm to differentiate into neural tissue is inhibited by BMPs

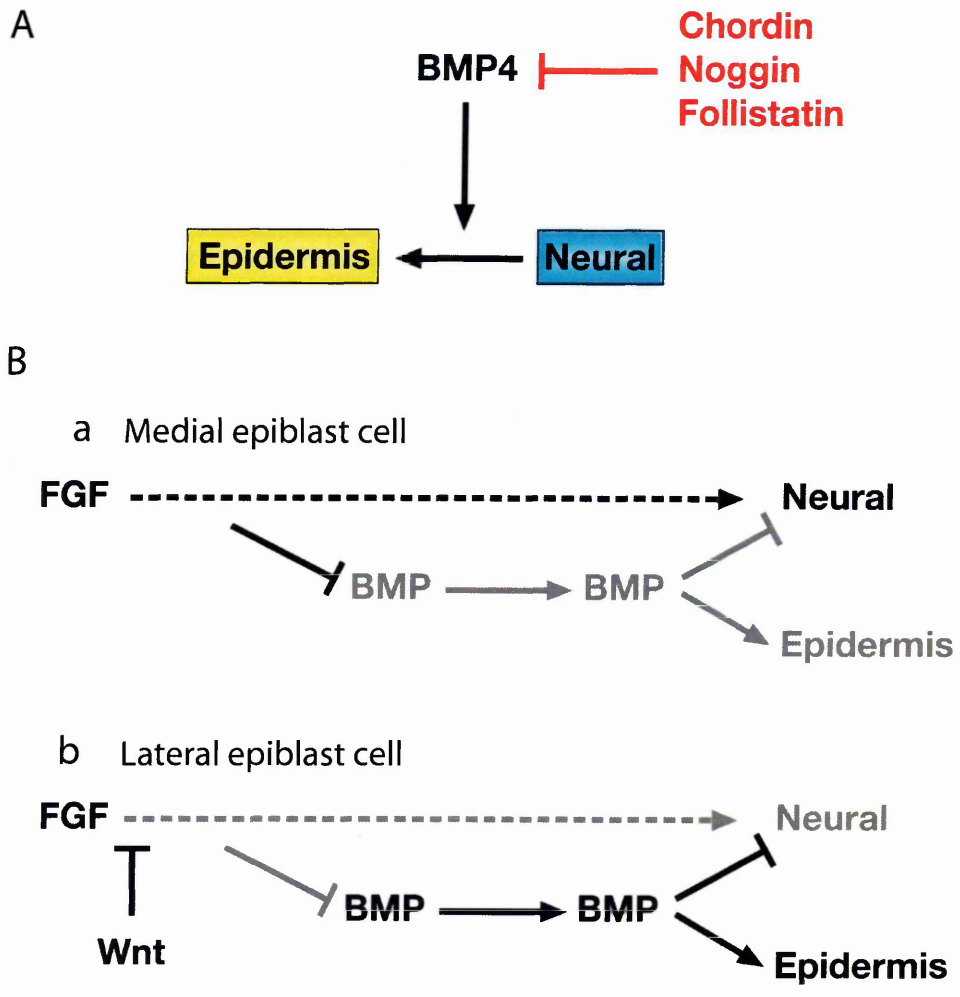


Figure 1. Classical and revised views of neural induction. (A) The default model in *Xenopus:* ectodermal cells that would otherwise become neural are driven toward an epidermal fate by BMP4, which is expressed ubiquitously. BMP inhibitors diffusing from the organiser block BMP4 signalling, permitting the neighbouring ectodermal cells to develop according to their "default" neural fate. (B) Model based on results of explant experiments in chick. (a) At the blastula stage, medial epiblast cells (prospective neural cells) express FGFs but not Wnts. FGF signalling represses BMP expression and also promotes neural fate by an independent pathway in medial epiblast cells (dashed line from FGF). (b) Lateral epiblast cells express both FGFs and Wnts. High Wnt levels block the response of epiblast cells to FGFs, thus BMPs are expressed and promote epidermal fate and repress neural fate. (Adapted from Wilson and Edlund, 2001 and Stern, 2005).

(Bone Morphogenic Proteins), in particular, BMP4, which acts as an epidermal inducer. BMP activity is antagonised by organiser-derived neuralising signals: Follistatin (Hemmati-Brivanlou and Melton, 1994), Noggin (Lamb et al., 1993; Smith and Harland, 1992; Smith et al., 1993) and Chordin (Sasai et al., 1995; Sasai et al., 1994). However, subsequent studies in several vertebrates have demonstrated that BMP inhibition is not sufficient for neural induction. For instance, in zebrafish embryos mutant for *chordin* neural tissue is formed, and although the explanation of this may be that other BMP antagonists still present in these mutants are responsible for neural induction, surgical removal of the organiser also does not prevent nervous system formation (Driever et al., 1996; Schulte-Merker et al., 1997; Shih and Fraser, 1996). Similarly, in zebrafish mutants in which the organiser is lost, including double mutants for *squint* and *cyclops* (Nodal- related genes essential for organiser development), and mutants for *one-eyed pinhead* (a protein required for Nodal function), the neural plate is reduced but still forms (Feldman et al., 1998; Gritsman et al., 1999). In chick, misexpression of BMP antagonists, Noggin or Chordin, in non-neural epiblast is not sufficient to induce neural tissue (Streit et al., 1998; Streit and Stern, 1999b; Streit and Stern, 1999c).

Roles of FGF, Wnt and BMP signalling in neural induction

Recent data from studies in chicken and mice provided evidence that neural induction is initiated in the pre-gastrulation embryo before organiser formation, and FGF (Fibroblast Growth Factor) and Wnt signals are involved in this process (Bainter et al., 2001; De Robertis and Kuroda, 2004; Linker and Stern, 2004; Stern, 2002; Stern, 2005; Wilson and Edlund, 2001). Based on chick explant experiments it is postulated that FGF signalling is required for neural induction, while the selection of neural versus epidermal fate is regulated by the status of Wnt signalling (Fig. 1B) (Wilson and Edlund, 2001; Wilson et al., 2001). At the blastula stage, in the absence of Wnt expression in the medial epiblast (prospective neural plate) FGF signalling promotes neural fate by a dual mechanism (Fig. 1Ba). It activates a pathway

necessary for the progression of the neural fate, and represses BMP expression in an independent pathway. In the lateral epiblast, high-level Wnt signals prevent cells from responding to FGF signalling, which in turn permits BMP expression and directs cells to an epidermal fate (Fig. 1Bb). Therefore, in the presence of high-level Wnt signalling, neither BMP antagonists nor FGF signals alone or in combination are able to induce neural fate in the prospective epidermal cells (Wilson et al., 2001). Studies in *Xenopus* showed that prior to organiser formation at the blastula stage, there is a transient expression of BMP antagonists, Chordin and Noggin, triggered by maternal β-catenin, which is required for neural predisposition in the prospective neuroectoderm (Kuroda et al., 2004). In addition, FGF signalling cooperates with BMP antagonists to induce a neural fate by blocking BMP signalling via phosphorylation of the linker region of a Smad BMP effector, which inhibits Smad activity (De Robertis and Kuroda, 2004; Delaune et al., 2005; Pera et al., 2003). Specific targets of FGF signalling in the neural induction have been identified in chick, including *ERNI* (early response of neural induction), *churchill* (Sheng et al., 2003; Streit et al., 2000) and *Sox3* (Uwanogho et al., 1995).

In zebrafish, both BMP inhibition and FGF signalling have been proposed to function as direct neural inducers. The BMP antagonists act to induce the anterior CNS, FGF signals induce the posterior neural plate and a combination of both specify intermediate regions (Furthauer et al., 2004; Kudoh et al., 2004; Rentzsch et al., 2004).

PATTERNING OF NEUROGENESIS - DEFINING DOMAINS OF NEUROGENESIS BY PREPATTERN FACTORS

Neural induction leads to the commitment of precursors to a neural fate. These precursors within the neural plate will further differentiate into neurons or glia in a spatially and temporary coordinated manner. In vertebrates, several transcription factors have been identified that promote neural fate downstream of neural induction. These neural effectors include

members of Sox, Gli, POU and Iroquois families and are initially broadly expressed throughout the prospective neural plate (Bainter et al., 2001; Bally-Cuif and Hammerschmidt, 2003). They are likely to connect neural induction with later neuronal cell fate determination and differentiation processes. In zebrafish, *irx1b* (previously *iro1*), *irx7* (prev. *iro7*), and *pou5f1* are expressed in the presumptive midbrain and hindbrain regions and Irx1b1, Irx7, and Pou5F1 are required for the expression of the proneural gene *neurogenin1* (*neurog1*) (Hauptmann and Gerster, 1995; Itoh et al., 2002; Lecaudey et al., 2001). Neurogenesis in the anterior epiphyseal proneural field is controlled by *flh*, which permits expression of *neurog1* as well as another proneural factor Ash1 (Cau and Wilson, 2003).

Neurogenesis is initiated at specific sites along the anterior-posterior (AP) and dorsal-ventral (DV) neural plate axes, in distinct differentiation-competent domains, that are believed to represent the equivalent of the *Drosophila* proneural clusters (Campos-Ortega, 1993). In *Drosophila*, the proneural fields are established due to the expression of neurogenesis activators (proneural genes) consisting of Achaete-Scute proteins, and neurogenesis inhibitors, which include Hairy-like factors. These genes are expressed following earlier patterning events to establish a prepattern of neurogenesis within the neuroectoderm. Similarly, in vertebrates, domains of early neurogenesis are established by a prepatterning effect of inhibitory factors that actively block differentiation of neural precursors (Fig. 2). In the zebrafish and *Xenopus* neural plate, the neurogenesis-free domains include the anterior neural plate, the midbrain-hindbrain boundary (MHB), and the posterior longitudinal inter-proneuronal stripes in the hindbrain and spinal cord that separate the columns of precursors of sensory neurons, motorneurons and interneurons. Studies in *Xenopus* revealed that the inter-proneuronal stripes are defined by the expression of transcription factors such as *Zic2*, *Xiro3*, *Xdbx* (Fig. 2) (Bae et al., 2005; Bellefroid et al., 1998; Brewster et al., 1998; Gershon et al., 2000; Hans et al., 2004). In zebrafish, these areas are labelled by *her3* and *her9* that inhibit neurogenesis (Bae et al., 2005). *her3* and *her9* are regulated by positional information in which BMP

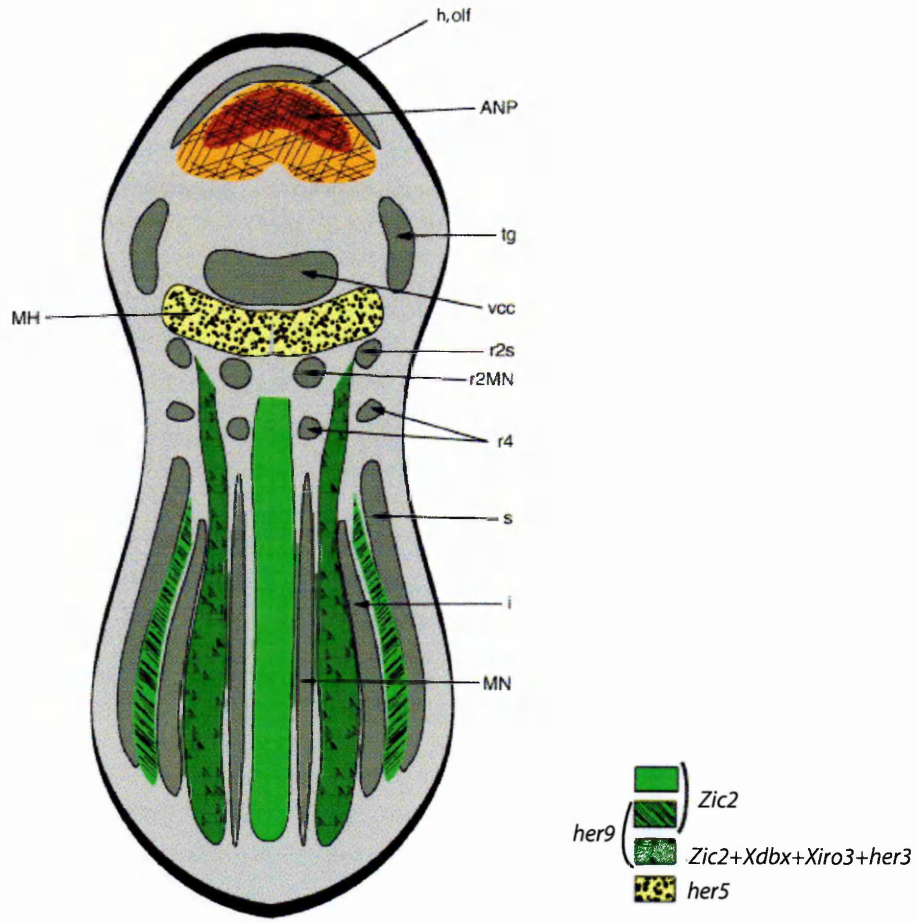


Figure 2. Patterning of neurogenesis. Schematic representation of the spatial pattern of neurogenesis (depicted in grey) and the expression of neurogenesis inhibitors (designated in coloured patterns) in the early neural plate (light grey) in zebrafish and *Xenopus*. In the domains of neurogenesis in the posterior neural plate, the presumptive spinal sensory neurons (s), interneurons (i) and motorneurons (MN) are born. ANP: anterior neural plate; h, olf: hypophysis and olfactory placodes; MH: midbrain-hindbrain domain; r4: motor- and sensory neurons of rhombomere 4; r2MN: motor- and sensory neurons of rhombomere 2; r2s: sensory neurons of rhombomere 2; tg: trigeminal placodes; vcc: ventro-caudal cluster. (Modified from Bally-Cuif, 2003).

signalling is involved (Bae et al., 2005). In the zebrafish MHB, neurogenesis is prevented by expression of *her5* and inhibition of Her5 function leads to an ectopic formation of a proneural field in the MHB area (Geling et al., 2003; Geling et al., 2004). *her5* expression is positioned within the MHB by factors patterning the midbrain-hindbrain domain, including Wnts and FGFs (Geling et al., 2003; Reifers et al., 1998).

MOLECULAR REGULATION OF NEUROGENESIS

Proneural genes

As a consequence of prepatterning, sites of neurogenesis are established and prefigured by the expression of proneural genes, which are basic helix-loop-helix (bHLH) transcription factors that function to promote formation of neurons (Bertrand et al., 2002). The vertebrate proneural genes are homologues of their invertebrate counterparts, which in *Drosophila* are both necessary and sufficient for the commitment of ectodermal cells to a neural progenitor fate (Campos-Ortega, 1993; Modolell, 1997). The bHLH transcription factors identified in vertebrates fall into two families, those related to the *Drosophila Achute-Scute* (*asc*) genes, and those related to *Drosophila atonal* (*ato*) (Guillemot, 1999; Lee, 1997). The *asc* family includes *ascl1*, which have been found in mouse (*Ascl1*, previously *Mash1*), chick (*Cash1*), *Xenopus* (*Xash1*) and zebrafish (*ascl1a* and *ascl1b*, previously *ash1a* and *ash1b*, respectively), and three other genes: *Ascl2* (previous *Mash2*) in mammals, *Xash3* in *Xenopus* and *Cash2* in chick. Based on the sequence homology of the bHLH domain only two true orthologues of *Drosophila ato* have been identified, mouse *Math1* and *Math5*. Other vertebrate *ato*-related genes have been classified as belonging to the neurogenin, NeuroD, or the Olig families, each characterised by the presence of family-specific residues in their bHLH region (Lee, 1997; Massari and Murre, 2000).

All vertebrate *asc*- and *ato*-related bHLH transcription factors are expressed in the developing nervous system but only some of them have a proneural

function in the context of neural tissue to specify progenitors that are limited to a neuronal fate. In mammals, the proneural genes include *Ascl1*, *Neurog1* and *Neurog2* (Fode et al., 1998; Guillemot et al., 1993; Ma et al., 1996). In zebrafish, only one neurogenin gene, *neurog1* and two *Ascl1* homologues, *ascl1a* and *ascl1b*, genes has been identified (Allende and Weinberg, 1994; Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998). Proneural genes are transcriptional activators and have multiple roles during neurogenesis that are discussed below.

Selection of neuronal progenitors

In both vertebrates and invertebrates, the selection of neuronal progenitors within the proneural domains of the neuroepithelium relies on the process of Delta-Notch-mediated lateral inhibition (Fig. 3). The Notch signalling mechanism is an evolutionarily conserved and universally used to direct equivalent cells (or equivalent groups) to acquire the appropriate cell fate during development (Heitzler and Simpson, 1991; Louvi and Artavanis-Tsakonas, 2006; Schweisguth, 2004). Interaction of the extracellular domain of Delta on the surface of one cell with the extracellular domain of the Notch receptor on an adjacent cell results in receptor activation. This leads to the cleavage of the Notch intracellular domain, NICD, which relocates to the nucleus, where it interacts with a DNA-binding protein, Supressor of Hairless (Su[H]). Su(H) binds to regulatory sequences of the Hairy/Enhancer of Split E(spl)-related (Hes and Hairy in mouse and chick, Her in zebrafish) genes and upregulates their expression (Bray and Furriols, 2001; Davis and Turner, 2001; Kageyama and Nakanishi, 1997). The Hes/Her bHLH transcription factors, in turn, downregulate downstream targets, for example proneural genes, which prevents the cell from undergoing differentiation.

In the context of neurogenesis, proneural genes drive expression of Notch ligands, and thereby positively regulate their own expression (Bertrand et al., 2002). Proneural genes are initially uniformly expressed in cells of the

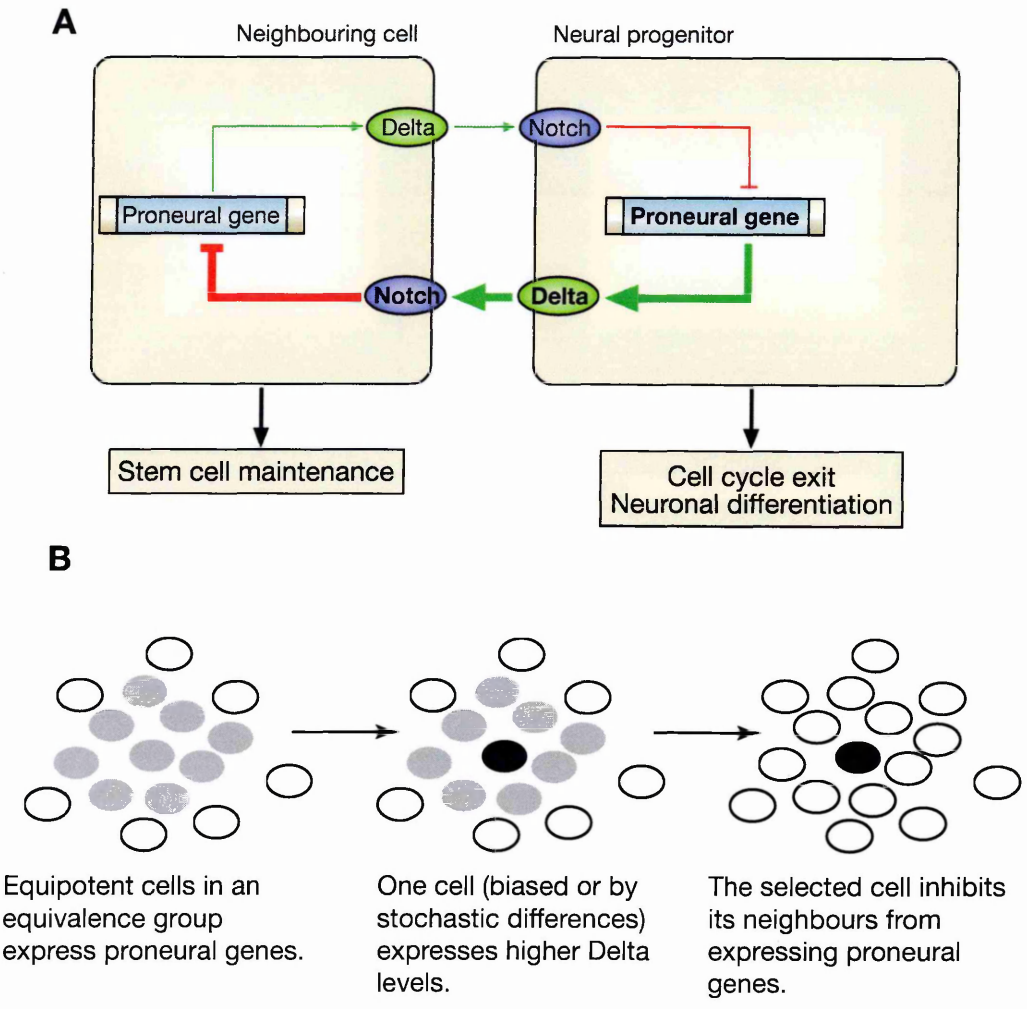


Figure 3. Neurogenesis and the principle of lateral inhibition. (A) Molecular pathway of lateral inhibition. Proneural genes positively regulate their own expression and that of the Notch ligand delta (green arrows). Delta activates Notch in adjacent cells, which inhibits proneural gene expression in the receiving (left) cell. This effect is reinforced over time as the signalling cell receives less inhibition, due to Delta expression being downregulated in the receiving cells. Consequently, the signalling cell initiates a programme of neuronal differentiation, while its neighbours remain undifferentiated. (B) Selection of a neuronal progenitor from an neurogenic (proneuronal) field of cells. Groups of cells (called equivalence groups) express equal levels of proneural genes. Lateral inhibition ensures that only one cell is selected to become a neuronal precursor. (Adapted from Bertrand et al., 2002).

neurogenic (proneuronal) regions, such that all cells have the same potential to activate the Notch pathway in their neighbours and thus both deliver and receive lateral inhibition. If one cell expresses the ligand at higher levels, either randomly or by some bias, it will activate the Notch signalling more strongly, that is, deliver more lateral inhibition in the adjacent cells causing them to express less ligand and consequently deliver back less lateral inhibition to the former cell. Thus, the more inhibition a cell receives from its neighbours, the less it is able to deliver back to them. This gives rise to a feedback loop that tends to amplify any initial difference between neighbouring cells and allows for selection of cells expressing higher levels of *delta* and proneural genes, which are thus fated to become neuronal progenitors (Fig. 3) (Artavanis-Tsakonas et al., 1999; Bray and Furriols, 2001; Collier et al., 1996).

During early neurogenesis in zebrafish embryos, the Notch mediated selection of neuronal progenitors takes place in proneuronal domains of the neural plate where the proneural gene, *neurog1,* is expressed. *neurog1* induces expression of *deltaA* and *deltaD* which activate Notch pathway in the neighbouring cells (Appel and Eisen, 1998; Haddon et al., 1998). This leads to expression of at least one of the Hairy/Enhancer of Split E(spl)-related genes, *her4,* which inhibits *neurog1,* thus preventing cells from acquiring neuronal fate (Appel and Eisen, 1998; Haddon et al., 1998; Takke et al., 1999).

A number of zebrafish mutants have been identified in which components of the Notch signalling pathway are affected. These include the *after eight* (*deltaD*) and *deadly seven* (*notch1a*) mutants that show weak neurogenic phenotypes, with a moderate excess of early-born neurons (Gray et al., 2001; Holley et al., 2000; Holley et al., 2002; Riley et al., 1999). The mild effects of these mutations most probably reflects partial redundancy of the multiple Delta and Notch homologs present in zebrafish. In contrast, another mutant called *mind bomb* (*mib*) is characterised by a severe neurogenic phenotype (Itoh et al., 2003).

In *mib* mutant, a major decrease in Notch signalling results in an excess of early differentiating neurons in expense of the later-differentiating neuronal cell types (Itoh et al., 2003; Jiang et al., 1996; Park and Appel, 2003; Schier et al., 1996). Positional cloning identified *mib* as a gene encoding an E3 ubiquitin ligase that interacts with the intracellular domain of Delta in the signalling cell to promote its ubiquitination and internalisation (Itoh et al., 2003). Delta endocytosis, accompanied by transendocytosis of the Notch extracellular domain, facilitates final cleavage of the Notch intracellular domain from the membrane of the receiving cell and its release to the nucleus where it activates target genes (De Strooper et al., 1999; Parks et al., 2000). Thus, mib activity is essential in the signalling cell for efficient activation of Notch in the neighbouring cells.

Autoregulation of proneural genes

Commitment of selected progenitors to a neuronal fate is reversible and has to be stabilised until determination is attained. Therefore, the levels of proneural gene expression have to be increased and/or maintained in progenitors and this is achieved by a positive feedback mechanism. This involves either direct autoregulation of proneural genes own promoters or indirect regulation via the induction of factors such as vertebrate Hes6 and Xcoe2, which in turn upregulate proneural gene expression (Bae et al., 2000; Bally-Cuif et al., 1998; Koyano-Nakagawa et al., 2000). X-MyT1, a Xenopus zinc finger protein, is also activated by proneural genes and, in cooperation with bHLH factors, promotes neuronal differentiation and confers insensitivity to lateral inhibition to the selected progenitors (Bellefroid et al., 1996).

Molecular cascade of neurogenesis

Proneural genes are both necessary and sufficient to induce neuronal development. For example, targeted mutations in the proneural genes such as mouse *Ascl1*, *Neurog1*, *Neurog2* and *Math1*, lead to a block in neuronal

differentiation (Ben-Arie et al., 1997; Fode et al., 1998; Guillemot et al., 1993; Ma et al., 1998). Conversely, misexpression of these genes promotes neurogenesis, as seen for example when the neurogenins are ectopically expressed in chick, zebrafish, *Xenopus* embryos or in cell lines (Blader et al., 1997; Farah et al., 2000; Lee et al., 1995; Ma et al., 1996; Perez et al., 1999; Takebayashi et al., 1997). Therefore, proneural genes promote the switching from the growth phase to the neuronal differentiation phase.

When the activity of the proneural genes is sufficiently high in progenitor cells, they activate expression of downstream differentiation genes, which then act to promote exit from the cell cycle and neuronal differentiation (Ma et al., 1996). The expression of proneural genes in individual progenitors is transient and downregulated before the differentiation programme is initiated, and therefore the induction of downstream regulatory genes is prerequisite for neurogenesis to occur (Gradwohl et al., 1996; Ma et al., 1996). These genes, many of them structurally related to proneural genes, act in unidirectional cascades and their expression correlates with sequential steps of neuronal determination and differentiation (Bertrand et al., 2002; Kintner, 2002; Lee, 1997). A number of these later-acting bHLH transcription factors have been identified in vertebrates and are capable of driving neuronal differentiation when expressed ectopically. One example are the *NeuroD* bHLH family, that when misexpressed induce production of ectopic neurons (Farah et al., 2000; Lee et al., 1995; Liao et al., 1999; Mueller and Wullimann, 2002b). Overexpression experiments in *Xenopus* and loss-of-function analysis in mouse have confirmed the epistatic relationship between the proneural and differentiation genes. In *Xenopus*, ectopic expression of Xngnr1 (homologue of mouse Neurog1) induces *NeuroD* expression as well as expression of another bHLH gene, *Xath3*, which has an intermediate position between *Xngnr1* and *NeuroD* in the gene expression cascade (Ma et al., 1996; Perron et al., 1999). In contrast, neither NeuroD nor Xath3 can induce *Xngnr1* expression, indicating that *Xngnr1*, *Xath3* and *NeuroD* constitute an unidirectional bHLH gene cascade (Perron et al., 1999). Similarly in mouse, Neurog1 and Neurog2 are necessary for the expression of *Math3* and

NeuroD in cranial sensory neurons and *Mash1* (*Ascl1*) and *Neurog1/NeuroD* mark successive stages of development of olfactory neurons precursors (Cau et al., 2002; Fode et al., 1998; Ma et al., 1998).

The differentiation process is linked to the exit from cell cycle but it is not known whether proneural genes themselves regulate cyclin-dependent kinase inhibitors or whether the downstream differentiation genes are responsible. Committed postmitotic neuronal progenitors also express markers identified after cell cycle arrest, a late delta factor, *deltaB*, and RNA-binding proteins of the Hu family (Haddon et al., 1998; Kim et al., 1996; Mueller and Wullimann, 2002a; Park et al., 2000a). During neurogenesis, proneural genes activate a number of other genes that modify cell metabolism, and rearrange the cytoskeletal architecture (Mattar et al., 2004; Reeves and Posakony, 2005). Expression of these genes enables the generation of basic neuronal characteristics.

Specification of neuronal subtypes

In addition to initiating the neurogenic gene program, proneural factors promote differentiation to neuronal subtypes. The ability to specify subtypes may be dependent on regional determinants and/or structural differences between proneural factors that promote interactions with cofactors that determine neuronal subtype (Chien et al., 1996; Powell et al., 2004).

Interestingly, while the proneural factors often function redundantly in driving neuronal differentiation, they have unique roles in specifying neuronal subtype identity. The redundant function of different bHLH factors in inducing neurogenesis has been shown in studies of *Mash1(Ascl1)/Math3* or *Mash1(Ascl1)/Neurog2* double mutants, in which neurogenesis is blocked, while it is not significantly altered in the single mutants (Tomita et al., 2000). On the other hand, in the dorsal embryonic spinal cord, *Math1*, *Neurog1* and *Mash1(Ascl1)* are each expressed in discrete progenitor populations and are required for specifying the distinct types of interneurons arising from

progenitors marked by their expression (Bermingham et al., 2001; Gowan et al., 2001).

In addition, bHLH genes that have no proneural function have been implicated in specification of neuronal identity. For example, *Xenopus Xath5* expression occurs in differentiating cells after expression of *Xath3* and prior to *NeuroD* expression (Kanekar et al., 1997). *Xath5* is expressed in the pineal gland, retina and olfactory placode and promotes both retinal ganglion and olfactory receptor cell fates (Burns and Vetter, 2002; Kanekar et al., 1997).

Inhibition of glial fates

The generation of neurons and glia occurs from a common multipotent precursor pool (Temple, 2001). Neurons are formed first and the glia precursors are neuroepithelial cells that are kept back from neuronal differentiation. Proneural genes both promote neuronal fates and inhibit glial fates, and thus have a significant role in controlling the switch between neurogenesis and gliogenesis (Nieto et al., 2001; Vetter, 2001). For example, in double mutant mice for *Mash1(Ascl1)/Math3* and *Mash1(Ascl1)/Neurog2*, a block of neuronal differentiation is accompanied by premature gliogenesis, and overexpression of Neurog1 in cultured rat cortical stem cells causes inhibition of astrocytic differentiation (Sun et al., 2001; Tomita et al., 2000).

Negative regulators of neurogenesis

Regulation of the levels of the expression and/or activity of proneural genes is critical for the temporally and spatially controlled generation of the appropriate number of neurons. This is achieved by antagonistically acting "anti-neuronal" proteins that repress the function of transcription factors and thereby negatively control neurogenesis.

Hairy and Enhancer of Split transcriptional repressors

One class of proneural gene inhibitors is represented by the Hairy and E(spl) (Her)-related family of proteins (Davis and Turner, 2001; Kageyama and Nakanishi, 1997). While many vertebrate Her proteins function as effectors of the Notch signalling pathway and restrict the differentiation of neurons from neural precursors cells, some act independently of Notch signalling as prepatterning factors that spatially control neurogenesis (Artavanis-Tsakonas et al., 1999; Bae et al., 2005; Geling et al., 2003). For example, in the developing mammalian nervous system, *Hes1* and *Hes5* are expressed by precursor cells, and in the absence of *Hes1* differentiation of precursors in the telencephalon, olfactory placode, inner ear and retina is accelerated (Akazawa et al., 1992; Cau et al., 2000; Ishibashi et al., 1995; Sasai et al., 1992; Tomita et al., 1996; Zheng et al., 2000). Targeted disruption of both the *Hes1* and *Hes5* genes results in disorganisation of the structural integrity of the nervous system due to premature neuronal differentiation (Hatakeyama et al., 2004). Conversely, constitutive expression of Hes1 in neural precursors, using a retroviral vector, inhibits neuronal differentiation in the brain and the retina (Ishibashi et al., 1994; Tomita et al., 1996). Similarly, ectopic expression of Her4 protein in zebrafish suppresses differentiation of primary neurons (Takke et al., 1999).

The principal mechanism underlying the suppression of proneural bHLH factors by Her proteins involves a conventional DNA-binding transcriptional repression. Once bound to DNA, Her proteins recruit a transcriptional co-repressor known as Groucho (or TLE proteins in vertebrates) (Fisher and Caudy, 1998; Grbavec et al., 1998; Grbavec and Stifani, 1996; Paroush et al., 1994). This interaction is required for Her protein to function as transcriptional repressors and is mediated through a conserved tetrapeptide sequence, WRPW/Y, present at the extreme C terminus of all Her proteins. Most bHLH proteins, including the ones encoded by proneural and proneural-related genes, bind as either hetero- or homodimers to a

consensus DNA sequence CANNTG, known as an E-box. A number of Her proteins, such as *Drosophila* E(spl) and mammalian Hes1 and Hes5, were initially found to bind to an alternate sequence, CACNAG, known as an N-box (Akazawa et al., 1992; Sasai et al., 1992; Tietze et al., 1992). However, subsequent studies have demonstrated that some Her proteins (for example mammalian *Hes2* and *Hes1*) can bind to E-boxes as well as N-boxes (Hirata et al., 2000; Jennings et al., 1999). This raises the possibility that Her proteins can compete with positive-acting bHLH neuronal genes for binding to E-box target sequences. While competition for binding sites was shown in *in vitro* DNA binding assays between Drosophila E(spl) proteins and heterodimers of daughterless and lethal of scute bHLH activators, it has not yet been demonstrated in vertebrates (Jennings et al., 1999).

The other proposed mechanism of repression of neuronal bHLH activators is suppression of functional heterodimer formation by protein-protein interactions. Like other bHLH factors showing a tissue-restricted pattern of expression, proneural and proneural-related proteins bind DNA as heterodimers that are formed with ubiquitously expressed E proteins (Cabrera and Alonso, 1991; Johnson et al., 1992; Massari and Murre, 2000). It has been suggested that Her proteins can inhibit activity of neuronal bHLH proteins through competitive binding to the E-proteins and formation of heterodimers which are incapable of activating transcription through E-box sequences (Davis and Turner, 2001; Hirata et al., 2000; Kageyama and Nakanishi, 1997; Sasai et al., 1992).

Id proteins - inhibitors of differentiation/DNA binding

The activities of bHLH proneural genes are also regulated by Id proteins that act through a dominant negative mechanism. These proteins contain a HLH domain but lack the adjacent basic motif necessary for DNA binding (Benezra et al., 1990; Ellis et al., 1990). They have a high affinity for E proteins and can compete with bHLH proteins, forming heterodimers that cannot bind DNA (Campuzano, 2001; Massari and Murre, 2000; Yokota, 2001). Thus,

Id proteins, acting as negative regulators of bHLH factors, inhibit differentiation. The regulatory role of Id proteins is not limited to the developing nervous system, and occurs in many organs and tissues where bHLH factors are expressed.

There are four related members of the Id family, Id1 through Id4 (Benezra et al., 1990; Christy et al., 1991; Riechmann et al., 1994; Sun et al., 1991) which in mouse all are expressed in the CNS. *Id1* and *Id3* have similar expression patterns restricted to proliferating neuroblasts in embryonic stages. Their expression is generally reduced when neuronal differentiation progresses and not detectable in mature differentiated neurons. No apparent phenotype is detected in the CNS of mice lacking either Id1 or Id3, but when both *Id1* and *Id3* genes are inactivated, the mice die *in utero* and have small brains, indicative of functional redundancy (Lyden et al., 1999). The neuroblasts in *Id1-/-Id3-/-* foetal brains prematurely withdraw from cell cycle and upregulate post-mitotic neuronal cell markers. Similar to *Id1* and *Id3*, a high expression level of *Id2* is observed in proliferating neural precursor cells at early stages of CNS development but, interestingly, also occurs in migrating postmitotic neurons and in specific neurons in the adult brain (Jen et al., 1997; Neuman et al., 1993; Tzeng and de Vellis, 1998). Although *Id2-/-* mice do not show an obvious phenotype, Id2 overexpression inhibits the induction of neuron-specific genes (Toma et al., 2000; Yokota, 2001). In addition, Id2 has a role in neural crest cell fate specification, since retroviral overexpression of Id2 within the chick embryonic surface induces the disappearance of the ectoderm overlying the neural tube and conversion of these cells to neural crest (Jen et al., 1997). Like *Id2, Id4* is expressed in neural precursor cells as well as postmitotic/immature cells in the CNS (Riechmann et al., 1994). Adult *Id4-/-* animals exhibit a drastic alteration of brain morphology, with fewer neurons and glia, a reduction in brain size and abnormally enlarged ventricules (Ruzinova and Benezra, 2003). Therefore, Id proteins are required as inhibitors of differentiation to maintain the immaturity of neuroblasts and permit their proliferation until the appropriate time during development.

GENERATION OF NEURONAL DIVERSITY

The vertebrate CNS is composed of large variety of different types of neurons each defined by distinct morphology, axon projection and function. Understanding how neural progenitors acquire specific neuronal characteristics is an important issue. Several mechanisms have been identified that control this process. Some aspects of the involvement of proneural factors in specification of neuronal subtypes have been discussed previously. Here, I will introduce how neuronal determination is regulated by spatial patterning.

The CNS is patterned during development along the AP and DV axes. This patterning is initiated prior to the neural plate stage and becomes evident in the neural tube. Spatial patterning of the neuroepithelium is controlled by inductive signals provided in adjacent tissues by specialised cellular groups that act as organising centres (Echevarria et al., 2003). These signalling centres operate independently along the AP and DV axes of the neural tube. As a consequence, a grid-like coordinate system is established that provides positional information for every cell within the neuroepithelium (Lumsden and Krumlauf, 1996).

Dorsal-ventral patterning

The developing spinal cord is one of the most investigated and understood models of neural patterning along the DV axis. In the spinal cord, motor neurons are positioned ventrally, and neurons that process sensory inputs dorsally, and several types of interneurons are found in various dorso-ventral locations. DV patterning of the spinal cord is controlled by two signalling centres: the medio-ventrally located floor plate and the medio-dorsally positioned roof plate. The floor plate, composed of specialised glial cell, is induced by the notochord, a mesodermal rod of cells underneath the neural tube. Both the notochord and floor plate secrete Sonic hedgehog (Shh), a morphogen, found to be necessary and sufficient in vivo and in vitro

to induce differentiation of most ventral neuronal subtypes (Chiang et al., 1996; Ericson et al., 1996; Marti et al., 1995; Placzek, 1995; Roelink et al., 1995). Shh acts in a graded fashion over a long distance thereby providing positional cues to ventral neural progenitors. Neural progenitors express homeodomain proteins that function as intermediary factors in the interpretation of Shh graded signalling. Based on their expression domains and regulation by Shh, these proteins are divided into two classes: class I proteins (including members of the Pax, Dbx and Irx families) are repressed by different thresholds of Shh activity and, consequently, their ventral limits of expression delineate progenitor domains; class II proteins (including the genes of Nkx family) require Shh signalling for their expression, and their dorsal boundaries of expression define progenitor domains. Selective cross-repressive interactions between pairs of class I and class II proteins function to refine boundaries, establishing five cardinal progenitor cell domains that give rise to distinct neuronal subtypes. Although Shh signalling is required for generation of most ventral neuronal subtypes, it is not necessary for differentiation of two most dorsal classes of interneurons. These neurons are induced by retinoids derived from paraxial mesoderm (Pierani et al., 1999).

Six classes of interneurons are generated within the dorsal spinal cord. Neural progenitors that give rise to these neuronal subtypes are defined by non-overlapping expression domains of bHLH transcription factors as well as homeodomain-containing proteins (Bermingham et al., 2001; Chizhikov and Millen, 2005; Gowan et al., 2001; Mansouri and Gruss, 1998). In the roof plate, several members of the BMP family and the Wnt family (including *Wnt1* and *Wnt3a*) as well as Growth/Differentiation Factors (GDFs) are expressed that provide signals for dorsal spinal cord patterning. Genetic ablation of roof plate cells in mouse demonstrated that the roof plate is required for specification of only the three dorsal most classes of interneurons (Lee et al., 2000; Muller et al., 2002). The homeodomain transcription factor *Lbx1* which is specifically expressed in the three most ventral subtypes is critical for preventing these neurons from adopting more dorsal fates. Interestingly, even though the roof plate is responsible for

specification of only the most dorsal spinal neurons, it appears also to be important for patterning of the ventral spinal cord. BMP signalling controls the range of Shh activity and prevents ventralisation of the dorsal half of the spinal cord (Briscoe and Ericson, 2001; Jessell, 2000). For instance, exposure of neural progenitors cells in vitro to a fixed concentration of Shh in presence of BMP proteins results in a dorsal shift in the identity of neural progenitors and differentiated neuronal subtypes (Liem et al., 2000).

Anterior-posterior patterning

AP patterning operates in parallel with DV patterning to specify individual neuronal fates. This is evident in specification of spinal motor neurons that exhibit differences in their identities along the AP axis. All spinal motor neurons derive from a ventral progenitor domain and acquire distinct identities at different axial levels so that they innervate specific targets in the periphery. For instance, lateral motor neuron columns are produced at limb levels, whereas autonomic motor neuron columns are generated in the intervening thoracic region (Dasen et al., 2003; Jessell, 2000). The establishment of these regional neuronal subtypes is controlled by expression of distinct Hox genes at different AP locations, and this is induced by an posterior-to-anterior gradient of FGF signalling. For example, expression of either group 9 Hox genes or of group 6 Hox genes direct neurons to adopt forelimb lateral motor column or thoracic identity, respectively (Dasen et al., 2003).

AP patterning is also apparent in the specification of primary motor neurons in zebrafish. Zebrafish spinal motor neurons are serially distributed in bilateral clusters (intrasegmental) next to the adjacent somites. Three main subtypes can be identified by their rostrocaudal positions within each cluster as well as by their selective projections to different axial muscles, and by combinatorial expression of LIM homeodomain proteins, islet1, islet2 and lim3 (Appel et al., 1995). Transplanting individual motor neurons into the new location rostrocaudal position results in adoption of neuronal fate

appropriate for the new location, indicating the presence of positional cues along the AP axis (Appel et al., 1995; Eisen, 1991).

REGIONALISATION OF THE CNS

Regionalisation of the CNS is a critical phase in vertebrate neural development. The vertebrate nervous system that is initially induced is of an anterior character, and later transformed to the graded posterior character. Transformation occurs due to gradients of posteriorising signals, including FGFs, retinoic acid and Wnts that transform the anterior neuroectoderm in a planar way, that is from posterior to anterior (Cox and Hemmati-Brivanlou, 1995; Diez del Corral et al., 2002; Diez del Corral et al., 2003; Holowacz and Sokol, 1999; Kudoh et al., 2002; Maden, 2000; Yamaguchi, 2001). This involves localised signals that induce spatially-restricted expression of transcription factors that control regional identity. Regionalisation is initiated at early stages by signals from the organiser but other tissues, such as the extraembryonic anterior visceral endoderm in the mouse, are also involved in this process (Beddington and Robertson, 1998; Thomas and Beddington, 1996). Surgical ablation of the anterior visceral endoderm at early gastrulation stages leads to the loss of expression of forebrain markers such as *Hesx1* (Thomas and Beddington, 1996).

At neural plate and tube stages, local signalling centres form at specific AP locations within the neural tissue and control regional specification of the neuroepithelium. Three main signalling centres have been identified, the anterior neural ridge, the zona limitans intrathalamica and the isthmic organiser, which refine AP specification of domains in the brain primodium: the forebrain, the midbrain and the hindbrain (Echevarria et al., 2003; Kiecker and Lumsden, 2005; Wurst and Bally-Cuif, 2001). Two of these signalling centres, the zona limitans intrathalamica and the isthmic organiser, have been shown to be located at cell restriction boundaries.

The anterior neural ridge, identified in mouse and chick, is located at the border between the anterior neural plate and non-neural ectoderm and patterns the anterior forebrain by expressing FGF8 (Echevarria et al., 2003). Shh is also expressed in the proximity of the anterior neural ridge and is important for the regionalisation and specification of the ventral telencephalon (Crossley et al., 2001; Hammerschmidt et al., 1997). In zebrafish during gastrulation, the first row of cells of the neural plate acts as a local signalling centre and is essential for forebrain patterning through its secretion of Wnt antagonist, Tlc, which counteracts caudalising signals of Wnt8b emanating from the posterior diencephalon. (Houart et al., 2002; Houart et al., 1998).

The zona limitans intrathalamica lies in the caudal forebrain and separates the prethalamus from the thalamus. It expresses Shh which is essential for esablishment of the prethalamus anteriorly and thalamus posteriorly (Echevarria et al., 2003; Kiecker and Lumsden, 2005).

The isthmic organiser forms at the midbrain-hindbrain boundary and its position relies on interactions between midbrain that expresses genes of Otx family and the anterior hindbrain, expressing members of Gbx family (Echevarria et al., 2003; Kiecker and Lumsden, 2005). Ablation and grafting studies have demonstrated that the isthmic organiser is necessary for the development of whole midbrain, and of the anterior hindbrain, in particular of rhombomere 1 and its derived structures such as the cerebellum. The isthmic organiser expresses Wnt1 and FGF8, and FGF8 has been shown to be able to mimic the morphogenic activity of this signalling centre (Crossley and Martin, 1995; Crossley et al., 1996).

Although the forebrain exhibits some morphological subdivisions, out of three main regions of the vertebrate brain, forebrain, midbrain and hindbrain, only the hindbrain is characterised by overt segmental organisation.

HINDBRAIN PATTERNING

Metameric organisation of the hindbrain

The developing hindbrain is transiently segmented into 7 or 8 morphological units called rhombomeres (r). The formation of rhombomeres underlies the generation of a subsequent segmental pattern of differentiation of neurons and neural crest cells that have distinct positional values along the AP axis (Guthrie, 1996; Lumsden et al., 1991; Wilkinson, 1995).

Developing hindbrain neurons exhibit two patterns of cellular organisation: one of reticular neurons and the other of branchial motor neurons. Reticular neurons are repeated through sequential rhombomeres with little axial variation (Clarke and Lumsden, 1993). Branchial motor neurons are also present in each rhombomere but their differentiation and axon projections display a two-segment periodicity pattern (Lumsden and Keynes, 1989). These neurons first appear in the even-numbered rhombomeres, r2 (trigeminal), r4 (facial) and r6 (glossopharyngeal), and then in the odd-numbered rhombomeres. Neurons occupying a pair of segments project their axons laterally from even-numbered rhombomeres and innervate a single branchial arch lying in register with these segments. For instance, in the chick, the trigeminal nerve (Vth) made of r2 and r3 motor neurons, projects through the r2 exit point to the first branchial arch, while the facial (VIIth) nerve composed of r4 and r5 motor neurons, projects through the r4 exit point to the second branchial arch (Clarke and Lumsden, 1993; Lumsden and Keynes, 1989; Simon and Lumsden, 1993).

A segmental periodicity is also observed in hindbrain neural crest cell migration. Neural crest streams form only adjacent to even-numbered rhombomeres and migrate ventrolaterally into the first, second and third branchial arches, respectively (Lumsden et al., 1991). Here they give rise to cranial sensory ganglia and pharyngeal mesenchyme, and contribute to the formation of skeletal, mascular and vascular structures specific for the

architecture of each arch (Könteges and Lumsden, 1996; Le Douarin, 1983; Noden, 1988).

Although hindbrain segmentation is important for segmental specification and for proper patterning of cranial neural crest migration, grafting experiments in mouse and zebrafish have revealed plasticity of AP character in neural crest cells (Trainor and Krumlauf, 2000b; Trainor and Krumlauf, 2001). In heterotopic transpositions of cells within the hindbrain, graft-derived cells migrate into the nearest branchial arch without re-routing to their original axial level (Schilling et al., 2001; Trainor and Krumlauf, 2000a). In addition, expression of genes (*Hox* genes) characteristic of their original identity is downregulated in an ectopic branchial-arch environment but supported when mesoderm from their original axial level is co-transplanted. Therefore, segmental organisation of the hindbrain provides neural crest cells with an initial AP character, which prepares them to respond to a particular set of environment signals, e.g. from mesoderm, in each arch. Thus, neural crest cells are prepatterned but not committed.

Evidence of a functional importance for the metameric organisation of the hindbrain has been demonstrated in studies of respiratory rhythm regulation. In both chick and mouse, neuronal rhythm generators have been found that match the alternate rhombomere pattern. By isolating rhombomeres in ovo, inter-rhombomeric interactions have been identified that allow the formation or deletion of a specific rhythm-promoting module (Chatonnet et al., 2002b). Perturbations of the rhombomeric pattern irreversibly alter a modular organisation of the rhythmogenic network that can be reflected at birth by respiratory deficits (Chatonnet et al., 2002a; del Toro et al., 2001; Jacquin et al., 1996).

Segmentation transcription factors

Several transcription factors, including *Krox20* and *kreisler/Maf-b* have been implicated in the process of forming hindbrain segments, whereas *Hox*

transcription factors are involved both in segmentation and in specifying segment identity.

Krox20 encodes a zinc finger transcription factor that is expressed in r3 and r5 (Fig. 4) (Wilkinson et al., 1989a). Targeted inactivation of this gene results in the deletion of r3 and r5 which in consequence leads to the fusion of r2/r4/r6 territory (Schneider-Maunoury et al., 1997; Schneider-Maunoury et al., 1993). Overexpression of *Krox20* in chick can impose odd rhombomere character on r2, r4 and r6, indicating that *Krox20* is responsible for odd segment identity in the hindbrain (Giudicelli et al., 2001). *Krox20* is a key controller of the various regulatory genes in r3 and r5. It directly upregulates *Hoxb2*, *Hoxa2* and *EphA4*, and represses *Hoxb1* (Giudicelli et al., 2001; Nonchev et al., 1996; Sham et al., 1993; Theil et al., 1998; Vesque et al., 1996).

Another gene, *Mafb/kreisler* (*valentino* in zebrafish), a member of the basic domain-leucine zipper (bZip) family of transcription factors is expressed in r5 and r6 and acts upstream of *Krox20* in r5 (Fig. 4) (Eichmann et al., 1997; Manzanares et al., 1999a; Manzanares et al., 1999b; Moens et al., 1996). Loss of *Mafb* expression in the *kreisler* mouse mutant results in abnormalities in hindbrain segmentation with the developing hindbrain posterior to the r3/r4 boundary failing to exhibit morphological boundaries (Giudicelli et al., 2003; McKay et al., 1994).

Mafb/kreisler has been found to act as a direct transcriptional activator of *Hoxa3* and *Hoxb3* as well as a repressor of *Hoxb1* in r5 and r6 (Giudicelli et al., 2003; Manzanares et al., 2001; Manzanares et al., 1999a; Manzanares et al., 1997; Manzanares et al., 1999b). In addition, *Krox20* cooperates with *Mafb/kreisler* to regulate *Hoxb3* directly in r5 (Manzanares et al., 2002). *Hox* genes are responsible for AP patterning, whereas *Krox20* and *Mafb/kreisler* are segmentation genes, and thus regulation of *Hox* genes by *Krox20* and *Mafb/kreisler* couples hindbrain segmentation with the specification of A-P identity.

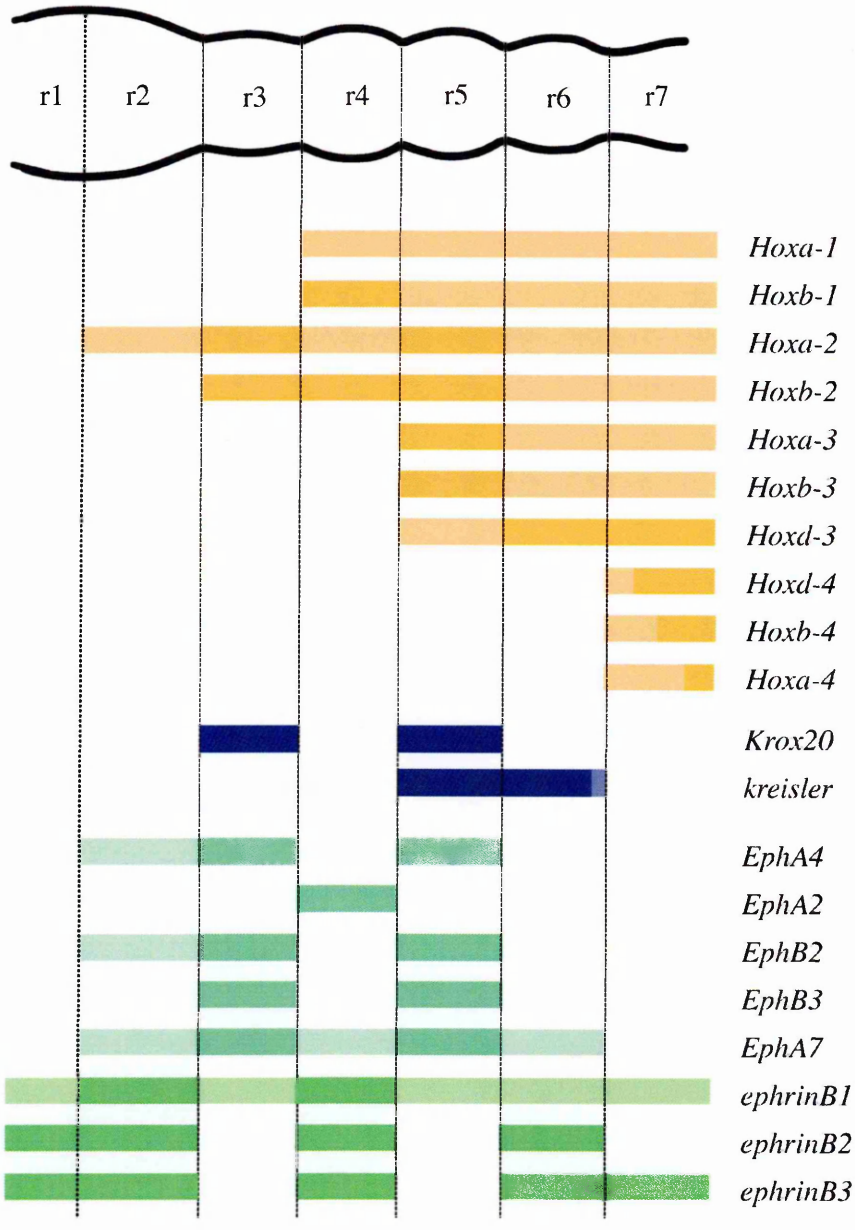


Figure 4. Summary of the correlation between gene expression and rhombomere boundary. Segmental gene expression in the hindbrain compiled from analyses in mouse and chick embryos. Rhombomeres are designated r1 to r7. The dashed vertical lines indicate rhombomere boundaries. Gene expression patterns are depicted in arbitrary colours, with the darkest colour indicating the highest level of expression. Related genes are indicated by the same colour: *Hox* homeobox genes in orange, other transcription factors in blue, *Eph* tyrosine kinase receptors in emerald green, *ephrin* ligands in green. (Modified from Lumsden and Krumlauf, 1996).

Recent studies in zebrafish have revealed components of the genetic hierarchy that regulates expression of *Mafb/kreisler* and *Krox20,* and consequently the patterning of the caudal hindbrain. Retinoic acid signals from the paraxial mesoderm establish, through the control of gene expression along the AP axis, a signalling centre in r4 where *FGF3* and *FGF8* are expressed (Gavalas and Krumlauf, 2000; Maves et al., 2002; Walshe et al., 2002). Retinoic acid induces a homeobox gene, *vhnf1,* which in cooperation with r4-derived FGF signals activates *valentino* expression in r5 and r6, and consequently *Krox20* expression in r5 (Hernandez et al., 2004). In this way *vhnf1* integrates local r4-FGF signals with global positional information provided by retinoic acid to specify r5 and r6 identities. Interestingly, while *vhnf1* is expressed posterior to the r4/r5 boundary and is involved in patterning of the posterior part of hindbrain in zebrafish, another transcription factor, *iro7* is expressed anterior to the r4/r5 boundary and promotes neurogenesis in the anterior hindbrain (Lecaudey et al., 2004). It is proposed that mutual repression between these two genes establishes the r4/r5 boundary.

Specifying rhombomere identity

The clustered homeobox containing transcription factors of the *Hox* family are key regulators which control the specification of positional identity within the hindbrain (Krumlauf, 1994). Vertebrate *Hox* genes are the orthologues of the homeotic complex (HOM-C) genes which govern parasegment identity in *Drosophila* (McGinnis et al., 1984; Scott and Weiner, 1984). They are organised in four chromosomal complexes and the genes of the first four paralogue groups of clusters a, b, c and d (with the exception of *Hoxd1*) are expressed in the hindbrain and the branchial region.

An important property of both *Hox* and *HOM* complexes is spatial colinearity, in which there is a correlation between the physical order of genes along the chromosome and their expression along the AP axis of the embryo (Lewis, 1978). In the vertebrate embryo, in general the more 3′ the

gene is in the complex, the more anterior is its limit of expression in the hindbrain, with the anterior expression boundary at the interface between rhombomeres. With a few exceptions, the rostral expression limits of paralogous groups vary with a two-rhombomere periodicity, whereas genes within a paralogous group usually have the same anterior limit (Fig. 4) (Wilkinson et al., 1989b). In addition to spatial colinearity there is temporal colinearity that is characteristic for *Hox* genes: the genes at the extreme 3'end of the clusters are activated the earliest (Izpisua-Belmonte et al., 1991).

Interfering with *Hox* gene expression can lead to homeotic transformations, and defects in the neural crest and its derivatives, establishing Hox genes as segment identity factors (Lumsden, 2004). In general, loss-of-function mutations lead to anteriorisation, whereas gain-of-function mutations have a posteriorising effect. For instance, inactivation of *Hoxb1* or of *Hoxb2* results in respecification of cells occupying r4 into an r2-like identity (Barrow and Capecchi, 1996; Goddard et al., 1996; Studer et al., 1996). Targeted disruption of *Hoxa2* leads to a homeotic transformation of the skeletal elements of the second pharyngeal arch that are derived from r4 neural crest into first arch derivatives (Gendron-Maguire et al., 1993; Rijli et al., 1993). Moreover, the cell fate of r2-r3 is partially switched towards an r1 identity (Taneja et al., 1996). Conversely, ectopic expression of *Hoxa1* or *Hoxb1* results in the transformation of r2 to an r4 identity (Alexandre et al., 1996; Bell et al., 1999; Zhang et al., 1994).

In addition to a role in specifying A-P identity, *Hox* genes play a role in segmentation and the maintenance of specific territories or cell populations. *Hoxa1* homozygous null embryos have a reduction of r4 and total or partial deletion of r5, *Hoxa1* $^{-/-}$/*Hoxb1*$^{-/-}$ embryos exhibit a loss of second arch neural crest leading to an absence of all second arch-derived structures, and *Hoxa3* mutation results in an absence of third arch derivatives (Barrow et al., 2000; Carpenter et al., 1993; Chisaka and Capecchi, 1991; Dollé et al., 1993).

In addition to discussed earlier regulation of specific subsets of *Hox* gene expression by *Mafb/kreisler* and *Krox20,* retinoids act as overall regulators of nested *Hox* expression, as many of the *Hox* genes contain retinoid-response elements within their regulatory regions (Dupé et al., 1999; Gould et al., 1998; Maconochie et al., 1996; Marshall et al., 1996; Marshall et al., 1994). Blocking retinoic acid signalling either by removing enzyme required for retinoic acid synthesis or by using antagonists to retinoic acid receptors results in anteriorisation of the hindbrain (Dupe and Lumsden, 2001; Niederreither et al., 2000). This is thought to reflect colinear differential sensitivities of 5′ and 3′ hox genes to increasing levels of retinoic acid (Papalopulu et al., 1991).

Restriction of cell mixing between rhombomeres

Subdivision of the hindbrain neuroepithelium into rhombomeres involves the formation of compartments as a result of cellular properties that prevent mingling between adjacent segments (Fraser et al., 1990; Guthrie et al., 1993; Wizenmann and Lumsden, 1997). The restriction of cell movements is achieved through the complementary expression of Eph receptors and their ligands, the ephrins, within the developing hindbrain. EphA4, EphB2 and EphB3 receptors are expressed in r3 and r5, while their interacting ligands, ephrin-B1, -B2 and -B3, are expressed in r2, r4 and r6 (Fig. 4) (Becker et al., 1994; Flenniken et al., 1996; Gale et al., 1996; Nieto et al., 1992). Evidence for the role of Eph receptors and ephrins in segmental restriction of cell intermingling comes from receptor blocking experiments in *Xenopus* and zebrafish and mosaic overexpression of these molecules in zebrafish embryos (Xu et al., 1995; Xu et al., 1999). This has been further confirmed by in vitro studies suggesting that bidirectional signalling between Eph receptors and ephrins at boundaries prevents intermingling of neighbouring cell populations (Mellitzer et al., 1999).

A SCREEN FOR NOVEL GENES EXPRESSED DURING HINDBRAIN PATTERNING

Despite the progress in gaining insight into the molecular and cellular mechanisms of hindbrain segmentation, large gaps remain in the understanding of this process. For instance, only a few components are known of the regulatory pathway of signalling and transcriptional factors that governs the process of hindbrain patterning and segmentation. The small number of known segmentation genes identified makes it difficult to understand how the hindbrain segmental organisation occurs and how this process is coupled with the specification of rhombomere identity by *Hox* genes, although *Mafb/kreisler* and *Krox20* are clearly involved. Little is also known how hindbrain segmentation is connected to cell differentiation and patterning of neural cell types. Therefore, there is a need for the identification and characterisation of further relevant genes and elucidation of how they are linked in cascades of cell signalling and transcriptional regulation, which lead to hindbrain patterning.

In order to identify novel genes with a potential role in hindbrain development, a subtractive cDNA library was made and screened by whole mount *in situ* hybridisation. The subtracted library consists of embryonic chick hindbrain cDNA library depleted of transcripts present in pre-gastrulation stage embryos (Harrison et al., 1995).

One of the clones, identified by this method, initially called V11, has a segmentally restricted expression pattern within the developing hindbrain.

AIM OF THE PROJECT

At the start, the focus of my project was to elucidate the potential role of V11 in hindbrain development. As the work had progressed it became clear that V11 encodes a novel BTB domain protein, whose involvement in vertebrate embryogenesis is not restricted to hindbrain patterning. Characterisation

and investigation of the function of this new gene in vertebrate development is the subject of my thesis.

MATERIALS AND METHODS

CLONING AND DNA METHODS

All molecular biology protocols were carried out as described in Sambrook *et al.* (Sambrook et al., 1989). Any alterations from this source are described below.

Bacterial strains and bacteriophage types

E. coli genotypes

DH5 α — *supE44* Δ*lac* U169(ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1

SOLR™ — *e14*$^-$*(McrA*$^-$*) Δ(mrcCB-hsdSMR-mrr)171sbcC recB recJ uvrC umuC::Tn5(Kan*^{r}*)lacgyrA96relA1thi-1 endA1*λ^R*[F'proABlacI*^{q}*Z ΔM15]*^{c}*Su*$^-$ (nonsuppressing)

XL1-Blue MRF' — *Δ(mrcA)183 Δ(mrcCB-hsdSMR-mrr)173 endA1 supE44 thi1recA1gyrA96relA1(lac)[F'proABlacIqZΔM15 Tn10(tet*^{R}*)*^{c} (Stratagene Cloning Systems)

Bacteriophage types

λZAPII — *λsbh*Iλ1°*chi*A131 (T *amp* ColE1 *ori* *lacZ* ' T3 promoter - polycloning site-T7promoter I) srIλ3°c*Its*857 *sr* Iλ4° *nin5* *srI*λ5° (Stratagene Cloning Systems)

ExAssist™ helper — (Stratagene Cloning Systems)

Production of competent bacterial cells

10ml of Luria-Bertani (LB) broth was inoculated with a single colony of DH5α cells and the culture was grown overnight on a gyratory shaker (set to 250rpm) at 37°C. Subsequently, the 10ml culture was sub-cultured into 200ml of fresh medium, prewarmed to 37°C, and incubated at 37°C until an OD_{550} of 0.45-0.55 was reached. Following storage on ice for 5 minutes the cells were pelleted by centrifugation at 2500rpm for 10 minutes at 4°C in a Sorvall GS-3 rotor. The supernatant was removed and the pellet was resuspended in 40ml of TfbI (30mM KOAc, 100mM RbCl, 10mM $CaCl_2.2H_2O$, 50mM $MnCl_2.4H_2O$, 15% glycerol, pH adjusted to 5.8 with 0.2M acetic acid, filter sterilised and stored at 4°C). The cells were chilled on ice for 5 minutes before further centrifugation at 2000rpm for 10 minutes at 4°C in a Sorvall HS4 rotor. The cells were resuspended in 8ml of TfbII (10mMOPS, 75mM $CaCl_2.2H_2O$, 10mM RbCl, 15% glycerol, pH adjusted to 6.5 with KOH, filter sterilised and stored at 4°C), placed on ice for 15 minutes, and then aliquoted and snap-frozen on dry ice. The tubes were stored at -70°C until use.

Transformation of bacterial cells

The cells were thawed on ice and 50µl dispensed into a prechilled 1.5ml microfuge tube. 1-5µl of DNA solution was added and mixed gently with the pipette tip. Following the 20 minutes incubation on ice, the sample was heat shock at 42°C for 90 seconds and then cooled on ice for 2 minutes. 200ml of LB broth was added and the cells were incubated for a further 20-30 minutes at 37°C before being plated out onto selective agar. The plates of bacteria were incubated at 37°C for 12-16 hours. If required, "blue/white" selection was performed according to Sambrook *et al* (Sambrook et al., 1989).

Isolation of plasmid DNA

Small scale preparation of plasmid DNA was performed according to the boiling plasmid 'mini-prep' method described by Holmes and Quigley (1981) or using the Qiagen™ Plasmid Miniprep purification kit. Large scale purifications utilised the Qiagen™ Maxi-kit, according to manufacturer's instructions.

Restriction enzyme digestion of DNA

All restriction enzymes were used according to the manufacturers' instructions (Promega, New England Biolabs), using the supplied buffer.

Blunt-ending, phosphatasing and ligation of DNA fragments

If insert and vector DNA fragments required for subcloning had incompatible cohesive ends, the following protocol was employed to convert both fragments to blunt ends. The single-stranded overhangs were removed using 0.1U of Klenow per µl in a solution of 10mM Tris (pH 7.4), 5mM $MgCl_2$ and 1mM of each of the four dNTPs, which was incubated at 37°C for 15 minutes. The DNA was then extracted and precipitated. Blunt-ended vector molecules (or those that had only been cut with a single restriction endonuclease) were treated with calf intestinal alkaline phosphatase from Boehouringer Mannheim according to the manufacturer's instructions to inhibit intramolecular ligation of vector without insert. Ligations were carried out using Rapid DNA Ligation Kit (Roche Molecular Biochemicals) according to the method of the supplier.

DNA sequencing and sequence analysis

DNA sequencing was performed at the Advanced Biotechnology Centre, Imperial College, London or in the sequencing facilities at the NIMR. Sequences were analysed for putative open reading frames using the Mac Vector 5.1 and 7.1 package (Oxford Molecular). Nucleic acid and protein

sequence similarity searches were performed using the gapped BLAST algorithms (Altschul et al., 1997) accessed via the internet at http://www.ncbi.nlm.nih.gov. Protein family searches (Pfam) were performed using http://pfam.wustl.edu, (TIGR) http://www.tigr.org/tdb/ and (Ensembl) http://www.ensembl.org/ web based software. Sequences were aligned with Lasergene (DNASTAR), CLUSTAL method, or Mac Vector 5.1/7.1 softwares.

RT-PCR and PCR

Preparation of RNA from embryonic tissue

For RT-PCR total RNA was extracted from chick or zebrafish embryos using TRIzol Reagent (GIBCO-BRL) according to the manufacturer's protocol.

First-strand cDNA synthesis

First-strand cDNA synthesis was generated with oligo(dT) or random hexamer primers using SuperScript™ First-Strand Synthesis System kit (Invitrogen) according to the manufacturer's manual.

PCR reaction

For PCR reactions, Expand High Fidelity (Roche) polymerase was used and the reaction mix prepared according to the manufacturer's instructions. Amplification was performed with approx 30 cycles of 30 seconds at 94°C, 1 minute at varying annealing temperature depending on the melting temperature of the primers, and 2 minutes at 72°C. The PCR reactions were gel electrophoresed to check for size and purity. For cloning, the band was excised and purified using either the QIAEX II Agarose Gel Extraction kit (Qiagen) or glass beads. The purified DNA was ligated into the plasmid and transformed. Clones with inserts were sequenced to confirm PCR accuracy.

Library screening

cDNA libraries in λZAPII: whole chick 12 - 15 somite stage and 8.5 dpc mouse embryo.

Both cDNA libraries constructed in λZAPII bacteriophage were screened with radiolabelled probe encompassing the region of the initially isolated chick V11 clone cDNA. XLI-Blue MRF' bacterial strain is a host for λZAPII phage. SOLR™ bacterial strain is a host for the excised phagemid containing an insert. The ExAssist™ interference resistant helper phage used to excise the phagemid from λZAPII vector is prevented from replicating in SOLR™ cells.

Preparation of XLI-Blue MRF' cells

A single colony of XLI-Blue MRF' cells was cultured overnight on a gyratory shaker (set to 250rpm) in 20ml LB broth supplemented with 0.2% maltose and 10mM $MgSO_4$. The cells were pelleted by centrifugation at 2000rpm for 10 minutes at 4°C in a Sorvall GS-3 rotor. The supernatant was discarded and the cells were resuspended at an OD_{600} of 1.0 (equal to $8x10^8$ bacteria/ml) in ice-cold 10mM $MgSO_4$.

Lambda bacteriophage library plating and lifts

Prior to plating, the titre of the library was determined. Tenfold serial dilutions of the phage in SM buffer were prepared. 400µl of XL1-Blue MRF' (OD=1.0) plating cells were mixed with an appropriate amount of phage stock in a 10ml tube. The cells were left at 37°C for 15 minutes to allow preadsorption of phage particles and then 6.5 ml of NZCYM top agarose at 42°C was added to each tube, quickly mixed by inversion of the tube and poured onto the surface of a prewarmed NZCYM plate (55°C) with swirling of the plate to ensure even coverage. The top agarose was allowed to set, and then the plates were inverted and incubated at 37°C overnight. The following morning plaques of dead cells caused by phage infection were seen in a bacterial lawn. The titre of the library was calculated as plaque

forming units (pfu) per unit of volume and then the library was plated at 7 x 10^4 pfu per 137mm plate. In total, 0.7 x 10^6 pfu were plated. Two replicas of each plate to be screened by hybridisation were taken by overlaying Colony/Plaque Screen™ hybridisation transfer membranes (DuPont NEN® Research Products) onto the plate and punching orientation marks through the filters and into the agar. The first filter was laid on the plate for 30 seconds, the second for 60 seconds. The filters were then processed according to the manufacturer's instructions.

Radioactive labelling of double stranded DNA

Fragments to be used as templates were excised from 1% agarose gels. These were labelled using the Megaprime DNA Labelling System (Amersham) according to the manufacturer's instructions. The labelling reaction was purified by gel exclusion chromatography through Sephadex G-50 (Pharmacia) equilibrated with TE (10mM Tris.Cl (pH 8.0), 1mM EDTA (pH 8.0), autoclaved).

Hybridisation of filters

Hybridisation was carried out overnight at 42°C in hybridisation buffer, containing 10% polyethylene glycol (PEG) 8000, 7% SDS, 4xSSPE, 100 μg/ml sheared denatured salmon sperm DNA and denatured radiolabelled chick V11 probe, at 42°C. Prior to the hybridisation step, filters were prehybridised in the same hybridisation mix (without a radiolabelled probe) for at least an hour. Following hybridisation, filters were washed consecutively in 4xSSC, 2xSSC and 1xSSC at 42°C, and finally in 1xSSC at 55°C for 1 hour each. Subsequently, the filters were wrapped in clingfilm and exposed to X-ray film (Kodak) at -70°C. Intensifying screens were used when required. Exposed film was developed in an automatic developing machine.

In vivo excision of the cloned insert from λZAPII phage

Positive phage plaques were cored from the agar plate and transferred to a sterile tube containing 500μl of SM buffer and 40μl of chloroform. In order to release phage particles into the SM buffer the tube was vortexed and incubated for 1-2 hours at room temperature or overnight at 4^0C. 200μl of XLI-Blue MRF$^-$ cells (OD_{600}=1.0), 200μl of λZAPII phage stock (containing > $1x10^5$ phage particles) and ExAssist™ helper phage (> $1x10^6$ pfu/ml) were combined in the tube and incubated at 37^0C. Following addition of 3ml of LB, the mixture was cultured for further 3 hours. Then the tube was heated at 65^0C for 20 minutes and spun down at 4000g for 5 minutes. The supernatant, containing the pBluescript phagemids packed as filamentous phage particles, was retained. 10 and 100μl of phagemid supernatant were combined with 200μl of SOLR cells (OD_{600}=1.0) in two separate tubes. Following 37^0C incubation for 15 minutes, 100μl of the mixture was plated on LB/Ampicillin plate. Colonies appearing after overnight culture contained the pBluescript double stranded phagemid with the cloned DNA insert.

Screening of libraries provided as high-density gridded filters: st. 3-6 (HH) chick embryonic cDNA (RZPD) and chicken genomic BAC (UK HGMP)

After the prehybridisation step, filters were hybridised with the ^{32}P radiolabelled probe in Church buffer (7% SDS, 0.5M sodium phosphate pH 7.2, 1mM EDTA, 0.1 mg/ml yeast t-RNA) at 65°C. For screening st. 3-6 (HH) chick cDNA library two probes were used, one generated from the entire initial chick V11 clone (cV11) and the other encompassing the 1.2 kb (between SmaBI and ScaI) of the 3'UTR region. For screening the chicken genomic BAC library only the first one was employed. After hybridisation, stringent consecutive washes were carried out with 1xSSC/0.5%SDS, 0.5xSSC/0.5%SDS and 0.1xSSC/0.5%SDS at 65°C for 1 hour each. Subsequently, the filters were wrapped in cling film and exposed to X-ray film (Kodak) at -70°C. Exposed film was developed in an automatic developing machine. Filters were scored for positive signals and the

potential positive clones depicted by the calculated coordinates were obtained either from RZPD or UK HGMP. The sequences of the clones identified in the chick embryonic cDNA library were obtained by sequence walking using the vector primers and gene specific primers. In case of the clones isolated from the chicken genomic BAC library, in order to acquire chick V11 5′ sequence, the sequencing was performed using three different upstream outwardly oriented primers:

5′-GCCTTGCCGTTTGACCTGACGCTC-3′

5′-CCCACGATGAAGTGGACGTCAG-3′

5′-TTGCTGTTGGCGATATTGGTGTTG-3′

To verify that the obtained sequence did not contain introns, two sets of primers (two 5′ end primers upstream of a putative V11 ATG codon and one 3′ end primer located within the initial clone V11 cDNA sequence downstream of a unique XhoI restriction site) were designed and used for PCR on stage HH16 whole chick embryo cDNA.

5′ end primers (with SalI restriction site):

5′-ACGCGTCGACCCCGCACTGCCCGGGTTC-3′

5′-ACGCGTCGACAGCCGGCGGTCCTCTCGC-3′

3′ end primer

5′-GCGCTGCGGCACCAGTCC-3′

The correct sized PCR products were gel purified, as described, digested with XhoI and ligated into the SalI/XhoI linearised V11/pSPORT1 plasmid for sequencing.

Hybridisation of filters for Northern, Southern and colony screens

For Northern blot analysis, the procedure was essentially the same as for the library screen filters (Hybond-N nylon membranes, Amersham) except for the following differences. Hybridisation was performed using hybridisation buffer comprised of: 5xSSC, 5xDenhardt's, 50% formamide, 1% SDS. Following overnight incubation at 42°C, the filters were washed with increasing stringency up to 0.1xSSC at 65°C. For Southern blots and colony

screens, hybridisation solution consisted of 0.2 M sodium phosphate (pH 7.2), 7%SDS, 1% BSA. Filters were hybridised overnight at 65° C.

Generation of constructs

The zBTBD6a1WT, ΔBTB and ΔPHR constructs were created by PCR-amplifying the *zBTBD6a1* cDNA with the following primers:

zBTBD6a1WT

5'-CCGGAATTCCGTTCTCATGCCCGCTGC-3'

5'-TACGTAACCCCCCTACTCTCTCTTC-3'

ΔBTB

5'-CCGGAATTCGGAGGCGCGAAATGCATG-3'

5'-TACGTAACCCCCCTACTCTCTCTTC-3'

ΔPHR

5'-CCGGAATTCCGTTCTCATGCCCGCTGC-3'

5'-TACGTACACAGCGTCCACGGTAGC-3'

The primers introduced restriction sites for EcoRI and SnaBI at the 5'- and 3'-ends, respectively. The PCR products were digested with EcoRI and SnaBI and subcloned into the corresponding sites of the pCS2+MT vector, each creating the constructs pCS2+MT/zBTBD6a1WT, pCS2+MT/ΔBTB and pCS2+MT/ΔPHR.

The following constructs have been already described:

Constitutively active Su(H), Su(H)-Ank (Wettstein et al., 1997)

lacZ (Xu et al., 1999)

neurog1 (Blader et al., 1997)

ZEBRAFISH EMBRYO CULTURE AND MANIPULATION

Fish maintenance

Adult zebrafish were maintained in 10h night/14h day cycles. Wild-type, *mib*^{ta52b} mutant (Jiang et al., 1996), and *elav3/HuC-GFP* transgenic (Park et al., 2000b) zebrafish embryos were obtained by natural spawning and raised at 28°C, as described (Westerfield, 1993). For stages earlier than 24h, embryos

were raised at 22°C or incubated at 28°C for first 9 hours and then at 18°C until the right stage. The stage was determined by number of somites and hours postfertilisation (Kimmel et al., 1995). Embryos were manually dechorionated after fixation.

Morpholino oligonucleotide and RNA injections

Injection protocol

0.8-6.5 ng of morpholino oligonucleotide was injected into the yolk of 1-4-cell stage embryos. For misexpression experiments, 0.1-0.4 ng of capped RNA was injected into one cell at either the 2-cell or the 1-cell stage. Injections were performed using a Picospritzer II (General Valve Corporation). The needles used for injection were constructed from 1.0mm external diameter glass capillaries with an internal filament (Harvard Apparatus, Kent) with a David Kopf Instruments needle puller.

Morpholino oligonucleotides

Morpholino oligonucleotides (MOs) were purchased from Gene Tools, LLC (Oregon). MOs were kept as 1mM stock solutions by adding pure water (Sigma) and further dilutions were made in pure water for injections. The following MO sequences were used:

zBTBD6a1 MO: 5'-CGCAGCGGGCATGAGAACGAGCGAG-3'
zBTBD6a2 MO: 5'-GTACAGTTCCGCCGCCATCCTCTTC-3'
neurog1 MO: 5'-ATACGATCTCCATTGTTGATAACCT-3'
her4 MO: as described (Pasini et al., 2004)
standard control MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3'

Generation of capped RNA for injection

Synthesis of capped mRNA for *in vivo* injection was carried out as previously described (Moon and Christian, 1989). Briefly, sense transcripts were

synthesised *in vitro* using 5 μg of linearised plasmid DNA and 50 units of SP6 RNA Polymerase (Roche) in the presence of: 10 mM DTT; 1 mM of ATP, CTP and UTP; 0.1 mM GTP (Pharmacia Biotech); 0.5 mM RNA cap analog (Roche); 10 μl 5x Transcription Optimised buffer (Promega) and 40 units of RNasin Ribonuclease Inhibitor (Promega) in a final volume of 50 μl. The reaction was incubated at 37 °C for at least 2 hours. To remove the DNA template after the RNA synthesis, 5 units of RQ1 DNase (Promega) were added to the reaction and incubated at 37 °C for 30 minutes. The synthetic RNA was purified from unincorporated nucleotides using Microspin G-50 columns (Amersham), ethanol precipitated, resuspended in Sigma pure water and analysed by agarose gel electrophoresis.

Detection of transcripts by in situ hybridisation

In situ hybridisation was performed using "Protocol Four" as previously described (Xu and Wilkinson, 1998), with the following modifications. In some cases BM Purple (Roche) was used instead of NBT/BCIP in the colour reaction. To detect transcripts with a fluorescent signal, TSA™ (Tyramide Signal Amplification) Plus Flourescence Systems (PerkinElmer) was used. This required usage of anti-digoxigenin (DIG) Horseradish Peroxidase antibodies (Roche). TSA system employs HRP to catalyse the deposition of a flourophore-labeled tyramide amplification reagent onto tissue. After the colour was developed, embryos were briefly fixed in 4% paraformaldehyde in PBS, then rinsed in PBS-0.1% Tween20 (PBST), stored and flat-mounted in 70% glycerol in PBST. For two-colour in situ hybridisation, both DIG- and fluorescein-labelled probes were hybridised simultaneously. The fluorescein-labelled probe was detected with anti-fluorescein-AP antibodies (Roche). Incubations with anti-DIG and anti-fluorescein-AP antibodies, and the colour development for each probe were performed sequentially. After the first colour reaction carried out with BM Purple, the alkaline phosphatase was inactivated by treating the embryos with 100% methanol for 20 minutes. Then the embryos were incubated with antibodies against the second probe and the signal was detected with Fast Red tablets (Roche).

Anti-sense RNA probes

The RNA probes used for detection of *BTBD6/BTBD3* transcripts are listed below. The source of the DNA template used for probe generation, the vector, the restriction site used for linearization and the RNA polymerase used for transcription reaction are tabulated below.

Gene	DNA source	Vector	linearisation/ polymerase
cBTBD6	cloned	pSPORT1	SalI/SP6
cBTBD3	BU444464 EST	pBluescript II KS+	NotI/T3
	BU274818 EST	pBluescript II KS+	NotI/T3
	BU389269 EST	pBluescript II KS+	NotI/T3
zBTBD6a	BG308275 EST	pBluescript SK-	BamHI/T7
zBTBD6b	BI350992 EST	pZIPLOX	SalI/SP6
	AI437221 EST	pSPORT1	SalI/SP6
zBTBD3a	AI883008 EST	pME18S-FL3 subcloned to EcoRI/PstIDelta pCS2+	EcoRI/T7
zBTBD3b	RT-PCR	transcription of PCR product	T7

In the case of *zBTBD3b*, a DNA template was obtained using RT-PCR with primers based on the predicted cDNA sequence provided by Ensembl. The T7 RNA promoter sequence was included in the 3′ primer to enable generation of the RNA probe of the PCR product. Sequencing was carried out with a T7 primer to confirm PCR accuracy.

5′ end primer:

5′-CGACACCGTCTTGGCAAC-3′

3′ end primer:

5′-AATACGACTCACTATAGGGAGAACCACGCTGGCAGTG-3′

The following probes have been previously described:

krox20 (Oxtoby and Jowett, 1993)

neurog1 (Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998)
pax2a (Krauss et al., 1991)
deltaA, *deltaB* and *deltaD* (Haddon et al., 1998)
neurod (Korzh et al., 1998)
neurod4 (Park et al., 2003; Wang et al., 2003)
isl1 (Inoue et al., 1994; Korzh et al., 1993)

Fluorescent in situ hybridisation combined with immunochemistry

Fluorescent in situ hybridisation to detect *zBTBD6a* transcripts in *elav3/HuC-GFP* transgenic embryos was performed essentially as described above. GFP protein was detected with anti-GFP (1:500, Molecular Probes) rabbit primary and Alexa Flour 594 goat anti-rabbit (1:500, Molecular Probes) secondary antibodies. Following incubation with anti-DIG HRP antibodies, the embryos were blocked in PBST containing 5% goat serum for at least 1 hour, then incubated overnight at 4°C with the anti-GFP antibody diluted in PBST with 2% goat serum. After several washes in PBST, the embryos were incubated with Alexa Flour 594 antibodies in PBST with 2% goat serum, then washed in PBST and incubated with TSA-Plus flourescein dye for 10 minutes. Following several washes in PBST, the embryos were flat-mounted in Vectorshield medium (Vector) and observed with confocal microscopy.

Detection of β-galactosidase and in situ hybridisation

Embryos were fixed in 4% paraformaldehyde in PBS, then dechorionated. After washing several times in PBST, β-galactosidase was detected by staining in X-gal. The embyos were refixed in 4% paraformaldehyde in PBS for 15 minutes, then in situ hybridisation was carried out using BM Purple as substrate.

Detection of *zBTBD6a1* and *zBTBD6a2* transcripts by RT-PCR

Total RNA extraction was carried out, as previously described. PCR-amplification was performed as described previously with the annealing temperature of 56°C and the following primers:

zBTBD6a1 5'-GAAGCGGGCAAGCAAGCA-3'

5'-TTCACCAGGAGGTCCAAC-3'

zBTBD6a2 5'-CACAAGTCCAGCCCTCGT-3'

5'-AATACGACTCACTATAGGGCTCAGATTTTGTGGGTTAGT-3'

(includes T7 promoter – not utilised)

PRODUCTION AND DETECTION OF RECOMBINANT PROTEINS

Cell culture and stable transfection

Human embryonic kidney cells (HEK293) were grown in DMEM supplemented with 10% FCS, 2 mM glutamine, 50μg/ml gentamicin at 37°C, 5% CO_2. Stable cell lines expressing zBTBD6a1WT, ΔBTB, ΔPHR were generated by co-transfection of zBTBD6a1WT/pCS2+MT, ΔBTB/pCS2+MT, ΔPHR/pCS2+MT vectors along with pcDNA3 vector (Invitrogen) in 1:10 ratio. Cells were selected in the presence of 900μg/ml of G418 for 2 weeks. Expression of zBTBD6a1WT and its truncated versions was confirmed by Western blotting and immunocytochemistry using anti-myc antibodies.

Immunocytochemistry

Cell were fixed with 4% paraformaldehyde, 4% sucrose in Dulbecco-PBS (D-PBS) for 15 minutes at room temperature, rinsed once with D-PBS, then incubated with 50 mM ammonium chloride in Dulbecco-PBS (D-PBS) for 10 minutes at room temperature and rinsed again. Cells were permeabilised for 5 minutes with ice cold 0.1% Triton X-100 in D-PBS at 4°C. After washing, blocking was done for 30 minutes at room temperature or overnight at 4 °C with 2% bovine serum albumin, 4% donkey serum (Jackson Immuno Research). Followed by incubation with primary antibodies for 60 minutes at

room temperature and then by washing, and incubation with secondary antibodies for 30 minutes at room temperature. After washing, samples were mounted using the ProLong antifade kit (Molecular Probes). Images were acquired using an inverted fluorescent microscope IX70 (Olympus) equipped with a digital camera.

Cell lysis and immunoprecipitation.

Cells or embryos were lysed in lysis buffer: 1.0% Nonidet NP-40, 50 mM HEPES, pH 7.5, 100 mM NaCl, 50 mM NaF, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1x Halt-protease inhibitor cocktail (Pierce) for 10 minutes on ice and centrifuged at 10000g for 10 minutes For Western blot analysis 4x SDS sample buffer and 10x Reducing agent (Invitrogen) were added to the cell lysates and incubated 10 minutes at 65°C. For immunoprecipitation cell lysates were incubated with 2μg of antibodies pre-bound to 10μl of protein-G Sepharose (Amersham Biosciences) for 1 hour or overnight at 4°C on a rotator. To remove unbound proteins, sepharose was washed 4 times with lysis buffer and once with 10 mM Hepes/NaOH, pH 7.0. Then 10μl of 2x SDS sample buffer with or without 2x Reducing agent (Invitrogen) was added to sepharose and samples were incubated for 10 minutes at 65°C. Sepharose was settled by centrifugation and eluted proteins collected by aspiration using gel-loading tips. Immunoprecipitates were resolved by SDS-PAGE gel electrophoresis under reducing or non-reducing conditions as required.

Preincubation of protein G Sepharose with antibodies

Sepharose was washed 1-2 times with Tris Buffered Saline (TBS) and 2μg of antibodies were added to 10μl of Sepharose in 250-300μl of PBS or TBS containing 0.01% Triton X-100. Samples were incubated for 1 hour or overnight at 4°C on a rotator. Sepharose was washed 1-2 times with the lysis buffer remove unbound antibodies and can be used immediately or stored overnight at 4°C.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Polyacrylamide gel electrophoresis of proteins was carried out using XCell SureLock Mini-Cell (Invitrogen) on NuPage 4-12% Bis-Tris gels (Invitrogen) according to manufacturer's instructions. After SDS-PAGE electrophoresis, proteins were transferred to Immobilon-FL PVDF membrane (Millipore) using the XCell II Blot module (Invitrogen) according to manufacturers' instructions. The membrane was blocked in blocking buffer (LI-COR Biosciences) for 1 hour at 25°C or overnight at 4°C with agitation. The membrane was then incubated with primary antibodies in the blocking buffer containing 0.1% Tween 20 for 1 hour and then washed 4 times for 5 minutes in TBS containing 0.1 % Tween 20 with agitation. The membrane was then incubated 1 hour with IRDye800 or IRDye700DX-conjugated secondary antibodies in the blocking buffer containing 0.1 % Tween 20 and 0.01 % SDS and washed 4 times for 5 minutes in TBS containing 0.1 % Tween 20 followed by rinsing with TBS. The membrane was then dried and the stained proteins were detected using the Odyssey infrared Imaging system.

Antibodies

Monoclonal 9E10 anti-myc antibodies (Cat.No. sc-40), rabbit polyclonal anti-myc antibodies (Cat.No. sc-788), and goat polyclonal anti-Cul-3 antibodies (Cat.No. sc-8556) were obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-MAPK antibodies (Cat.No. M-5670) were from Sigma. IRDye800- and IRDye700DX-conjugated secondary antibodies were from Rockland.

IDENTIFICATION AND SEQUENCE ANALYSIS OF NOVEL BTB DOMAIN-CONTAINING GENES

This chapter describes molecular cloning and characterisation of a novel BTB domain-containing gene, first isolated from embryonic chick cDNA library under the provisional name, V11, and subsequently identified in mouse, human and zebrafish .

Initial work involving recovery of the full-length chick V11 cDNA and cloning of one of the mouse homologues is presented below in a chronological manner that explains the logic of undertaken steps.

CLONING AND MOLECULAR ANALYSIS OF CHICK V11

Isolation of chick clone V11

Clone V11 was initially isolated in our laboratory from a subtracted embryonic chick (HH stage 9 to 12 (Hamburger and Hamilton, 1951)) hindbrain cDNA library. This subtracted library was obtained by constructing a directionally cloned chick hindbrain cDNA library and removing ubiquitously expressed genes through a subtractive hybridisation, using as driver a chick cDNA library, constructed from embryos prior to formation of the primitive streak (Harrison et al., 1995).

The first step in the project was to obtain chick V11 (cV11) nucleotide sequence. The V11 clone was sequenced from each end, using the vector and gene specific primers, and a contiguous sequence of 2156 nucleotides in length was assembled. A putative stop codon and an accompanying upstream open reading frame of 882 nucleotides were present, but the ATG start codon was not identified. The termination codon was followed by a 1247 nucleotide long untranslated region (UTR) ending with a poly (A) tail that was preceded 40 bp upstream by the AATAAA consensus polyadenylation signal (Fig. 5). The absence of the initiation translation

Figure 5. Nucleotide sequence of the initial chick V11 clone isolated from a hindbrain cDNA library. The open reading frame is indicated in triplet codons and the predicted amino acids are shown by the single letter designation. A start codon (ATG) has not been identified and the termination codon (TGA) at position 879 is indicated by an asterix. A putative polyadenylation signal and a poly(A) tail are indicated in pink and green, respectively. The BACK domain and partial PHR motifs are labelled in red and blue, respectively.

```
                                        CC CAG AGC CGG CTC TTC GAG      20
                                           Q   S   R   L   F   E
GAG CCG GAG CTG ACG CAG CGC TGC TGG GAG GTG ATC GAC GCT CAG      88
 E   P   E   L   T   Q   R   C   W   E   V   I   D   A   Q
GCC GAG ATG GCG CTC AAG TCG GAA GGG TTC TGT GAG ATC GAC CTG     133
 A   E   M   A   L   K   S   E   G   F   C   E   I   D   L
CAG ACG CTG GAG ATC ATC GTG ACG CGG GAG GCC CTC AAC ACC AAG     178
 Q   T   L   E   I   I   V   T   R   E   A   L   N   T   K
GAG GTG GTG GTG TTC GAG GCC GTT CTC AAC TGG GCG GAG GCC GAG     223
 E   V   V   V   F   E   A   V   L   N   W   A   E   A   E
TGC AAG AGG CAG GGG CTG CCG GTG ACG CCG CGC AAC AAG AGG AAC     268
 C   K   R   Q   G   L   P   V   T   P   R   N   K   R   N
GTG CTG GGG AAG GCG CTG TAC CTG GTG CGG ATC CCC ACG ATG ACT     313
 V   L   G   K   A   L   Y   L   V   R   I   P   T   M   T
CTG GAA GAG TTC GCC AAC GGG GCG GCC CAG TCC GAC ATC CTC ACG     358
 L   E   E   F   A   N   G   A   A   Q   S   D   I   L   T
CTG GAG GAG ACG CAC AAC ATA TTC CTG TGG TAC ACG GCC GCC AAC     403
 L   E   E   T   H   N   I   F   L   W   Y   T   A   A   N
AAA CCC AAA CTC GAG TTT CCG CTG ACA AAG AGG AAA GGA CTG GTG     448
 K   P   K   L   E   F   P   L   T   K   R   K   G   L   V
CCG CAG CGC TGC CAT CGC TTT CAG TCG TCT GCG TAC CGC AGT AAC     493
 P   Q   R   C   H   R   F   Q   S   S   A   Y   R   S   N
CAG TGG AGG TAC CGG GGG CGG TGC GAC AGT ATT CAG TTT GCC GTA     538
 Q   W   R   Y   R   G   R   C   D   S   I   Q   F   A   V
GAC AAA CGG ATA TTT ATA GCG GGA CTG GGA TTG TAT GGG TCG AGT     583
 D   K   R   I   F   I   A   G   L   G   L   Y   G   S   S
TGT GGC AAA GCT GAA TAC AGC GTC AAA ATT GAA CTG AAG CGC TTA     628
 C   G   K   A   E   Y   S   V   K   I   E   L   K   R   L
GGA GTC GTC CTT GCT CAG AAT CTG ACA AAG TTT ACC TCC GAC GGC     673
 G   V   V   L   A   Q   N   L   T   K   F   T   S   D   G
TCC AGT AAT ACC TTC TCG GTG TGG TTT GAG CAC CCG GTG CAG GTT     718
 S   S   N   T   F   S   V   W   F   E   H   P   V   Q   V
GAG CAG GAC ACG TTC TAC AAT GTA AGT GCC ATT CTG GAT GGC AAT     763
 E   Q   D   T   F   Y   N   V   S   A   I   L   D   G   N
GAG CTC AGT TAC TTC GGG CAG GAG GGA ATG ACT GAA GTG CAG TGC     808
 E   L   S   Y   F   G   Q   E   G   M   T   E   V   Q   C
GGG AAA GTG ACC TTC CAG TTC CAG TGC TCC TCG GAC AGT ACC AAC     853
 G   K   V   T   F   Q   F   Q   C   S   S   D   S   T   N
GGG ACT GGG GTA CAA GGA GGA CAA ATC CCT GAG CTC ATT TTC TAT     898
 G   T   G   V   Q   G   G   Q   I   P   E   L   I   F   Y
GCA TGATGCATTTCACCTTGATTGTATTCCAGTACTGCAACGCTGCACATCTAAGGGAT    919
 A   *
TCTTCAGTTTTGCTACAGAACTGTCAAGCAGTATGGGAATGTTACGCTACTTACCTATCT
GCTACATCANGCTATACCCAATTAAGTGAAAGGAATGCTCTCAAATTTAATCTTATTTTA    979
TTTATTCATAAGCTATTTGACTTGATTAAGACTGCAGCGAGCAGAAAAATGTTAAATTTT   1039
GCACATGCAGTGCATTTATTTTGTATATAGATAACTAACTTGCAGACTGCAGCTGACTCA   1099
AACAGAATGTTCTAAACTAGAGCAGTTTATGAACCGGTGAAATATAAAACATTTCTACTT   1159
GCCCTAAATCCCGCGTACTGCCCCTTAATGATGTTAGGGCCTGTCTGCTAATGCAGCTAC   1219
AGCGTAGTTAACGGGTGGTAACACCGCTGGTTGCCTGTCCTCTGCAAGGTGTGAGCAGTG   1279
CTGCACGTAGAAAGGCTCCCCCACCTGCAGAGCCCATCCATCAGGCAGCCCTCATTGTCC   1399
TTCACCGAGAAGGACAGGAGGAAGGAAGCTCAGAGGTTGGCTATGGGTCGCCCAAGCTGA   1459
ACAGAAAAAACCTGAGCATAACCAGCACTGATAAAACCTCGTGCTTACTGCTTGTAAGCT   1519
TCTTCTGTCCCTTTGCATTCTGCTTTTGGCACCACCTGTGTTAACCTCCACCCTGCCCAG   1579
CAAACAGCAGCAGTAACACCTGCACTGTCAAAACACACCAGACTTGAGCAGCCAATGTCA   1639
GGACAGACAGATAAAGCCTCGTGCAGCCACAAGCTGTAGCACTGACAGCCACTACTTTAA   1699
ATATACATCACAGCTCGCCTTTAGGAGCAGACAGATTTACAATACGGTCACTCAAAATTA   1759
CCGAGGGAAGCCACCATAAGGAAAAGCTGCCAGGCCTTTTTTCTCCCTGTGTAGTTTTGG   1819
GGGTATCAGTCATTTTTATAGCTAGCATCCTTCAGTTTTCTCANAGACTGACTAAGATAG   1879
GTGTATTCCTCAAGCATCTGACCACCTTTACAGCTGAACCAATGTAAACCAATGTACAAA   1939
ACATTGTAATCATGATCCAATTTACCTTTTTTTTTTTGCACATAACAGGTACCTCTACCT   1999
TCAACTTCAAGTTTTCTGTTTGTTTTTTTTTTTTACCTGAGCTGCATTATTTTTTTACCT   2059
TGAAACTAAACTGTAATAAAATTTTGCCTGTTCTCTCAGAGAAAGTAAAAACAGGAAAAG   2119
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                 2156
```

sequences suggested that V11 represents an incomplete cDNA clone with 5' end sequences missing. To determine the expected transcript size of cV11, Northern analysis was performed. Total RNA isolated from HH stage 9-12 chick embryos was hybridised with radiolabelled V11 probe cDNA and a 3 kb transcript was detected, which suggested that about 800 nucleotides upstream of the known V11 sequence were missing (Fig. 6).

In order to characterise chick V11 it was essential to obtain the V11 full-length cDNA. Finding putative V11 homologues in other species and acquiring their nucleotide and amino acid sequences was helpful for a comparative analysis of the sequentially obtained V11 5' sequences.

The predicted, partial chick V11 peptide sequence was used for BLAST similarity searches in the NCBI nonredundant protein database (http://www.ncbi.nlm.nih.gov/BLAST/(Altschul et al., 1997). At the time, both searches identified two human (Gen-Bank Accession Nos: BAA76796, gi3334982) and one *Caenorhabditis elegans* (Acc. Nos: CAB01179) proteins with significant homology scores (Fig. 7).

The human protein gi3334982 had 46.2% identity with the chick V11 protein and represented a translation of a predicted ORF derived from a 3.5 Mb contiguous nucleotide sequence in human 19p13.3 chromosome. A similar level of homology was found between the chick (39.4% identity) V11 protein and the *C. elegans* CAB01179 protein, which represented a conceptual translation of the predicted coding sequences obtained from sequencing of randomly selected *C. elegans* genomic clones (Consortium, 1998).

Most related to the mouse and chick V11 predicted proteins (78% and 80% identity, respectively) was the human protein BAA76796, which was the conceptual translation of a randomly selected cDNA clone derived from a human brain cDNA library (Nagase et al., 1999). Comparison of the

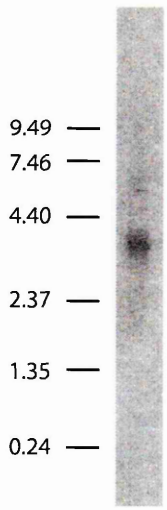


Figure 6. Northern blot analysis of total chick RNA. 10 μg of total RNA, isolated from HH stage 9-12 chick embryos was separated by denaturing agarose gel electrophoresis, blotted onto a filter and hybridised with a 32P labelled chick V11 cDNA probe. A single 3kb transcript was detected.

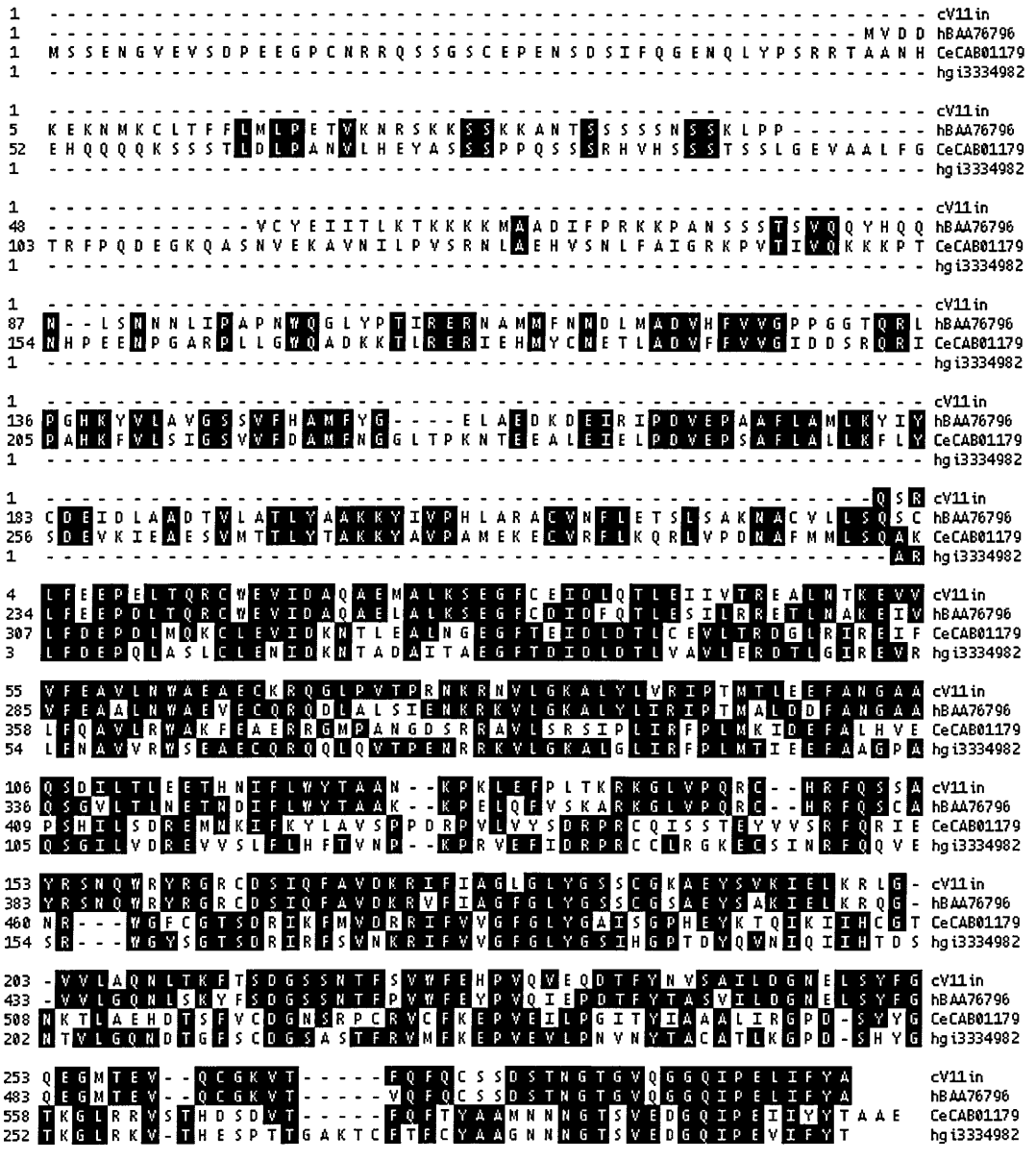


Figure 7. Multiple amino acid alignment of the initial cV11 and homologues. Multiple amino acid sequence alignment (MacVector™ software, ClustalW method) of the initial chick clone V11 (cV11in) and homologues identified in the sequence databases by a BLAST homology search. Black background indicates identical amino acids. Human protein BAA76796 (hBAA76796) is most related to the cV11in predicted partial protein (81.2% identity over the overlapping region). Ce, *Caenorhabditis elegans*.

BAA76796 amino acid sequence with the translation of the 5′ extended ORF of the successively obtained V11 clones was used to determine the full-length V11 cDNA.

Screening of other chick cDNA libraries

Initial attempts to recover further 5′ sequences of the chick V11 cDNA through RACE (rapid amplification of cDNA ends) proved to be unsuccessful. Therefore, screening of a number of chick cDNA libraries was undertaken.

Screening of an embryonic (HH stage 3-6) whole chick cDNA library (Resource Centre of the German Human Genome Project)

In order to isolate a cDNA clone with a longer ORF, two radiolabeled probes were generated from the initial clone V11, one covering the full length of the isolated V11 clone and the other spanning the 1.2 kb of the 3′ end UTR. These two probes were used independently to screen chick (HH3-6) cDNA library spotted filters.

The same eleven positively hybridising clones were identified using either of the probes and two longest ones, V11.9G and V11.2G, had inserts of approximately 2.7 and 2.9 kb, respectively. Nucleotide sequences of both inserts, by sequence walking, using vector primers and gene specific primers, were obtained. A comparative nucleotide sequence analysis of the original V11 and isolated clones, revealed that V11.9G extended a further 495 bp 5′ to original sequence, whereas V11.2G was longer by 730 bp. There were no initiation translation codons identified within the sequence of either of the clones and the analysis of the 5′ end sequences for splice donors and acceptors revealed that both sequences contained introns (one intron in V11.9G and two introns in the V11.2G sequence), suggesting that the isolated clones were of genomic origin. (Fig. 8) Therefore, effectively, the V11 cDNA

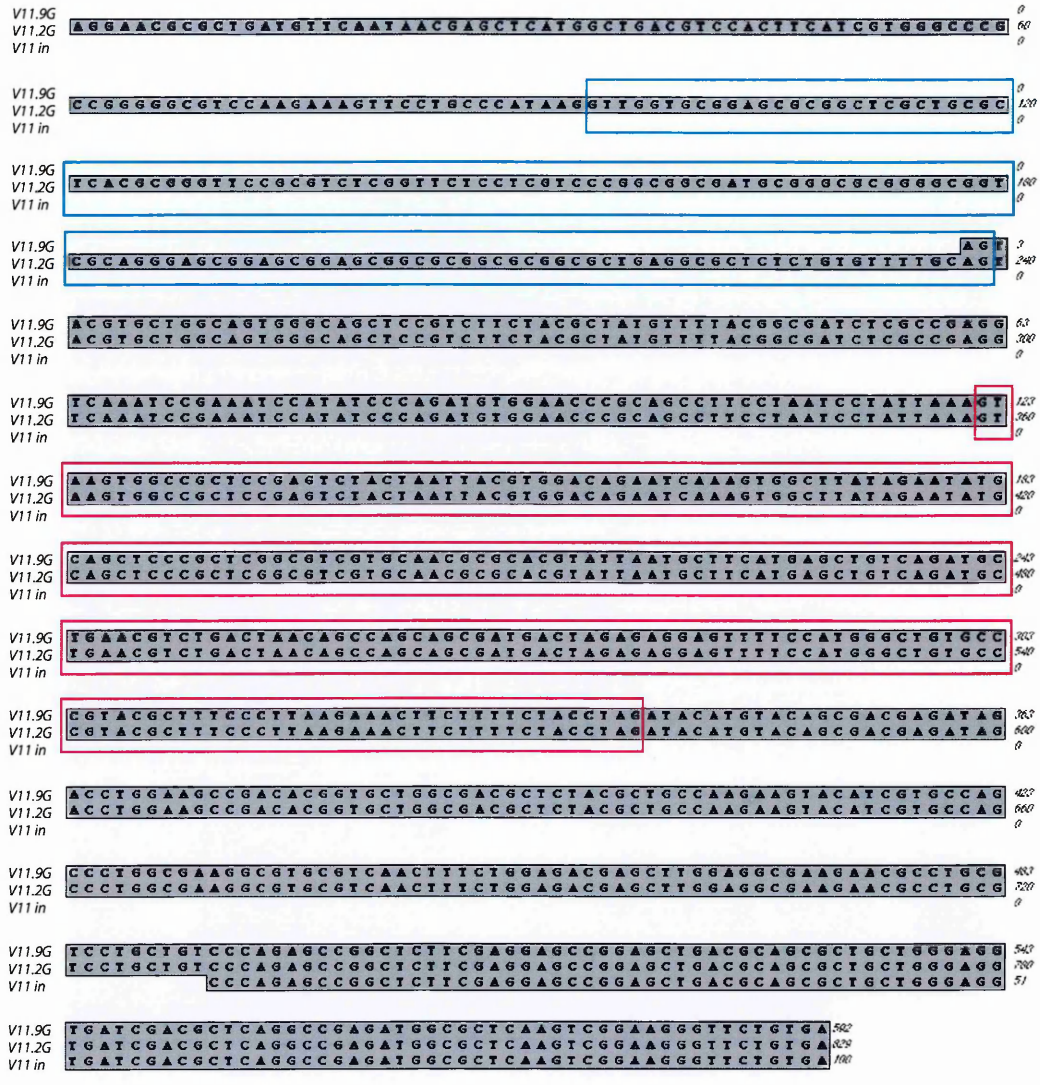


Figure 8. Partial nucleotide sequence alignment of chick V11in, V11.9G and V11.2G. Comparison of the 5′ end nucleotide sequences between the initial chick V11 (V11in) clone and the two longest clones isolated from a HH stage 3-5 whole chick cDNA library, V11.9G and V11.2G. Intron sequences identified in both V11.9G and V11.2G are indicated with pink boxes, and the intron present in the V11.2G clone only is depicted by blue box. The 5′ end sequence of the V11.2G transcript was extended beyond the initial V11 sequence by 364 bp.

sequence was extended at 5'end by only 364 bp and a further search for the missing 5' sequences was undertaken.

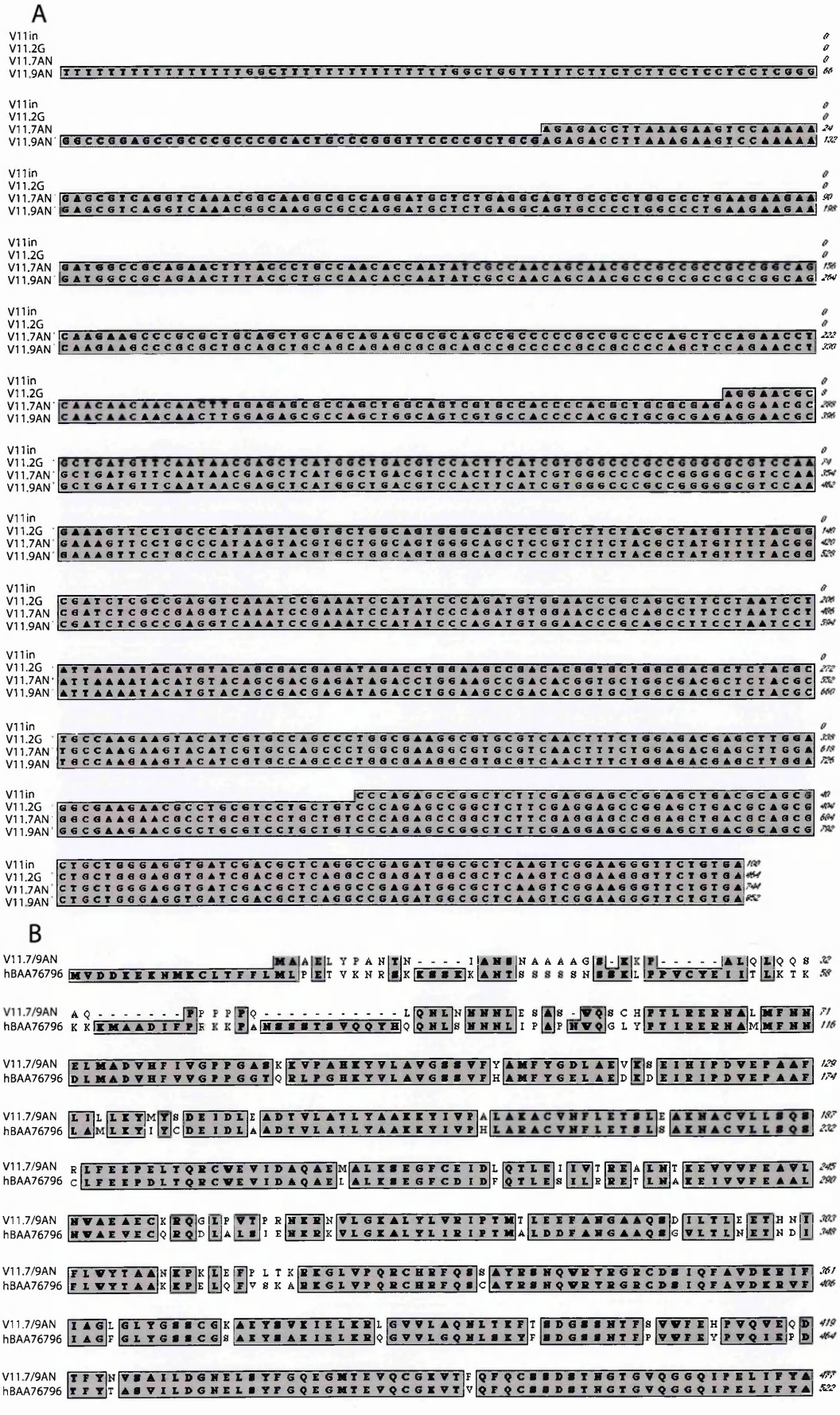
Screening of a whole chick 12 - 15 somite stage cDNA library

A whole chick 12 - 15 somite stage cDNA library, constructed in λZAPII (M.A.Nieto), was screened by filter hybridisation and five positively hybridising clones were identified. The two longest clones, V11.7AN and V11.9AN, were found to encompass the inserts of approximately 3.1 and 2.9 kb, respectively. The nucleotide sequence of clone V11.7AN was identical with the initial V11 sequence between the nucleotides 645 - 1884 and longer at the 5'end by 644 bp (Fig. 9A). However, the 3' end sequence starting from nucleotide 1884 did not have any homology with V11, suggesting that V11.7AN represented a hybrid clone. V11.9AN nucleotide sequence extended upstream beyond the sequence of the initial V11 clone by 753 bp (Fig. 5A) and was fully identical with it at the 3' end. Sequence analysis of both V11.7AN and V11.9AN established that the longest open reading frame identified was identical for both of them and a conceptual translation of this ORF, giving a product of 478 amino acid residues, was compared with the peptide sequence of the V11 closest human homologue, BAA76796. This comparison showed that the BAA76796 protein was longer than V11.7AN/V11.9AN in the N-terminus as there was another more upstream-located methionine (Fig. 9B). This drew a possibility that neither of the isolated clones covered the complete V11 mRNA sequence at the 5' end.

Screening of chick genomic BAC library

Final completion of the V11 transcript was achieved through the screening of the Wageningen chicken BAC (Bacterial Artificial Chromosome) library (Crooijmans et al., 2000) followed with the reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Four clones positively hybridising with a radiolabeled probe, generated from initial clone V11, were sequenced using three different upstream outwardly oriented primers specific to the

Figure 9. Partial nucleotide sequence alignment of chick V11in, V11.2G, V11.7AN and V11.9AN, and comparison of V11in and hBAA76796 proteins. (A) Comparison of the 5′ end nucleotide sequences between the initial V11 (V11in) clone, the longest clone isolated from the HH stage 3-5 whole chick cDNA library, V11.2G, and two longest clones identified in the whole chick 12 - 15 somite stage cDNA library, V11.7AN and V11.9AN. Clone V11.9AN is the longest and its sequence extends upstream beyond the V11in sequence by 753 bp. (B) Amino acid sequence alignment of the closest human (h) V11 homologue, hBAA76796, and V11.7AN/V11.9AN (predicted open reading frame identical for both chick clones). hBAA76796 protein is longer than V11.7/9AN peptide in the N-terminus suggesting that V11.7AN and V11.9AN may represent incomplete cDNA clones.


A
V11in
V11.2G
V11.7AN
V11.9AN
B
V11.7/9AN
hBAA76796

overlapping 5' end region of V11.7AN and V11.9AN sequences. The obtained DNA sequence allowed for the designing of primers used for amplification of the V11 5'end sequences in the PCR step on the 25-28ss (HH16) whole chick embryos cDNA.

Final construction of V11 full-length cDNA sequence

The final assembly of the clones isolated from different chick cDNA libraries and RT PCR products gave a transcript (cV11 isoform 1) of 2890 bp containing an ORF (from nt 21-1613) encoding a 531 amino acid product (Fig. 10). Completion of the Human Genome Project and recent availability of the entire chicken genome sequence in the databases, provided a new insight into the V11 cloning results obtained at that time. Revised analysis of the results suggests the presence of two splice variants of the cV11 transcript. The identified second transcript, cV11 isoform 2, is 2909 bp long and contains a 1430 bp long ORF encoding a predicted protein which, compared to the cV11 isoform 1 putative peptide, is shorter at the N-terminus by 53 amino acid residues (Fig. 11). Previously isolated clone V11.9AN represents cV11 isoform 2.

The presence of two splice variants of chick V11 is in correlation with my later findings, showing that V11 homologue in zebrafish also has two isoforms (see later chapter).

Identification of cV11 protein motifs

Analysis of the amino acid sequence of both cV11 splice variants with protein domain identification software (NCBI and PFAM; http://www.pfam.wustl.edu) revealed the presence of three motifs, BTB/POZ, BACK and PHR, spanning residues 118 to 228, 233 to 351 and 384 to 530 in cV11 isoform 1, and 65 to 175, 179 to 298 and 331 to 477 in cV11 isoform 2, respectively (Fig. 12).

Figure 10. Nucleotide sequence and predicted amino acid sequence of chick V11 isoform 1. The open reading frame is accompanied by the predicted amino acids shown by the single letter designation beneath it. A start codon (ATG) shown in bold is at nucleotide position 34 and the termination codon (TGA) at position 1611 is indicated by an asterix. A putative polyadenylation signal and a poly(A) tail are indicated in pink and light green, respectively. The BTB domain, BACK domain and PHR motif are labelled in green, red and blue, respectively.

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AGCCGGCGGTCCTCTCGCCG**ATG**CCACTGCCCCATGGGTGCCTCAATGGCAGGATCATGAAGTGTTTGAC   70
                      M  P  L  P  H  G  C  L  N  G  R  I  M  K  C  L
TTTTTTTCTTCTGCTTCCAGAGACCTTAAAGAAGTCCAAAAAAGAGCGTCAGGTCAAACGGCAAGGCGCCA  140
  F  F  L  L  L  P  E  T  L  K  K  S  K  K  S  V  R  S  N  G  K  A  P
GGATGCTCTGAGGCAGTGCCCCTGGCCCTGAAGAAGAAGATGGCCGCAGAACTTTACCCTGCCAACACCA  210
 G  C  S  E  A  V  P  L  A  L  K  K  K  M  A  A  E  L  Y  P  A  N
ATATCGCCAACAGCAACGCCGCCGCCGCCGGCAGCAAGAAGCCCGCGCTGCAGCTGCAGCAGAGCGCGCA  280
N  I  A  N  S  N  A  A  A  A  G  S  K  K  P  A  L  Q  L  Q  Q  S  A
GCCGCCCCCGCCGCCCCAGCTCCAGAACCTCAACAACAACAACTTGGAGAGCGCCAGCTGGCAGTCGTGC  350
  P  P  P  P  P  Q  L  Q  N  L  N  N  N  N  L  E  S  A  S  W  Q  S  C
CACCCCACGCTGCGCGAGAGGAACGCGCTGATGTTCAATAACGAGCTCATGGCTGACGTCCACTTCATCG  420
 H  P  T  L  R  E  R  N  A  L  M  F  N  N  E  L  M  A  D  V  H  F  I
TGGGCCCGCCGGGGGGCGTCCAAGAAAGTTCCTGCCCATAAGTACGTGCTGGCAGTGGGCAGCTCCGTCTT  490
V  G  P  P  P  G  A  S  K  K  V  P  A  H  K  Y  V  L  A  V  G  S  S  V  F
CTACGCTATGTTTTACGGCGATCTCGCCGAGGTCAAATCCGAAATCCATATCCCAGATGTGGAACCCGCA  560
  Y  A  M  F  Y  G  D  L  A  E  V  K  S  E  I  H  I  P  D  V  E  P  A
GCCTTCCTAATCCTATTAAAATACATGTACAGCGACGAGATAGACCTGGAAGCCGACACGGTGCTGGCGA  630
 A  F  L  I  L  L  K  Y  M  Y  S  D  E  I  D  L  E  A  D  T  V  L  A
CGCTCTACGCTGCCAAGAAGTACATCGTGCCAGCCCTGGCGAAGGCGTGCGTCAACTTTCTGGAGACGAG  700
T  L  Y  A  A  K  K  Y  I  V  P  A  L  A  K  A  C  V  N  F  L  E  T  S
CTTGGAGGCGAAGAACGCCTGCGTCCTGCTGTCCCAGAGCCGGCTCTTCGAGGAGCCGGAGCTGACGCAG  770
  L  E  A  K  N  A  C  V  L  L  S  Q  S  R  L  F  E  E  P  E  L  T  Q
CGCTGCTGGGAGGTGATCGACGCTCAGGCCGAGATGGCGCTCAAGTCGGAAGGGTTCTGTGAGATCGACC  840
 R  C  W  E  V  I  D  A  Q  A  E  M  A  L  K  S  E  G  F  C  E  I  D
TGCAGACGCTGGAGATCATCGTGACGCGGGAGGCCCTCAACACCAAGGAGGTGGTGGTGTTCGAGGCCGT  910
L  Q  T  L  E  I  I  V  T  R  E  A  L  N  T  K  E  V  V  V  F  E  A  V
TCTCAACTGGGCGGAGGCCGAGTGCAAGAGGCAGGGGCTGCCGGTGACGCCGCGCAACAAGAGGAACGTG  980
  L  N  W  A  E  A  E  C  K  R  Q  G  L  P  V  T  P  R  N  K  R  N  V
CTGGGGAAGGCGCTGTACCTGGTGCGGATCCCCACGATGACTCTGGAAGAGTTCGCCAACGGGGCGGCCC 1050
 L  G  K  A  L  Y  L  V  R  I  P  T  M  T  L  E  E  F  A  N  G  A  A
AGTCCGACATCCTCACGCTGGAGGAGACGCACAACATATTCCTGTGGTACACGGCCGCCAACAAACCCAA 1120
Q  S  D  I  L  T  L  E  E  T  H  N  I  F  L  W  Y  T  A  A  N  K  P  K
ACTCGAGTTTCCGCTGACAAAGAGGAAAGGACTGGTGCCGCAGCGCTGCCATCGCTTTCAGTCGTCTGCG 1190
  L  E  F  P  L  T  K  R  K  G  L  V  P  Q  R  C  H  R  F  Q  S  S  A
TACCGCAGTAACCAGTGGAGGTACCGGGGGCGGTGCGACAGTATTCAGTTTGCCGTAGACAAACGGATAT 1260
 Y  R  S  N  Q  W  R  Y  R  G  R  C  D  S  I  Q  F  A  V  D  K  R  I
TTATAGCGGGACTGGGATTGTATGGGTCGAGTTGTGGCAAAGCTGAATACAGCGTCAAAATTGAACTGAA 1330
F  I  A  G  L  G  L  Y  G  S  S  C  G  K  A  E  Y  S  V  K  I  E  L  K
GCGCTTAGGAGTCGTCCTTGCTCAGAATCTGACAAAGTTTACCTCCGACGGCTCCAGTAATACCTTCTCG 1400
  R  L  G  V  V  L  A  Q  N  L  T  K  F  T  S  D  G  S  S  N  T  F  S
GTGTGGTTTGAGCACCCGGTGCAGGTTGAGCAGGACACGTTCTACAATGTAAGTGCCATTCTGGATGGCA 1470
 V  W  F  E  H  P  V  Q  V  E  Q  D  T  F  Y  N  V  S  A  I  L  D  G
ATGAGCTCAGTTACTTCGGGCAGGAGGGAATGACTGAAGTGCAGTGCGGGAAAGTGACCTTCCAGTTCCA 1540
N  E  L  S  Y  F  G  Q  E  G  M  T  E  V  Q  C  G  K  V  T  F  Q  F  Q
GTGCTCCTCGGACAGTACCAACGGGACTGGGGTACAAGGAGGACAAATCCCTGAGCTCATTTTCTATGCA 1610
  C  S  S  D  S  T  N  G  T  G  V  Q  G  G  Q  I  P  E  L  I  F  Y  A
TGATGCATTTCACCTTGATTGTATTCCAGTACTGCAACGCTGCACATCTAAGGGATTCTTCAGTTTTGCT 1680
 *
ACAGAACTGTCAAGCAGTATGGGAATGTTACGCTACTTACCTATCTGCTACATCANGCTATACCCAATTA 1750
AGTGAAAGGAATGCTCTCAAATTTAATCTTATTTTATTTATTCATAAGCTATTTGACTTGATTAAGACTG 1820
CAGCGAGCAGAAAAATGTTAAATTTTGCACATGCAGTGCATTTATTTTGTATATAGATAACTAACTTGCA 1890
GACTGCAGCTGACTCAAACAGAATGTTCTAAACTAGAGCAGTTTATGAACCGGTGAAATATAAAACATTT 1960
CTACTTGCCCTAAATCCCGCGTACTGCCCCTTAATGATGTTAGGGCCTGTCTGCTAATGCAGCTACAGCG 2030
TAGTTAACGGGTGGTAACACCGCTGGTTGCCTGTCCTCTGCAAGGTGTGAGCAGTGCTGCACGTAGAAAG 2100
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TGAACCAATGTAAACCAATGTACAAAACATTGTAATCATGATCCAATTTACCTTTTTTTTTTTGCACATA 2730
ACAGGTACCTCTACCTTCAACTTCAAGTTTTCTGTTTGTTTTTTTTTTTTACCTGAGCTGCATTATTTTT 2800
TTACCTTGAAACTAAACTGTAATAAAATTTTGCCTGTTCTCTCAGAGAAAGTAAAAACAGGAAAAGAAAA 2870
AAAAAAAAAAAAAAAAAA                                                       2889
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Figure 11. Nucleotide and amino acid sequence of chick V11 isoform 2 and comparison of V11 isoform 1 and V11 isoform 2 proteins. (A) Nucleotide sequence and deduced amino acid sequence of chick V11 isoform 2. The open reading frame is accompanied by the predicted amino acids shown by the single letter abbreviations beneath it. The putative translation codon (ATG), highlighted in bold is at nucleotide position 200 and the stop codon (TGA) at position 1680 is indicated by an asterix. A putative polyadenylation signal and a poly(A) tail are indicated in pink and light green, respectively. The BTB domain, BACK domain and PHR motif are labelled in green, red and blue, respectively. (B) Alignment of chick V11 isomer 1 (cV11is1) and V11 isomer2 predicted polypeptide sequences showing that the V11 isomer 2 (cV11is2) deduced protein is shorter at the N-terminus by 53 amino acid residues.

A

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TTTTTTTTTTTTTTTTGGCTTTTTTTTTTTTTTTGGCTGGTTTTTCTTCTCTTCCTCCTCCTCGGGGGCC   70
GGAGCCGCCCGCCCGCACTGCCCGGGTTCCCCGCTGCGAGAGACCTTAAAGAAGTCCAAAAAGAGCGTCA  140
GGTCAAACGGCAAGGCGCCAGGATGCTCTGAGGCAGTGCCCCTGGCCCTGAAGAAGAAGATGGCCGCAGA  210
                                                          M  A  A  E
ACTTTACCCTGCCAACACCAATATCGCCAACAGCAACGCCGCCGCCGCCGGCAGCAAGAAGCCCGCGCTG  280
  L  Y  P  A  N  T  N  I  A  N  S  N  A  A  A  A  G  S  K  K  P  A  L
CAGCTGCAGCAGAGCGCGCAGCCGCCCCCGCCGCCCCAGCTCCAGAACCTCAACAACAACAACTTGGAGA  350
 Q  L  Q  Q  S  A  Q  P  P  P  P  P  Q  L  Q  N  L  N  N  N  N  L  E
GCGCCAGCTGGCAGTCGTGCCACCCCACGCTGCGCGAGAGGAACGCGCTGATGTTCAATAACGAGCTCAT  420
S  A  S  W  Q  S  C  H  P  T  L  R  E  R  N  A  L  M  F  N  N  E  L  M
GGCTGACGTCCACTTCATCGTGGGCCCGCCGGGGGCGTCCAAGAAAGTTCCTGCCCATAAGTACGTGCTG  490
  A  D  V  H  F  I  V  G  P  P  G  A  S  K  K  V  P  A  H  K  Y  V  L
GCAGTGGGCAGCTCCGTCTTCTACGCTATGTTTTACGGCGATCTCGCCGAGGTCAAATCCGAAATCCATA  560
  A  V  G  S  S  V  F  Y  A  M  F  Y  G  D  L  A  E  V  K  S  E  I  H
TCCCAGATGTGGAACCCGCAGCCTTCCTAATCCTATTAAAATACATGTACAGCGACGAGATAGACCTGGA  630
I  P  D  V  E  P  A  A  F  L  I  L  L  K  Y  M  Y  S  D  E  I  D  L  E
AGCCGACACGGTGCTGGCGACGCTCTACGCTGCCAAGAAGTACATCGTGCCAGCCCTGGCGAAGGCGTGC  700
  A  D  T  V  L  A  T  L  Y  A  A  K  K  Y  I  V  P  A  L  A  K  A  C
GTCAACTTTCTGGAGACGAGCTTGGAGGCGAAGAACGCCTGCGTCCTGCTGTCCCAGAGCCGGCTCTTCG  770
 V  N  F  L  E  T  S  L  E  A  K  N  A  C  V  L  L  S  Q  S  R  L  F
AGGAGCCGGAGCTGACGCAGCGCTGCTGGGAGGTGATCGACGCTCAGGCCGAGATGGCGCTCAAGTCGGA  840
E  E  P  E  L  T  Q  R  C  W  E  V  I  D  A  Q  A  E  M  A  L  K  S  E
AGGGTTCTGTGAGATCGACCTGCAGACGCTGGAGATCATCGTGACGCGGGAGGCCCTCAACACCAAGGAG  910
  G  F  C  E  I  D  L  Q  T  L  E  I  I  V  T  R  E  A  L  N  T  K  E
GTGGTGGTGTTCGAGGCCGTTCTCAACTGGGCGGAGGCCGAGTGCAAGAGGCAGGGGCTGCCGGTGACGC  980
 V  V  V  F  E  A  V  L  N  W  A  E  A  E  C  K  R  Q  G  L  P  V  T
CGCGCAACAAGAGGAACGTGCTGGGGAAGGCGCTGTACCTGGTGCGGATCCCCACGATGACTCTGGAAGA 1050
P  R  N  K  R  N  V  L  G  K  A  L  Y  L  V  R  I  P  T  M  T  L  E  E
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  F  A  N  G  A  A  Q  S  D  I  L  T  L  E  E  T  H  N  I  F  L  W  Y
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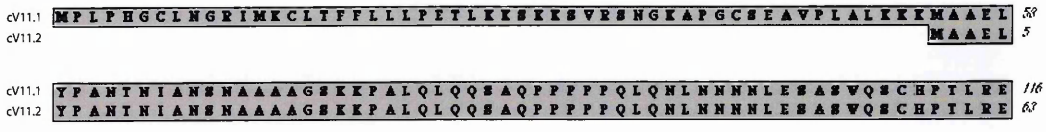
 T  A  A  N  K  P  K  L  E  F  P  L  T  K  R  K  G  L  V  P  Q  R  C
ATCGCTTTCAGTCGTCTGCGTACCGCAGTAACCAGTGGAGGTACCGGGGGCGGTGCGACAGTATTCAGTT 1260
H  R  F  Q  S  S  A  Y  R  S  N  Q  W  R  Y  R  G  R  C  D  S  I  Q  F
TGCCGTAGACAAACGGATATTTATAGCGGGACTGGGATTGTATGGGTCGAGTTGTGGCAAAGCTGAATAC 1330
  A  V  D  K  R  I  F  I  A  G  L  G  L  Y  G  S  S  C  G  K  A  E  Y
AGCGTCAAAATTGAACTGAAGCGCTTAGGAGTCGTCCTTGCTCAGAATCTGACAAAGTTTACCTCCGACG 1400
 S  V  K  I  E  L  K  R  L  G  V  V  L  A  Q  N  L  T  K  F  T  S
GCTCCAGTAATACCTTCTCGGTGTGGTTTGAGCACCCGGTGCAGGTTGAGCAGGACACGTTCTACAATGT 1470
G  S  S  N  T  F  S  V  W  F  E  H  P  V  Q  V  E  Q  D  T  F  Y  N  V
AAGTGCCATTCTGGATGGCAATGAGCTCAGTTACTTCGGGCAGGAGGGAATGACTGAAGTGCAGTGCGGG 1540
  S  A  I  L  D  G  N  E  L  S  Y  F  G  Q  E  G  M  T  E  V  Q  C  G
AAAGTGACCTTCCAGTTCCAGTGCTCCTCGGACAGTACCAACGGGACTGGGGTACAAGGAGGACAAATCC 1610
 K  V  T  F  Q  F  Q  C  S  S  D  S  T  N  G  T  G  V  Q  G  G  Q  I
CTGAGCTCATTTTCTATGCATGATGCATTTCACCTTGATTGTATTCCAGTACTGCAACGCTGCACATCTA 1680
P  E  L  I  F  Y  A  *
AGGGATTCTTCAGTTTTGCTACAGAACTGTCAAGCAGTATGGGAATGTTACGCTACTTACCTATCTGCTA 1750
CATCANGCTATACCCAATTAAGTGAAAGGAATGCTCTCAAATTTAATCTTATTTTATTTATTCATAAGCT 1820
ATTTGACTTGATTAAGACTGCAGCGAGCAGAAAAAATGTTAAATTTTGCACATGCAGTGCATTTATTTTGT 1890
ATATAGATAACTAACTTGCAGACTGCAGCTGACTCAAACAGAATGTTCTAAACTAGAGCAGTTTATGAAC 1960
CGGTGAAATATAAAACATTTCTACTTGCCCTAAATCCCGCGTACTGCCCCTTAATGATGTTAGGGCCTGT 2030
CTGCTAATGCAGCTACAGCGTAGTTAACGGGTGGTAACACCGCTGGTTGCCTGTCCTCTGCAAGGTGTGA 2100
GCAGTGCTGCACGTAGAAAGGCTCCCCCACCTGCAGAGCCCATCCATCAGGCAGCCCTCATTGTCCTTCA 2170
CCGAGAAGGACAGGAGGAAGGAAGCTCAGAGGTTGGCTATGGGTCGCCCAAGCTGAACAGAAAAAACCTG 2240
AGCATAACCAGCACTGATAAAACCTCGTGCTTACTGCTTGTAAGCTTCTTCTGTCCCTTTGCATTCTGCT 2310
TTTGGCACCACCTGTGTTAACCTCCACCCTGCCCAGCAAACAGCAGCAGTAACACCTGCACTGTCAAAAC 2380
ACACCAGACTTGAGCAGCCAATGTCAGGACAGACAGATAAAGCCTCGTGCAGCCACAAGCTGTAGCACTG 2450
ACAGCCACTACTTTAAATATACATCACAGCTCGCCTTTAGGAGCAGACAGATTTACAATACGGTCACTCA 2520
AAATTACCGAGGGAAGCCACCATAAGGAAAAGCTGCCAGGCCTTTTTTCTCCCTGTGTAGTTTTGGGGGT 2590
ATCAGTCATTTTTATAGCTAGCATCCTTCAGTTTTCTCANAGACTGACTAAGATAGGTGTATTCCTCAAG 2660
CATCTGACCACCTTTACAGCTGAACCAATGTAAACCAATGTACAAAACATTGTAATCATGATCCAATTTA 2730
CCTTTTTTTTTTTTGCACATAACAGGTACCTCTACCTTCAACTTCAAGTTTTCTGTTTGTTTTTTTTTTT 2800
ACCTGAGCTGCATTATTTTTTTACCTTGAAACTAAACTGTAATAAAATTTTGCCTGTTCTCTCAGAGAAA 2870
GTAAAAACAGGAAAAGAAAAAAAAAAAAAAAAAAAAAA                                   2909
```

B



BTB domain

The BTB/POZ (broad complex, tramtrack, and bric-a-brac/poxvirus and zinc finger) domain is an evolutionarily conserved protein-protein interaction motif present in proteins involved in a wide variety of biological processes, such as transcription regulation (Ahmad et al., 1998; Zollman et al., 1994), cytoskeletal arrangement (Kang et al., 2004; Melnick et al., 2000), ion conductance (Kreusch et al., 1998) and protein ubiquitination (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003). The BTB domain is around 115 amino acids long and typically found as a single copy near the N-terminus of a variety of proteins that have one other domain at the C terminus. Among the identified BTB-associated C-terminal motifs are zinc-finger (Bardwell and Treisman, 1994), kelch (Adams et al., 2000; Bork and Doolittle, 1994; Prag and Adams, 2003) and recently reported PHR (Xu et al., 2003b) domains. A general property of the BTB domain is to mediate homomeric dimerisation and in some instances heteromeric dimerisation (Bardwell and Treisman, 1994; Collins et al., 2001). The crystal structure of the BTB domain of human promyelocytic leukaemia zinc-finger protein (PLZF) has been solved and revealed that BTB monomers are tightly interwined as dimers (Ahmad et al., 1998). BTB domain from PLZF and several other zinc finger transcription proteins have been shown to mediate transcriptional repression (Dhordain et al., 1997; Wong and Privalsky, 1998). However, there have been reports of the BTB domain being capable of mediating transcriptional activation as well (Kobayashi et al., 2000a).

BACK domain

The BACK domain has recently been identified and is predominantly found in the BTB-kelch proteins in the intervening region separating N-terminal BTB from C-terminal kelch domain (Stogios and Prive, 2004). The length of this region is around 130 residues, and the strongest conserved sequence features are in the first 70 residues that immediately follow the BTB domain. The function of the BACK motif is not defined yet, however BTB-kelch

Figure 12. Alignment of functional domains in chick BTBD6. Alignment of the consensus BTB (A), BACK (B), and PHR (C) domains (Pfam) with the corresponding domains of chick BTBD6. The upper line represents the consensus BTB, BACK or PHR domain with capitals denoting residues that are absolutely conserved and lower case letters representing residues that are present in a majority of proteins containing a BTB domain in that position. The lower line represents chick BTBD6 and the middle line shows absolute matches to the consensus (shown in upper and lower case letters) or conservative amino acid substitutions (indicated by cross). Dashes denote gaps that have been introduced to achieve the alignment.


A
*->lnelreqgelcDVtLvvgdgsgrydvgkkfkAHKavLaacSpYFkal
+++++ + DV + vg + kk++AHK vLa S++F+a+
118 NALMFNNELMADVHFIVGPPGA----SKKVPAHKYVLAVGSSVFYAM 160
FtgkfkesiteeessvsseieledvspedfealLefiYtgelsitqdqks
F g++ e +s ei ++dv+p +f +lL+++Y++e++++
161 FYGDL-----AEVKS---EIHIPDVEPAAFLILLKYMYSDEIDLE----- 197
PsscksеenvedlLalAdllqipslvdaCeefliesl<-*
++v + L++A+++ +p+l +aC +fl +sl
198 ------ADTVLATLYAAKKYIVPALAKACVNFLETSL 228
B
*->NClgIrrFAdaygCkeLaeaAddfilqnFeeVskseEFLqLsfeeLi
N++ +++ + ++ +eL +++++ i e kse F +++++ L
232 NACVLLSQSRLFEEPELTQRCWEVIDAQAEMALKSEGFCEIDLQTLE 278
elLssDeLnVesEeqVfeAvlrWvkhDveeRkkhlpe.....LL
++ ++ Ln++ E +VfeAvl+W ++ e+ ++ lp +++++++ ++ L
279 IIVTREALNTK-EVVVFEAVLNW--AEAECKRQGLPVtprnkrnvlgkAL 325
snVRLpLLspeyLverVesepLiksdpeCrdlldEAmkyhllp<-*
VR+p ++ e + + + +++ l+
326 YLVRIPTMTLEEFANGAAQ----------SDILTLE 351
C
*->nRFqrvsaRggtWgySngsvDAIaFsVDkrGIfivGfGlYGgisGey
+RFq+ ++R+++W+y +g++D+I+F+VDkr Ifi+G+GlYG+++G +
384 HRFQSSAYRSNQWRY-RGRCDSIQFAVDKR-IFIAGLGLYGSSCGKA 428
eyklkllydlgtHagdldhsekwttLesvtteyssDcGasetaevrFdeP
ey++k+ +l+++++ +L+++ t+++sD G+s+t+ v+F++P
429 EYSVKI---------ELKRLGV--VLAQNLTKFTSD-GSSNTFSVWFEHP 466
VlikpnvwYavrarisGpslSdcGdkGmtsVstpdGkVtFqFsssslSnN
V+ + +++Y v+a ++G++lS++G++Gmt+V++ GkVtFqF++ss+S+N
467 VQVEQDTFYNVSAILDGNELSYFGQEGMTEVQC--GKVTFQFQCSSDSTN 514
GTtVqrGQIPeILYYl<-*
GT+Vq+GQIPe+++Y+
515 GTGVQGGQIPELIFYA 530

proteins have recently been implicated as substrate-specific adaptors for Cullin3-based protein ubiquitination with BACK domain directly involved in this process (Kobayashi and Yamamoto, 2005; Pintard et al., 2003; Xu et al., 2003a).

PHR motif

The PHR motif was first identified as a 150aa-long sequence repeat found in the proteins: human **P**am ("protein associated with *Myc*")(Guo et al., 1998), *Drosophila* **h**ighwire (Wan et al., 2000) and *C. elegans* **R**PM-1 (Zhen et al., 2000). These proteins are putative ubiquitin ligases and have a conserved role as presynaptic regulators of synapse formation and/or growth (DiAntonio et al., 2001; Fischer and Overstreet, 2002). The PHR family proteins are unusually large (around 500-kDA) and apart of PHR domain, contain several functional domains, including a guanine nucleotide exchange factor (GEF) domain and a RING H2 finger that likely confers E3 ubiquitin ligase activity. The function of the PHR domain, duplicated in PAM, highwire and RPM-1, is unclear. A single copy of PHR motif has also been identified in two human BTB-containing proteins, BTBD1 and BTBD2 (Xu et al., 2003b).

HUMAN BTBD6 and BTBD3 - CHICK V11 IS BTBD6 IN HUMAN

Initial BLAST homology searches with the partial chick V11 predicted amino acid sequences identified three related proteins and the one with the highest homology scores (81.2% identity over the overlapping region) was an unnamed hypothetical human protein (GenBank Acc. No. BAA76796) (Nagase et al., 1999; Strausberg et al., 2002). This protein is currently designated BTBD3 (BTB Domain-containing protein 3) with a new GenBank Accession Number CAC22147. Comparison of the entire cV11 amino acid sequence with the hBTBD3 peptide sequence reveals 69.2% identity between these two proteins.

Routine sequence database analysis undertaken during this project identified, at the later date, a second unknown human BTB domain-containing protein, BTBD6 (Acc. No. AAK39520) which is more closely related to chick V11 than hBTBD3 (Wistow et al., 2002). The retrieved hBTBD6 predicted amino acid sequence (which appears to be incomplete in the N-terminus with the initial methionine lacking), when compared with the chick V11 isoform1 putative polypeptide, exhibits 87.1% identity (over the overlapping region) (Fig. 13). Therefore, as BTBD6 appears to represent a chick V11 homologue in human, following the Hugo Nomenclature Committee instructions (http://www.gene.ucl.ac.uk/nomenclature/), the name V11 has been replaced by BTBD6 and this name will be used from now on in this thesis.

IDENTIFICATION OF CHICK BTBD3

Further sequence database searches using chick BTBD6 cDNA identified another novel chick BTB-containing gene with a high homology score. The predicted cDNA of this gene obtained from Ensembl database [http://www.ensembl.org/; (Hubbard et al., 2005)] contains an ORF that produces a deduced protein of 520 amino acids (Fig. 14). This protein exhibits 95.6%, 72.8% and 69.9% identity with hBTBD3, hBTBD6 (overlapping region) and cBTBD6, respectively, the highest being with hBTBD3, suggesting that the characterised gene represents the chick equivalent of human BTBD3 (cBTBD3) (Fig. 18). None of the ESTs for cBTBD3 available in the databases cover the predicted full-length cDNA. The ones acquired (GeneBank Acc. Nos: BU444464, BU274818, BU389269) and sequenced, encompass the regions: nt 327 - 1100 and nt 1340 - 1570 of a putative cDNA and also extend 586 bp downstream into the 3′ UTR. The entire sequence of the cBTBD3 3′ UTR has not been identified as there is no poly(A) tail detected in any of the 3′ end extended ESTs.

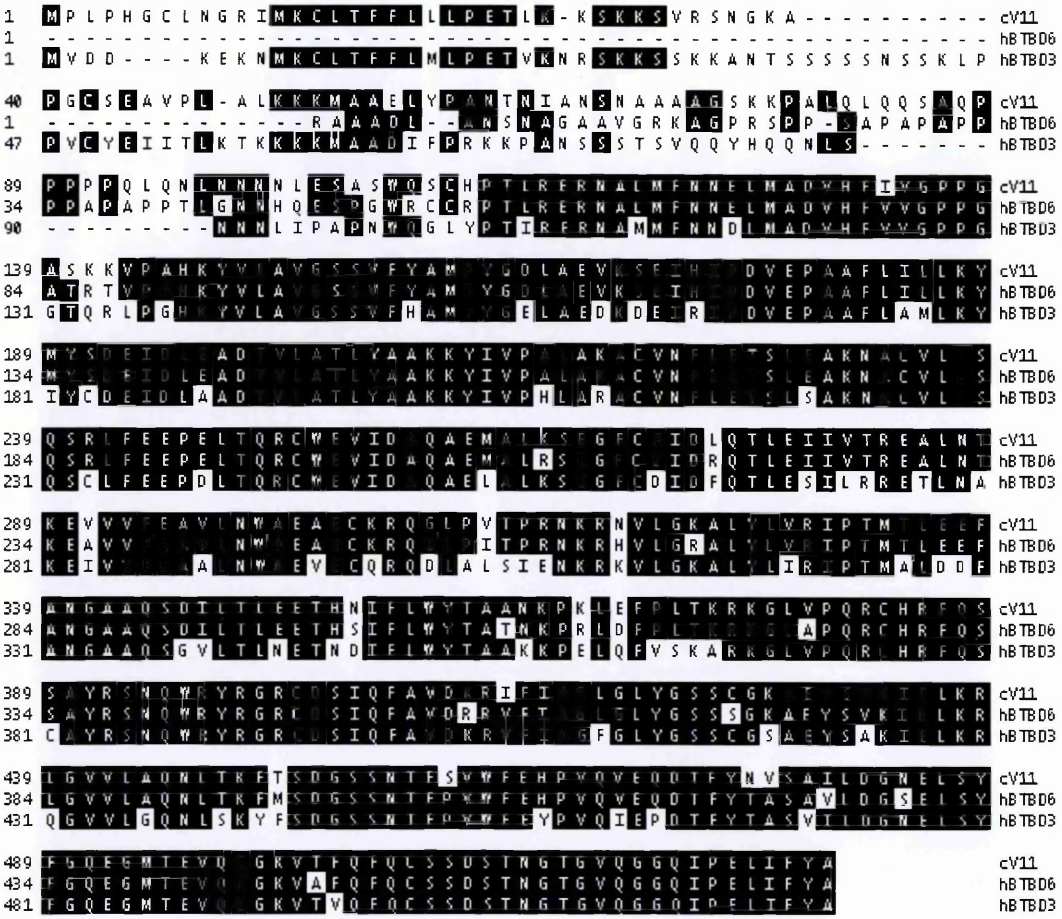


Figure 13. Multiple amino acid alignment of chick V11, human BTBD6 and human BTBD3. The sequences were aligned using the ClustalV algorithm (Higgins and Sharp, 1989). Identical residues are boxed in black.

```
ATACTC**ATG**GTAGATGAGAAAGGAAAGAACATGAAATGTCTCACCTTTTTCTTGATGCTTCCAGAGACGG    70
        M  V  D  E  K  G  K  N  M  K  C  L  T  F  F  L  M  L  P  E  T
TCAAGAACAGGTCCAAGAAGAGCTCTAAGAAGGGAAATAGCAACAGCAGCAGCAGCAAATTGCCTCCCGT   140
V  K  N  R  S  K  K  S  S  K  K  G  N  S  N  S  S  S  S  K  L  P  P  V
TTGCTATGAAATCATTACCTTGAAGACCAAAAAGAAGAAGAAGATGGCTGCTGATATTTTTCCCCGAAAG   210
  C  Y  E  I  I  T  L  K  T  K  K  K  K  K  M  A  A  D  I  F  P  R  K
AAACCAGCCAACACTAATACAACTGCTGTCCAGCAGTACCACCAGCAAAATCTCAATAACAACAACACTA   280
 K  P  A  N  T  N  T  T  A  V  Q  Q  Y  H  Q  Q  N  L  N  N  N  N  T
TTCCAGCACCAAACTGGCAAGGACTTTATCCGACCATCAGAGAGAGAAATGCAGTGATGTTCAACAATGA   350
I  P  A  P  N  W  Q  G  L  Y  P  T  I  R  E  R  N  A  V  M  F  N  N  D
CTTGATGGCAGATGTTCATTTTGTGGTCGGGCCACCAGGTGGGACCCAGCGGCTGCCAGGACATAAATAT   420
  L  M  A  D  V  H  F  V  V  G  P  P  G  G  T  Q  R  L  P  G  H  K  Y
GTCCTGGCTGTTGGGAGCTCTGTATTCCATGCGATGTTCTATGGAGAACTTGCTGAGGACAAAGATGAAA   490
 V  L  A  V  G  S  S  V  F  H  A  M  F  Y  G  E  L  A  E  D  K  D  E
TCCGTATACCAGATGTTGAGCCTGCTGCTTTTCTTGCAATGCTGAAATACATATATTGTGATGAAATTGA   560
I  R  I  P  D  V  E  P  A  A  F  L  A  M  L  K  Y  I  Y  C  D  E  I  D
TTTGGCTGCAGATACCGTCCTGGCCACTCTTTATGCTGCCAAGAAGTACATCGTCCCTCATCTTGCCCGT   630
  L  A  A  D  T  V  L  A  T  L  Y  A  A  K  K  Y  I  V  P  H  L  A  R
GCCTGCGTCAACTTCCTAGAAACAAGTCTAAGTGCAAAGAACGCCTGCGTGCTGCTTTCCCAAAGCTGCT   700
 A  C  V  N  F  L  E  T  S  L  S  A  K  N  A  C  V  L  L  S  Q  S  C
TATTCGAGGAACCCGACCTGACCCAGCGCTGTTGGGAAGTGATTGATGCCCAAGCCGAGCTAGCTTTGAA   770
L  F  E  E  P  D  L  T  Q  R  C  W  E  V  I  D  A  Q  A  E  L  A  L  K
GTCCGAGGGCTTCTGTGACATTGATTTTCAGACGCTTGAAAGCATTCTCCGAAGGGAGACTCTGAATGCC   840
  S  E  G  F  C  D  I  D  F  Q  T  L  E  S  I  L  R  R  E  T  L  N  A
AAAGAAATTGTTGTTTTTGAAGCGGCTCTGAATTGGGCTGAAGTGGAGTGTCAGCGACAGGAGCTAACTG   910
 K  E  I  V  V  F  E  A  A  L  N  W  A  E  V  E  C  Q  R  Q  E  L  T
CCACCATAGAGAATAAGCGCAAGGTCCTCGGGAAGGCGCTGTACTTGATACGTATCCCTACCATGGCACT   980
A  T  I  E  N  K  R  K  V  L  G  K  A  L  Y  L  I  R  I  P  T  M  A  L
CGACGACTTTGCAAATGGCGCTGCTCAGTCCGGGATTCTGACTCTCAACGAAACCAATGATATCTTCCTT  1050
  D  D  F  A  N  G  A  A  Q  S  G  I  L  T  L  N  E  T  N  D  I  F  L
TGGTATACAGCTGCCAAAAAGCCAGAGCTGCAGTTTGTAAGCAAGCCCCGGAAAGGCCTTGTTCCTCAGC  1120
 W  Y  T  A  A  K  K  P  E  L  Q  F  V  S  K  P  R  K  G  L  V  P  Q
GGTGCCATCGTTTCCAGTCCTGTGCTTACCGCAGCAACCAGTGGCGTTACAGGGGCCGGTGTGACAGCAT  1190
R  C  H  R  F  Q  S  C  A  Y  R  S  N  Q  W  R  Y  R  G  R  C  D  S  I
CCAGTTTGCTGTTGATAAGAGAGTGTTTATAGCTGGCTTTGGGCTATACGGCTCCAGCTGCGGATCAGCA  1260
  Q  F  A  V  D  K  R  V  F  I  A  G  F  G  L  Y  G  S  S  C  G  S  A
GAATACAGTGCCAAGATTGAACTCAAACGACAAGGAGTTATCCTAGGCCAGAACTTGAGCAAATATTTCT  1330
 E  Y  S  A  K  I  E  L  K  R  Q  G  V  I  L  G  Q  N  L  S  K  Y  F
CAGATGGTTCTAGTAACACTTTCCCTGTGTGGTTTGAATATCCAGTACAGATTGAGCCTGACACCTTCTA  1400
S  D  G  S  S  N  T  F  P  V  W  F  E  Y  P  V  Q  I  E  P  D  T  F  Y
CACAGCTAGTGTGATTTTGGATGGTAATGAACTCAGCTATTTTGGACAGGAAGGAATGACAGAAGTTCAG  1470
  T  A  S  V  I  L  D  G  N  E  L  S  Y  F  G  Q  E  G  M  T  E  V  Q
TGTGGGAAAGTGACTGTTCAGTTTCAGTGCTCTTCGGACAGTACAAATGGCACTGGGGTACAGGGAGGGC  1540
 C  G  K  V  T  V  Q  F  Q  C  S  S  D  S  T  N  G  T  G  V  Q  G  G
AAATTCCTGAACTCATATTTTATGCTTGA                                            1569
Q  I  P  E  L  I  F  Y  A  *
```

Figure 14. Putative cDNA and amino acid sequence of cBTBD3. The open reading frame is indicated by the predicted amino acids shown by the single letter designation beneath it. An initiation translation codon (ATG) is shown in bold and the stop codon (TGA) is indicated by an asterix. The BTB domain, BACK domain and PHR motif are labelled in green, red and blue, respectively.

IDENTIFICATION OF MOUSE BTBD6 AND BTBD3

In order to identify a murine homologue of chick BTBD6, at the beginning of this project, screening of a 8.5 dpc. mouse cDNA library with a radiolabelled probe encompassing the initial chick V11 clone cDNA, was undertaken.

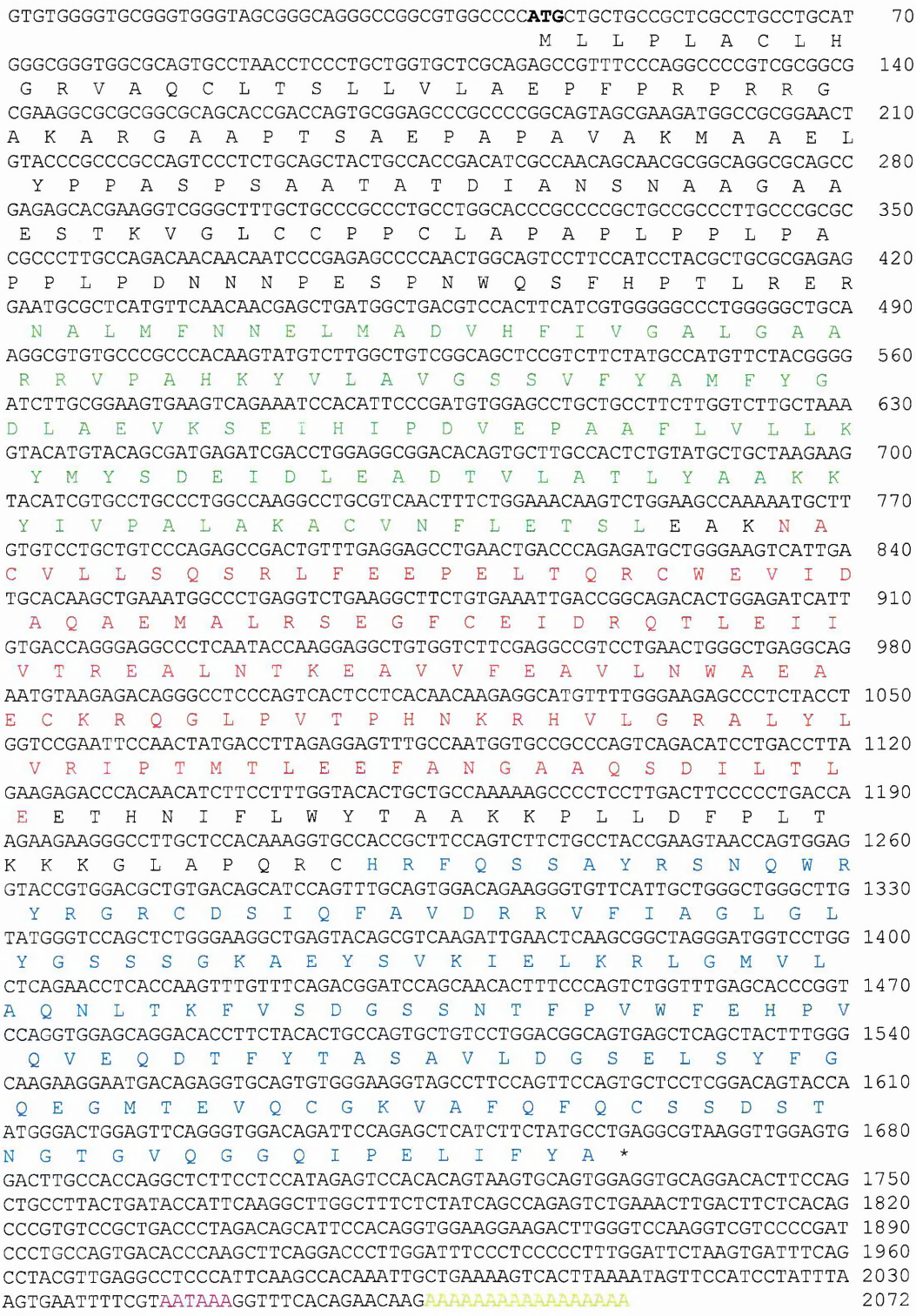
Three clones, 1 kb, 1.3kb and 1.9 kb in size were isolated and sequenced. The sequence analysis revealed that the 1.3 kb insert represented a hybrid clone and that the 1 kb clone was a shorter version of the 1.9 kb insert. The 1.9 kb clone, named at the time mouse V11 (mV11), was chosen for further analysis.

Examination of the mV11 nucleotide sequence established that it was 1851 nucleotides long and, whilst lacking a start codon, contained a partial ORF ending with a putative stop codon at position 1429.

The comparison of the mouse and chick V11 partial sequences revealed that the mouse coding sequence is extended 5′ in relation to the chick clone and that there is 92% identity over the overlapping region at both nucleotide and amino acid levels, suggesting that mouse V11 is very closely related to chick V11.

The entire mV11 cDNA, including missing 5′ end sequences, was assembled using the EST sequences (GeneBank Acc. Nos. AW226647.1 and BY123280.1) identified later through the database BLAST searches. Within the obtained mV11 nucleotide sequence there is a full-length ORF detected that encodes a 539 amino acid-deduced protein exhibiting 81.3% identity with the chick BTBD6 protein (Fig. 15). When compared with the human BTBD6 and human BTBD3 proteins, mV11 shows 90.5% and 66.6% identity, respectively, indicating that mV11 is a homologue of hBTBD6 and currently designated mBTBD6.

Figure 15. Nucleotide sequence and predicted amino acid sequence of mouse BTBD6. The open reading frame is indicated by the predicted amino acids designated by the single letter abbreviations underneath. The putative translation codon (ATG) is highlighted in bold and a stop codon (TGA) is designated by an asterix. A putative polyadenylation signal and a poly(A) tail are indicated in pink and light green, respectively. The BTB domain, BACK domain and PHR motif are labelled in green, red and blue, respectively.


GTGTGGGGTGCGGGTGGGTAGCGGGCAGGGCCGGCGTGGCCCCATGCTGCTGCCGCTCGCCTGCCTGCAT 70
M L L P L A C L H
GGGCGGGTGGCGCAGTGCCTAACCTCCCTGCTGGTGCTCGCAGAGCCGTTTCCCAGGCCCCGTCGCGGCG 140
G R V A Q C L T S L L V L A E P F P R P R R G
CGAAGGCGCGCGGCGCAGCACCGACCAGTGCGGAGCCCGCCCCGGCAGTAGCGAAGATGGCCGCGGAACT 210
A K A R G A A P T S A E P A P A V A K M A A E L
GTACCCGCCCGCCAGTCCCTCTGCAGCTACTGCCACCGACATCGCCAACAGCAACGCGGCAGGCGCAGCC 280
Y P P A S P S A A T A T D I A N S N A A G A A
GAGAGCACGAAGGTCGGGCTTTGCTGCCCGCCCTGCCTGGCACCCGCCCCGCTGCCGCCCTTGCCCGCGC 350
E S T K V G L C C P P C L A P A P L P P L P A
CGCCCTTGCCAGACAACAACAATCCCGAGAGCCCCAACTGGCAGTCCTTCCATCCTACGCTGCGCGAGAG 420
P P L P D N N N P E S P N W Q S F H P T L R E R
GAATGCGCTCATGTTCAACAACGAGCTGATGGCTGACGTCCACTTCATCGTGGGGGCCCTGGGGGCTGCA 490
N A L M F N N E L M A D V H F I V G A L G A A
AGGCGTGTGCCCGCCCACAAGTATGTCTTGGCTGTCGGCAGCTCCGTCTTCTATGCCATGTTCTACGGGG 560
R R V P A H K Y V L A V G S S V F Y A M F Y G
ATCTTGCGGAAGTGAAGTCAGAAATCCACATTCCCGATGTGGAGCCTGCTGCCTTCTTGGTCTTGCTAAA 630
D L A E V K S E I H I P D V E P A A F L V L L K
GTACATGTACAGCGATGAGATCGACCTGGAGGCGGACACAGTGCTTGCCACTCTGTATGCTGCTAAGAAG 700
Y M Y S D E I D L E A D T V L A T L Y A A K K
TACATCGTGCCTGCCCTGGCCAAGGCCTGCGTCAACTTTCTGGAAACAAGTCTGGAAGCCAAAAATGCTT 770
Y I V P A L A K A C V N F L E T S L E A K N A
GTGTCCTGCTGTCCCAGAGCCGACTGTTTGAGGAGCCTGAACTGACCCAGAGATGCTGGGAAGTCATTGA 840
C V L L S Q S R L F E E P E L T Q R C W E V I D
TGCACAAGCTGAAATGGCCCTGAGGTCTGAAGGCTTCTGTGAAATTGACCGGCAGACACTGGAGATCATT 910
A Q A E M A L R S E G F C E I D R Q T L E I I
GTGACCAGGGAGGCCCTCAATACCAAGGAGGCTGTGGTCTTCGAGGCCGTCCTGAACTGGGCTGAGGCAG 980
V T R E A L N T K E A V V F E A V L N W A E A
AATGTAAGAGACAGGGCCTCCCAGTCACTCCTCACAACAAGAGGCATGTTTTGGGAAGAGCCCTCTACCT 1050
E C K R Q G L P V T P H N K R H V L G R A L Y L
GGTCCGAATTCCAACTATGACCTTAGAGGAGTTTGCCAATGGTGCCGCCCAGTCAGACATCCTGACCTTA 1120
V R I P T M T L E E F A N G A A Q S D I L T L
GAAGAGACCCACAACATCTTCCTTTGGTACACTGCTGCCAAAAAGCCCCTCCTTGACTTCCCCCTGACCA 1190
E E T H N I F L W Y T A A K K P L L D F P L T
AGAAGAAGGGCCTTGCTCCACAAAGGTGCCACCGCTTCCAGTCTTCTGCCTACCGAAGTAACCAGTGGAG 1260
K K K G L A P Q R C H R F Q S S A Y R S N Q W R
GTACCGTGGACGCTGTGACAGCATCCAGTTTGCAGTGGACAGAAGGGTGTTCATTGCTGGGCTGGGCTTG 1330
Y R G R C D S I Q F A V D R R V F I A G L G L
TATGGGTCCAGCTCTGGGAAGGCTGAGTACAGCGTCAAGATTGAACTCAAGCGGCTAGGGATGGTCCTGG 1400
Y G S S S G K A E Y S V K I E L K R L G M V L
CTCAGAACCTCACCAAGTTTGTTTCAGACGGATCCAGCAACACTTTCCCAGTCTGGTTTGAGCACCCGGT 1470
A Q N L T K F V S D G S S N T F P V W F E H P V
CCAGGTGGAGCAGGACACCTTCTACACTGCCAGTGCTGTCCTGGACGGCAGTGAGCTCAGCTACTTTGGG 1540
Q V E Q D T F Y T A S A V L D G S E L S Y F G
CAAGAAGGAATGACAGAGGTGCAGTGTGGGAAGGTAGCCTTCCAGTTCCAGTGCTCCTCGGACAGTACCA 1610
Q E G M T E V Q C G K V A F Q F Q C S S D S T
ATGGGACTGGAGTTCAGGGTGGACAGATTCCAGAGCTCATCTTCTATGCCTGAGGCGTAAGGTTGGAGTG 1680
N G T G V Q G G Q I P E L I F Y A *
GACTTGCCACCAGGCTCTTCCTCCATAGAGTCCACACAGTAAGTGCAGTGGAGGTGCAGGACACTTCCAG 1750
CTGCCTTACTGATACCATTCAAGGCTTGGCTTTCTCTATCAGCCAGAGTCTGAAACTTGACTTCTCACAG 1820
CCCGTGTCCGCTGACCCTAGACAGCATTCCACAGGTGGAAGGAAGACTTGGGTCCAAGGTCGTCCCCGAT 1890
CCCTGCCAGTGACACCCAAGCTTCAGGACCCTTGGATTTCCCTCCCCCTTTGGATTCTAAGTGATTTCAG 1960
CCTACGTTGAGGCCTCCCATTCAAGCCACAAATTGCTGAAAAGTCACTTAAAATAGTTCCATCCTATTTA 2030
AGTGAATTTTCGTAATAAAGGTTTCACAGAACAAG 2072

The BLAST homology searches have also identified the mouse BTBD3 (mBTBD3) protein (97.7% identity to hBTBD3), which also exhibits 93.8%, 73.1%, 65.9% and 69.1% identity with cBTBD3, hBTBD6, mBTBD6 and cBTBD6, respectively (Fig. 20).

IDENTIFICATION OF ZEBRAFISH BTBD6 AND BTBD3 GENES

The chick BTBD6 cDNA sequence was used to search for related zebrafish EST sequences using The Institute for Genomic Research Zebrafish Gene Index (TIGR; http://www.tigr.org), NCBI and Washington University Zebrafish Genome Resources (WUZGR; http://www.genetics.wustl.edu/fish_lab/) websites. Four highly related ESTs (GeneBank Acc. No. AI437221, BI350992, BG308275, AI883008) were identified, and their corresponding cDNAs obtained and sequenced. Sequencing results combined with the information obtained in the sequence databases as well as the data so far collected in this study allow for identification of four zebrafish BTB-containing genes, two of them most related to BTBD6 and another two homologous to BTBD3.

Sequence analysis of the 2358 bp cDNA clone equivalent to the BG308275 EST revealed that it contains a full-length ORF (nt 273-1895) for a 541 amino acids putative polypeptide that exhibits a 93.9%, 68.2% identity with the cBTBD6, cBTBD3 proteins, respectively (Fig. 16&20). This novel zebrafish gene is highly similar to cBTBD6 and was designated zBTBD6a.

The nucleotide sequences of two clones represented by BI350992 EST and AI437221 EST were partially overlapping and, when aligned, produced a 2056 nucleotide long contiguous sequence with an ORF (nt 174-1622) encoding deducted protein of 482 amino acid. This novel gene is also highly related to cBTBD6 and moderately related to cBTBD3 with 90.8% and 78.3% identity at the amino acid level, respectively, and was named zBTBD6b (Fig. 17&20).

Figure 16. Nucleotide sequence and predicted amino acid sequence of zebrafish BTBD6a. The open reading frame is indicated by the predicted amino acids shown by the single letter designation beneath it. A start codon (ATG) shown in bold is at nucleotide position 273 and the termination codon (TGA) at position 1895 is indicated by an asterix. A polyadenylation signal is not identified and a poly(A) tail is shown in light green. The BTB domain, BACK domain and PHR motif are labelled in green, red and blue, respectively.

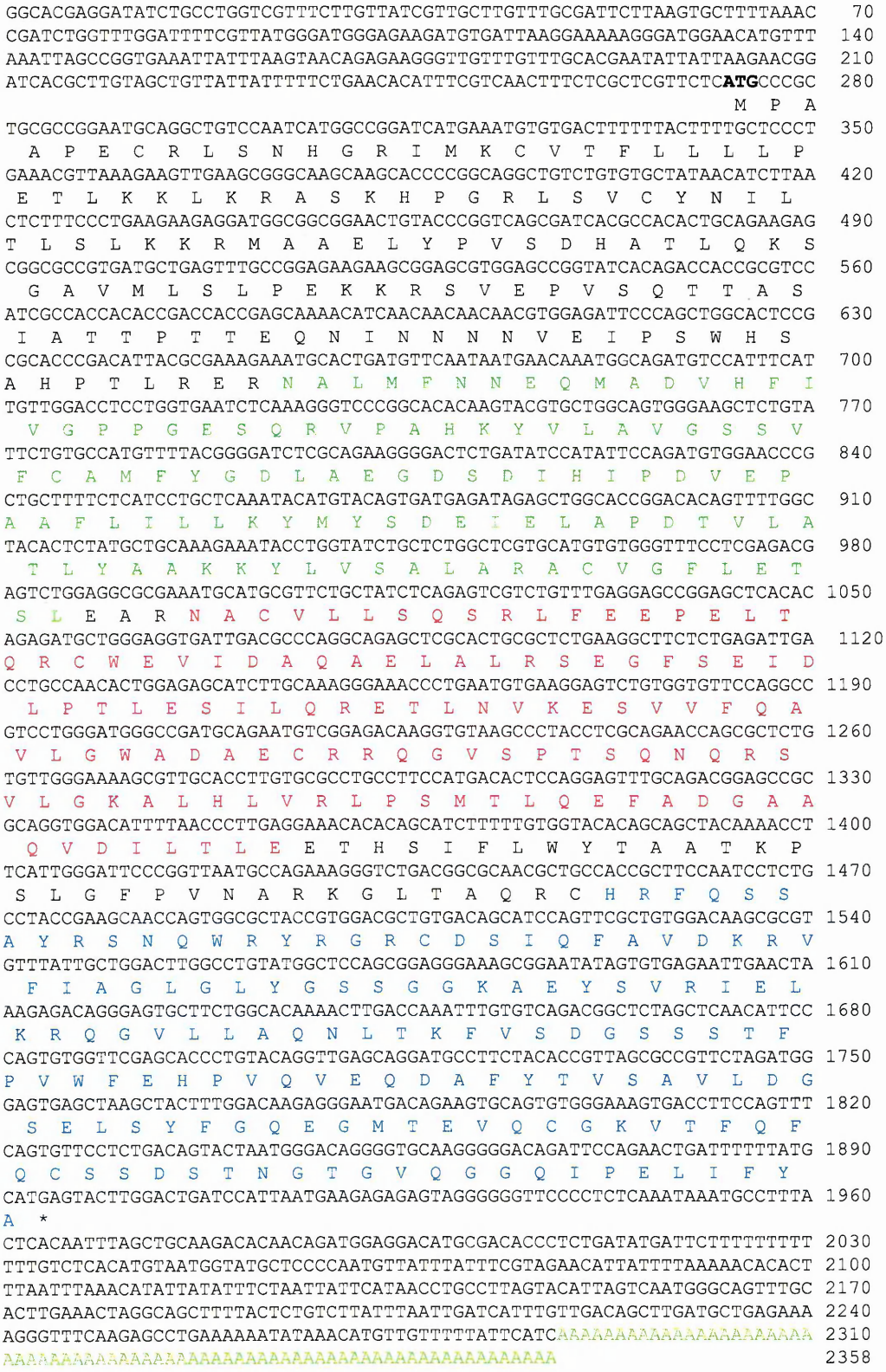

GGCACGAGGATATCTGCCTGGTCGTTTCTTGTTATCGTTGCTTGTTTGCGATTCTTAAGTGCTTTTAAAC 70
CGATCTGGTTTGGATTTTCGTTATGGGATGGGAGAAGATGTGATTAAGGAAAAAGGGATGGAACATGTTT 140
AAATTAGCCGGTGAAATTATTTAAGTAACAGAGAAGGGTTGTTTGTTTGCACGAATATTATTAAGAACGG 210
ATCACGCTTGTAGCTGTTATTATTTTTCTGAACACATTTCGTCAACTTTCTCGCTCGTTCTCATGCCCGC 280
M P A
TGCGCCGGAATGCAGGCTGTCCAATCATGGCCGGATCATGAAATGTGTGACTTTTTTACTTTTGCTCCCT 350
A P E C R L S N H G R I M K C V T F L L L L P
GAAACGTTAAAGAAGTTGAAGCGGGCAAGCAAGCACCCCGGCAGGCTGTCTGTGTGCTATAACATCTTAA 420
E T L K K L K R A S K H P G R L S V C Y N I L
CTCTTTCCCTGAAGAAGAGGATGGCGGCGGAACTGTACCCGGTCAGCGATCACGCCACACTGCAGAAGAG 490
T L S L K K R M A A E L Y P V S D H A T L Q K S
CGGCGCCGTGATGCTGAGTTTGCCGGAGAAGAAGCGGAGCGTGGAGCCGGTATCACAGACCACCGCGTCC 560
G A V M L S L P E K K R S V E P V S Q T T A S
ATCGCCACCACACCGACCACCGAGCAAAACATCAACAACAACAACGTGGAGATTCCCAGCTGGCACTCCG 630
I A T T P T T E Q N I N N N N V E I P S W H S
CGCACCCGACATTACGCGAAAGAAATGCACTGATGTTCAATAATGAACAAATGGCAGATGTCCATTTCAT 700
A H P T L R E R N A L M F N N E Q M A D V H F I
TGTTGGACCTCCTGGTGAATCTCAAAGGGTCCCGGCACACAAGTACGTGCTGGCAGTGGGAAGCTCTGTA 770
V G P P G E S Q R V P A H K Y V L A V G S S V
TTCTGTGCCATGTTTTACGGGGATCTCGCAGAAGGGGACTCTGATATCCATATTCCAGATGTGGAACCCG 840
F C A M F Y G D L A E G D S D I H I P D V E P
CTGCTTTTCTCATCCTGCTCAAATACATGTACAGTGATGAGATAGAGCTGGCACCGGACACAGTTTTGGC 910
A A F L I L L K Y M Y S D E I E L A P D T V L A
TACACTCTATGCTGCAAAGAAATACCTGGTATCTGCTCTGGCTCGTGCATGTGTGGGTTTCCTCGAGACG 980
T L Y A A K K Y L V S A L A R A C V G F L E T
AGTCTGGAGGCGCGAAATGCATGCGTTCTGCTATCTCAGAGTCGTCTGTTTGAGGAGCCGGAGCTCACAC 1050
S L E A R N A C V L L S Q S R L F E E P E L T
AGAGATGCTGGGAGGTGATTGACGCCCAGGCAGAGCTCGCACTGCGCTCTGAAGGCTTCTCTGAGATTGA 1120
Q R C W E V I D A Q A E L A L R S E G F S E I D
CCTGCCAACACTGGAGAGCATCTTGCAAAGGGAAACCCTGAATGTGAAGGAGTCTGTGGTGTTCCAGGCC 1190
L P T L E S I L Q R E T L N V K E S V V F Q A
GTCCTGGGATGGGCCGATGCAGAATGTCGGAGACAAGGTGTAAGCCCTACCTCGCAGAACCAGCGCTCTG 1260
V L G W A D A E C R R Q G V S P T S Q N Q R S
TGTTGGGAAAAGCGTTGCACCTTGTGCGCCTGCCTTCCATGACACTCCAGGAGTTTGCAGACGGAGCCGC 1330
V L G K A L H L V R L P S M T L Q E F A D G A A
GCAGGTGGACATTTTAACCCTTGAGGAAACACACAGCATCTTTTTGTGGTACACAGCAGCTACAAAACCT 1400
Q V D I L T L E E T H S I F L W Y T A A T K P
TCATTGGGATTCCCGGTTAATGCCAGAAAGGGTCTGACGGCGCAACGCTGCCACCGCTTCCAATCCTCTG 1470
S L G F P V N A R K G L T A Q R C H R F Q S S
CCTACCGAAGCAACCAGTGGCGCTACCGTGGACGCTGTGACAGCATCCAGTTCGCTGTGGACAAGCGCGT 1540
A Y R S N Q W R Y R G R C D S I Q F A V D K R V
GTTTATTGCTGGACTTGGCCTGTATGGCTCCAGCGGAGGGAAAGCGGAATATAGTGTGAGAATTGAACTA 1610
F I A G L G L Y G S S G G K A E Y S V R I E L
AAGAGACAGGGAGTGCTTCTGGCACAAAACTTGACCAAATTTGTGTCAGACGGCTCTAGCTCAACATTCC 1680
K R Q G V L L A Q N L T K F V S D G S S S T F
CAGTGTGGTTCGAGCACCCTGTACAGGTTGAGCAGGATGCCTTCTACACCGTTAGCGCCGTTCTAGATGG 1750
P V W F E H P V Q V E Q D A F Y T V S A V L D G
GAGTGAGCTAAGCTACTTTGGACAAGAGGGAATGACAGAAGTGCAGTGTGGGAAAGTGACCTTCCAGTTT 1820
S E L S Y F G Q E G M T E V Q C G K V T F Q F
CAGTGTTCCTCTGACAGTACTAATGGGACAGGGGTGCAAGGGGGACAGATTCCAGAACTGATTTTTTATG 1890
Q C S S D S T N G T G V Q G G Q I P E L I F Y
CATGAGTACTTGGACTGATCCATTAATGAAGAGAGAGTAGGGGGGTTCCCCTCTCAAATAAATGCCTTTA 1960
A *
CTCACAATTTAGCTGCAAGACACAACAGATGGAGGACATGCGACACCCTCTGATATGATTCTTTTTTTTT 2030
TTTGTCTCACATGTAATGGTATGCTCCCCAATGTTATTTATTTCGTAGAACATTATTTTAAAAACACACT 2100
TTAATTTAAACATATTATATTTCTAATTATTCATAACCTGCCTTAGTACATTAGTCAATGGGCAGTTTGC 2170
ACTTGAAACTAGGCAGCTTTTACTCTGTCTTATTTAATTGATCATTTGTTGACAGCTTGATGCTGAGAAA 2240
AGGGTTTCAAGAGCCTGAAAAAATATAAACATGTTGTTTTTATTCATCAAAAAAAAAAAAAAAAAAAAA 2310
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2358

Figure 17. Nucleotide sequence and predicted amino acid sequence of zebrafish BTBD6b. The open reading frame is accompanied by the predicted amino acids shown by the single letter designation beneath it. A start codon (ATG) shown in bold is at nucleotide position 174 and the termination codon (TGA) at position 1620 is indicated by an asterix. A putative polyadenylation signal and a poly(A) tail are indicated in pink and light green, respectively. The BTB domain, BACK domain and PHR motif are labelled in green, red and blue, respectively.

```
ATGGCTCCTGGTTGCCAGCGAGCCTCCAAACATCAAACCTGAATGTATTTTTCCTGGCAGCTGCGCTTCG   70
CTCGGCTCAATAAGAAACCTTAAAAAGGACTAGAAAGAGTGGGAAACAGCTCAGCAAGCTGCCAGTATGC  140
TATGAAATCGTGACTTTGTCCTTGAGGAAGAAGATGGCTGCAGAACTCTATCCTGCCAGCATAAATACCA  210
                                    M  A  A  E  L  Y  P  A  S  I  N  T
ACCTTCCAAACAGTAACAGCACAGCAGTCACAGCTGCCAGCAAAAAGACCATCGTCCAAGTCACTCAAAC  280
N  L  P  N  S  N  S  T  A  V  T  A  A  S  K  K  T  I  V  Q  V  T  Q  T
GGTGACCACACCGACTACAACTGCCACTCAGCAGAACATCAACAATAATAACGTCGAGACTGCCAGCTGG  350
   V  T  T  P  T  T  T  A  T  Q  Q  N  I  N  N  N  N  N  V  E  T  A  S  W
CAGTCCACTCACCCGACATTGCGAGAAAGGAATGCTTTGATGTTCAATAACGAACTCATGGCAGATGTTC  420
 Q  S  T  H  P  T  L  R  E  R  N  A  L  M  F  N  N  E  L  M  A  D  V
ATTTTGTTGTGGGTCCTCCTGGCGCATCCCAAAAAGTTCCAGCACACAAGTATGTGCTGGCAGTGGGGAG  490
H  F  V  V  G  P  P  G  A  S  Q  K  V  P  A  H  K  Y  V  L  A  V  G  S
TTCTGTTTTTGGTGCCATGTTTTATGGAGATCTAGCAGAAGGAGAATCTGAGATTCATATTCCTGATGTG  560
   S  V  F  G  A  M  F  Y  G  D  L  A  E  G  E  S  E  I  H  I  P  D  V
GAACCTGCTGCTTTTTTAATTCTTTTGAAGTACATGTACAGCGATGAGATTGAACTTGAAGCGGACACAG  630
 E  P  A  A  F  L  I  L  L  K  Y  M  Y  S  D  E  I  E  L  E  A  D  T
TGCTGGCCACTCTGTACGCTGCCAAAAAATATATAGTGCCTGCGCTGGCTAAGGCCTGTGTCACCTTTCT  700
V  L  A  T  L  Y  A  A  K  K  Y  I  V  P  A  L  A  K  A  C  V  T  F  L
GGAGACAAGCCTGGAGGCCAAAAATGCCTGTGTGTTGTTGTCCCAGAGTAGACTATTTGAGGAGCCTGAG  770
   E  T  S  L  E  A  K  N  A  C  V  L  L  S  Q  S  R  L  F  E  E  P  E
CTGACCCTGCGGTGTTGGGAGGTCATTGATGCTCAGGCTGAACTGGCGCTTCACTCTGAGGGCTTCTGTG  840
 L  T  L  R  C  W  E  V  I  D  A  Q  A  E  L  A  L  H  S  E  G  F  C
AGATTGATCTTCAAACGCTGGAGATCATACTAAAGAGAGAGACTCTGAATACCCGGGAGGCAGTGGTCTT  910
E  I  D  L  Q  T  L  E  I  I  L  K  R  E  T  L  N  T  R  E  A  V  V  F
TCAGGCAGCTCTTGATTGGGCTGTGGCTGAATGCAAAAGGCAAGGACTGGGACCGACCGCTCGTAATAAA  980
   Q  A  A  L  D  W  A  V  A  E  C  K  R  Q  G  L  G  P  T  A  R  N  K
AGGGCAGTGCTGGGCAAGGCTCTCTACTTGGTTCGCATCCCAACCATGACCCTGGAGGAGTTCGCCAATG 1050
 R  A  V  L  G  K  A  L  Y  L  V  R  I  P  T  M  T  L  E  E  F  A  N
GAGCTGCACAGTCAGATGTATTGACTCTGGAAGAGACTCATGATGTCTTCCTTTGGTACACTGCAGCCAA 1120
G  A  A  Q  S  D  V  L  T  L  E  E  T  H  D  V  F  L  W  Y  T  A  A  N
TAAACCCAAGCTGGAATTCCCACTACAAAAAAGAAAAGGTTTGACGCCACAGCGATGCCATCGCTTCCAG 1190
   K  P  K  L  E  F  P  L  Q  K  R  K  G  L  T  P  Q  R  C  H  R  F  Q
TCTTCGGCCTACCGCAGTAACCAGTGGCGCTACCGTGGACGTTGCGATAGCATCCAGTTTGCAGTGGACA 1260
 S  S  A  Y  R  S  N  Q  W  R  Y  R  G  R  C  D  S  I  Q  F  A  V  D
AGAGGATTTTCATCGCAGGACTTGGCTTGTATGGTTCAAGTGGTGGAAAGGCAGAGTACAGCGTCAAGAT 1330
K  R  I  F  I  A  G  L  G  L  Y  G  S  S  G  G  K  A  E  Y  S  V  K  I
CGAACTCAAGCGCCAAGGAGTGACTCTGGCCCAGAACCTAACAAAATTTATCTCAGATGGATCCAGCAAC 1400
   E  L  K  R  Q  G  V  T  L  A  Q  N  L  T  K  F  I  S  D  G  S  S  N
ACCTTCTCCGTATGGTTTGAACACCCCGTCCAAGTGGAGCAGGACACCTTCTACACAGTCAGTGCTGTAT 1470
 T  F  S  V  W  F  E  H  P  V  Q  V  E  Q  D  T  F  Y  T  V  S  A  V
TGGATGGGAATGAACTTAGTTATTTTGGACAGGAGGGTATGACAGAAGTGCAGTGTGGAAAAGTGACCTT 1570
L  D  G  N  E  L  S  Y  F  G  Q  E  G  M  T  E  V  Q  C  G  K  V  T  F
CCAGTTCCAGTGTTCCTCGGACAGTACCAATGGAACTGGAGTCCAGGGGGGTCAGATCCCAGAGCTGGTC 1610
   Q  F  Q  C  S  S  D  S  T  N  G  T  G  V  Q  G  G  Q  I  P  E  L  V
TTCTATGCGTGAAATTGTCAGGATGTCCCATTGTAGTGCATTCCGCTCACTGGAATTAAGATGGATTGAA 1680
 F  Y  A  *
TACAGGGTCTCGTGAGGCTCTTGCCTTCATTATGTAGCAGTTGTGAACTCAATGGTTATGGCTCCTCTGT 1750
ACCACAATGGACTCAGATGGAAGGAATGTTCTTCAGCTTTATTTATTGCAAAAAAATATCTATATTTGAT 1820
TTTAATTATTGACGCTGCTGCAAGCAGATCTATATATAAATATATATAAATGAGATAGTTGTACTGCTCT 1890
GATAGTGGACGGAAGCCACTAGTGTGTCATGTTTTTGTACATAACGTATTTGTTTTCAGCTTGATGCTGA 1960
TTGACAAATAAAATGTCTTGTATTAGTGAACCGCTTCCCGAACTGCAGATTATAAATGTTTTTGTTCCTC 2030
GAGTCCAAAAAAAAAAAAAAAAAAA                                               2056
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Nucleotide sequence of the 2029 bp cDNA clone represented by AI883008 contained an ORF (nt 80-1465) encoding a putative protein of 461 amino acids, which shows 74% identity with the cBTBD6 protein and 88.5% with the cBTBD3 protein. Based on the close homology to human and chick BTBD3 this gene is designated zBTBD3a (Fig. 18&20).

Further BLAST searches of the Ensembl zebrafish sequence database performed at a later date, using chick BTBD6 and BTBD3 cDNA, identified another novel gene that encodes a putative highly related protein (68.9 and 90.1% identity, respectively). No corresponding ESTs were available for this gene. However the RT PCR using primers located within the coding region conducted on 2-13 somite stage zebrafish embryos, generated a 850 bp single product the sequence of which corresponds to the one obtained from the database. The purpose of amplifying these sequences was to generate a DNA template for the RNA in situ hybridisation probe (see chapter on the expression patterns) rather than cloning the full gene. Using the predicted cDNA sequence provided by Ensembl, the full ORF was identified which produces a deduced polypeptide of 517 amino acids (Fig. 19). This gene was designated zBTBD3b.

Thus, two of the identified novel zebrafish genes, corresponding to ESTs: BG308275 and BI350992/AI437221, are very highly related to cBTBD6 and less related to cBTBD3 and are designated zBTBD6a and zBTBD6b, respectively. Two other identified genes, one equivalent to EST AI883008 and the other found in the Ensembl database, are very highly related to cBTBD3 and less related to cBTBD6 and are designated zBTBD3a and zBTBD3b, respectively.

PHYLOGENETIC ANALYSIS OF THE VERTEBRATE BTBD6 AND BTBD3 PROTEINS

To determine the relationship between the human, mouse, chick and zebrafish BTBD6 and BTBD3 predicted proteins, the amino acid sequences

```
TGGCCTACTGTCTGAACTCCATCCGTTGCCAGATCCCCTGCCATGAAATCCTCCTTATGACTTGACATGT   70
GTTGGTAAAATGGCTGCTGAGCTGTTCCCTACCAAAAAACTGCCCACCGTCAGTGCCAGCGCCGTCCAGC  140
          M  A  A  E  L  F  P  T  K  K  L  P  T  V  S  A  S  A  V  Q
AGTTCCAGCAGCAGAACGTCAGCAACAACAACACCATTCAGGGATGTAACTGGCAAGGCTTGTATCCCAC  210
Q  F  Q  Q  Q  N  V  S  N  N  N  T  I  Q  G  C  N  W  Q  G  L  Y  P  T
AATCAGAGAAAGAAACTCAGTCATGTTCAACAACGAGCTGATGGCCGATATTCATTTTGTGGTGGGACCT  280
  I  R  E  R  N  S  V  M  F  N  N  E  L  M  A  D  I  H  F  V  V  G  P
CCGGGAGGAACCCAGAGAGTGCCGGGACACAAGTATGTCCTGGCTGTGGGTAGCTCAGTTTTTCATGCCA  350
  P  G  G  T  Q  R  V  P  G  H  K  Y  V  L  A  V  G  S  S  V  F  H  A
TGTTTTATGGAGAACTGGCTGAAGATAAAGATGAGATCCGGATCCCTGATGTGGAGCCACCCTCATTCCT  420
M  F  Y  G  E  L  A  E  D  K  D  E  I  R  I  P  D  V  E  P  P  S  F  L
GGCCATGCTGAAGTACATCTACTGTGATGAGATCGACCTCTGCGCAGACACTGTACTGGCCACACTGTAC  490
  A  M  L  K  Y  I  Y  C  D  E  I  D  L  C  A  D  T  V  L  A  T  L  Y
GCCGCCAAAAAGTACATCGTCCCCCATTTAGCCCGTGCTTGCGTCAACTTCCTAGAGACCAGCCTGAGCG  560
  A  A  K  K  Y  I  V  P  H  L  A  R  A  C  V  N  F  L  E  T  S  L  S
CCAGAAACGCCTGCGTTCTGCTGTCCCAGAGCTGCCTGTTCGAGGAGCCTGACCTGACGCAGCGATGCTG  630
A  R  N  A  C  V  L  L  S  Q  S  C  L  F  E  E  P  D  L  T  Q  R  C  W
GGAGGTGATTGACGCGCAGGCTGAACTTGCGCTCCGCTCTGAAGGATTCTGCGACATTGACACTCAAACG  700
  E  V  I  D  A  Q  A  E  L  A  L  R  S  E  G  F  C  D  I  D  T  Q  T
TTAGAAAGCATCCTACGGCGTGAAACGCTCAACGCTAAAGAGATGGTGGTTTTCGAAGCGACGTTGAGCT  770
  L  E  S  I  L  R  R  E  T  L  N  A  K  E  M  V  V  F  E  A  T  L  S
GGGCTGAAGCCGAATGTCACCGACAGGAACTCCAACCCACAATCGAAAACAAGCGACTTGTTTTGGGAAA  840
W  A  E  A  E  C  H  R  Q  E  L  Q  P  T  I  E  N  K  R  L  V  L  G  K
GTCTATTTATCTAATACGAATCCCTGCGATGGCGCTTGATGATTTTGCTAATGGCGTCGCTCAGTCAGGA  910
  S  I  Y  L  I  R  I  P  A  M  A  L  D  D  F  A  N  G  V  A  Q  S  G
GTGCTAACGCTCAACGAAACAAACGATATTTTCTTATGGTACACAGCAGCCAAAAAACCAGAGCTGAAGT  980
  V  L  T  L  N  E  T  N  D  I  F  L  W  Y  T  A  A  K  K  P  E  L  K
TTGTGTGCAAACCGCGAAAAGGTTTAACGCCGCAAAAGTGCCATCGTTTTCAATCGTGCGCTTATCGTAG 1050
F  V  C  K  P  R  K  G  L  T  P  Q  K  C  H  R  F  Q  S  C  A  Y  R  S
CAATCAGTGGCGCTACCGTGGGCGCTGCGATAGCATTCAGTTCGCCGTGGACAAACGTGTGTTTATTGCT 1120
  N  Q  W  R  Y  R  G  R  C  D  S  I  Q  F  A  V  D  K  R  V  F  I  A
GGATTTGGTTTGTATGGCTCTAGCTGCGGTTCAGCGGAGTACCAGGCCAAGATTGAATTGAAGCGGCAGG 1190
  G  F  G  L  Y  G  S  S  C  G  S  A  E  Y  Q  A  K  I  E  L  K  R  Q
GAGTCACGCTCGGCATCGCCATCATCAAATATTTCTCCGACGGCTCTAGCAACACCTTCTCTGTGTTCTT 1260
G  V  T  L  G  I  A  I  I  K  Y  F  S  D  G  S  S  N  T  F  S  V  F  F
CGAGTATCCGGTGCAGATCGAGCCGGACACTTTCTACACTGCCAGCGTCATTTTGGACGGCAACGAGTTG 1330
  E  Y  P  V  Q  I  E  P  D  T  F  Y  T  A  S  V  I  L  D  G  N  E  L
AGTTATTTTGGGCAGGAGGGCATGACGGAGGTGCAGTGTGGTAAGGTGACCTTCCAGTTCCAGTGTTCGT 1400
  S  Y  F  G  Q  E  G  M  T  E  V  Q  C  G  K  V  T  F  Q  F  Q  C  S
CCGACAGCACCAACGGGACGGGTGTGCAGGGTGGCCAGATCCCCGAACTCATATTTTACGCCTGAGAGAG 1470
S  D  S  T  N  G  T  G  V  Q  G  G  Q  I  P  E  L  I  F  Y  A  *
AACAAAAGTCCTATTTAGGATTATTCAGAAACAAAAAGGTTTCTGTTCCTTAATGAAGTGCTGCTTTGAT 1540
TTTGCACAAAGGCTGCAAAAAGAATTGTAGAGATTACCGACAATCTATATATAGACTGTGTTAGAAGTAG 1610
GGTTGTCAAAAGTATCGAATTCGGTGCAAAAATTTAAAAAATGTCCACTTCCTGCTAGGATTTGAGTGCA 1680
TACTTAAACATTGGCTGATCGGCCACTGTGTTCAAATGCTCAACATAAATGACTGTGACTGGCTGTGAAG 1750
ATCATCGGTTCATCACTGTTCACCGAGTGCAAATGTTTCTAAAATTTTAGAACCGATTGGTATCGAACTC 1820
GATACTTTTGACAACCCTGGCTAGAATTGTTAGAAGCGCAAAGGAAAAGAGGAAAATTTGGTCATTTTAC 1890
TCAACCTCATTTAGTTATAAACCTGAAGATGATTTTAATTTTTTTTAAGTTTATTCATGCAGGTCAGTCG 1960
AATTTCAGAAAAAAAATCTAACTTAATAAATTAAAGATATAGTTAACCCAAAAAAAAAAAAAAAAAAAA   2027
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Figure 18. Nucleotide and deducted amino acid sequence of zebrafish BTBD3a. The open reading frame is accompanied by the predicted amino acids shown by the single letter designation beneath it. A start codon (ATG) shown in bold is at nucleotide position 80 and the termination codon (TGA) at position 1465 is indicated by an asterix. A polyadenylation signal and a poly(A) tail are is shown in pink and light green, respectively. The BTB domain, BACK domain and PHR motif are labelled in green, red and blue, respectively.

```
CTCAGCATCTTCTCTGCATGCTTTTCCACTGCTCTGCAGGTGAACACACTCATGGTGGATGCCAAGGGAA   70
                                                      M  V  D  A  K  G
GGAACATGAAATGTCTGACGTTCTTGTTGATGCTTCCAGAATCAGTTAAGAGCAGGTCCAGTAAAGGCTC  140
R  N  M  K  C  L  T  F  L  L  M  L  P  E  S  V  K  S  R  S  S  K  G  S
CAAAAAGGGGAGTCCCAGCAGCTCGTCCAAGCTGCCCCCTGTGTGCTATGAGATCATCACTTTGAAGACC  210
   K  K  G  S  P  S  S  S  S  K  L  P  P  V  C  Y  E  I  I  T  L  K  T
AAGAAGAAGAAGAAGATGGCAGCGGAGATCTTTCCTACCAAGAAGCCGGCGTCGGCCACCACGGTGCAGC  280
 K  K  K  K  K  M  A  A  E  I  F  P  T  K  K  P  A  S  A  T  T  V  Q
AGTACCAGCAGCAGAACTTGAACAATAATAACACTATCCAATGCTGCAACTGGCAGGGTCTCTACTCCAC  350
Q  Y  Q  Q  Q  N  L  N  N  N  N  T  I  Q  C  C  N  W  Q  G  L  Y  S  T
CATCAGGGAGAGAAATTCTGTGATGTTTAATAATGAACTGATGGCTGACGTTCACTTTGTTGTCGGTCAG  420
   I  R  E  R  N  S  V  M  F  N  N  E  L  M  A  D  V  H  F  V  V  G  Q
TCTGGAGGGACTCAAAGGCTCCCGGGACACAAGTATGTCCTTGCTGTGGGAAGCTCTGTTTTCCATGCCA  490
 S  G  G  T  Q  R  L  P  G  H  K  Y  V  L  A  V  G  S  S  V  F  H  A
TGTTCTATGGAGAGCTGGCGGAAGACACGGATGAGATTCGTATTCCTGATGTGGAACCTCCAGCTTTTCT  560
M  F  Y  G  E  L  A  E  D  T  D  E  I  R  I  P  D  V  E  P  P  A  F  L
GGCTATGCTAAAGTACATTTACTGTGACGAAATTGACTTAAGTGCCGACACCGTCTTGGCAACTTTATAC  630
   A  M  L  K  Y  I  Y  C  D  E  I  D  L  S  A  D  T  V  L  A  T  L  Y
GCAGCCAAAAAGTACATAGTCCCACACCTGGCACGGGCTTGCGTCAACTTCTTGGAAACAAGTTTGAGTG  700
 A  A  K  K  Y  I  V  P  H  L  A  R  A  C  V  N  F  L  E  T  S  L  S
CCAAAAACGCGTGTATTCTTTTATCTCAAAGCTGTCTGTTCGAAGAGCCAGACCTGACACAACGCTGCTG  770
A  K  N  A  C  I  L  L  S  Q  S  C  L  F  E  E  P  D  L  T  Q  R  C  W
GGAAGTCATAGACGCTCAGGCAGAGCTGGCGCTTAAATCCGATGGCTTTTGTGACATTGACTCTCAGACC  840
   E  V  I  D  A  Q  A  E  L  A  L  K  S  D  G  F  C  D  I  D  S  Q  T
TTGGAGAGCATCCTCAGACGGGAGACGCTGAATGCAAAGGAGATTGTAGTGTTTGAAGCAGCACTAAGCT  910
 L  E  S  I  L  R  R  E  T  L  N  A  K  E  I  V  V  F  E  A  A  L  S
GGGCAGATGCCGAGTGCCAACGGAGGGAGATGAACACCTCTATTGACAACAAACGCAAGGTGTTGGGTCA  980
W  A  D  A  E  C  Q  R  R  E  M  N  T  S  I  D  N  K  R  K  V  L  G  Q
GTCCATATATCTAATACGTATCCCAACAATGGGTCTTGATGATTTCGCAAATGGTGCCGCGCAGTCAGGA 1050
   S  I  Y  L  I  R  I  P  T  M  G  L  D  D  F  A  N  G  A  A  Q  S  G
GTGTTGACGTTAAACGAAACCAATGACATTTTCTTATGGTACACAGCGGCTAAGAAACCCGAACTGCAGT 1120
 V  L  T  L  N  E  T  N  D  I  F  L  W  Y  T  A  A  K  K  P  E  L  Q
TTGCCAGTCAACCTCGTAAAGGCTTGACACCACAGAAGTGCCACCGCTTCCAGTCGTGCGCCTACCGCAG 1190
F  A  S  Q  P  R  K  G  L  T  P  Q  K  C  H  R  F  Q  S  C  A  Y  R  S
CAATCAATGGCGATACCGCGGACGCTGCGACAGCATTCAGTTCGCTGTTGACAAGCGGGTGTTCATCGCC 1260
   N  Q  W  R  Y  R  G  R  C  D  S  I  Q  F  A  V  D  K  R  V  F  I  A
GGTTTTGGGCTTTACGGCTCAAGCTGTGGGTCAGCTGAGTACACCGCCAAAATCGAGCTCAAACGGCAAG 1330
 G  F  G  L  Y  G  S  S  C  G  S  A  E  Y  T  A  K  I  E  L  K  R  Q
GGGTGAATTTGGGAACCAACCTCAGCAAGTACTTTTCCGATGGATCTAGTAACACCTTCCCCGTTTGGTT 1400
G  V  N  L  G  T  N  L  S  K  Y  F  S  D  G  S  S  N  T  F  P  V  W  F
TGAGTATCCAGTTCAAATCGAGCCGGACACTTTCTACACTGCCAGCGTGGTTCTGGATGGAAATGAACTG 1470
   E  Y  P  V  Q  I  E  P  D  T  F  Y  T  A  S  V  V  L  D  G  N  E  L
AGCTATTTCGGACAAGAGGGTATGACGGAAGTGCAGTGTGGAAAGGTGACCTTTCAGTTTCAGTGCTCTT 1540
 S  Y  F  G  Q  E  G  M  T  E  V  Q  C  G  K  V  T  F  Q  F  Q  C  S
CGGACAGTACCAACGGCACAGGTGTGCAAGGCGGCCAGATCCCAGAGCTCATTTTCTATGCCTGA      1605
S  D  S  T  N  G  T  G  V  Q  G  G  Q  I  P  E  L  I  F  Y  A  *
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Figure 19. Nucleotide sequence and deduced amino acid sequence of a putative zebrafish BTBD3b cDNA. The open reading frame is accompanied by the predicted amino acids designated by the single letter abbreviations shown underneath. The putative translation start codon (ATG) is highlighted in bold and a stop codon (TGA) is designated by an asterix. The BTB domain, BACK domain and PHR motif are labelled in green, red and blue, respectively.

were aligned with the ClustalW algorithm (Thompson et al., 1994) of the Megalign (DNAstar Inc.) program. The most notable feature of this alignment is that all the above-mentioned proteins are outstandingly related, especially in the region of the BTB, BACK and PHR domains all present in each of these proteins (Fig. 20). The N-terminus diverges most in all analysed proteins, however it is more conserved in the BTBD3 proteins.

The analysis of the phylogenetic tree constructed on the basis of the peptide alignment indicates that the BTBD6 proteins are clustered together as are the BTBD3 proteins, suggesting that they represent two subgroups of the same protein family. There are single human, mouse and chick homologues and two zebrafish homologous genes within each subgroup. In both subgroups the human BTBD6 and BTBD3 proteins are closest to their mouse and chick counterparts (Fig. 20).

DISCUSSION

Chick V11 is BTBD6 and contains BTB, BACK and PHR domains

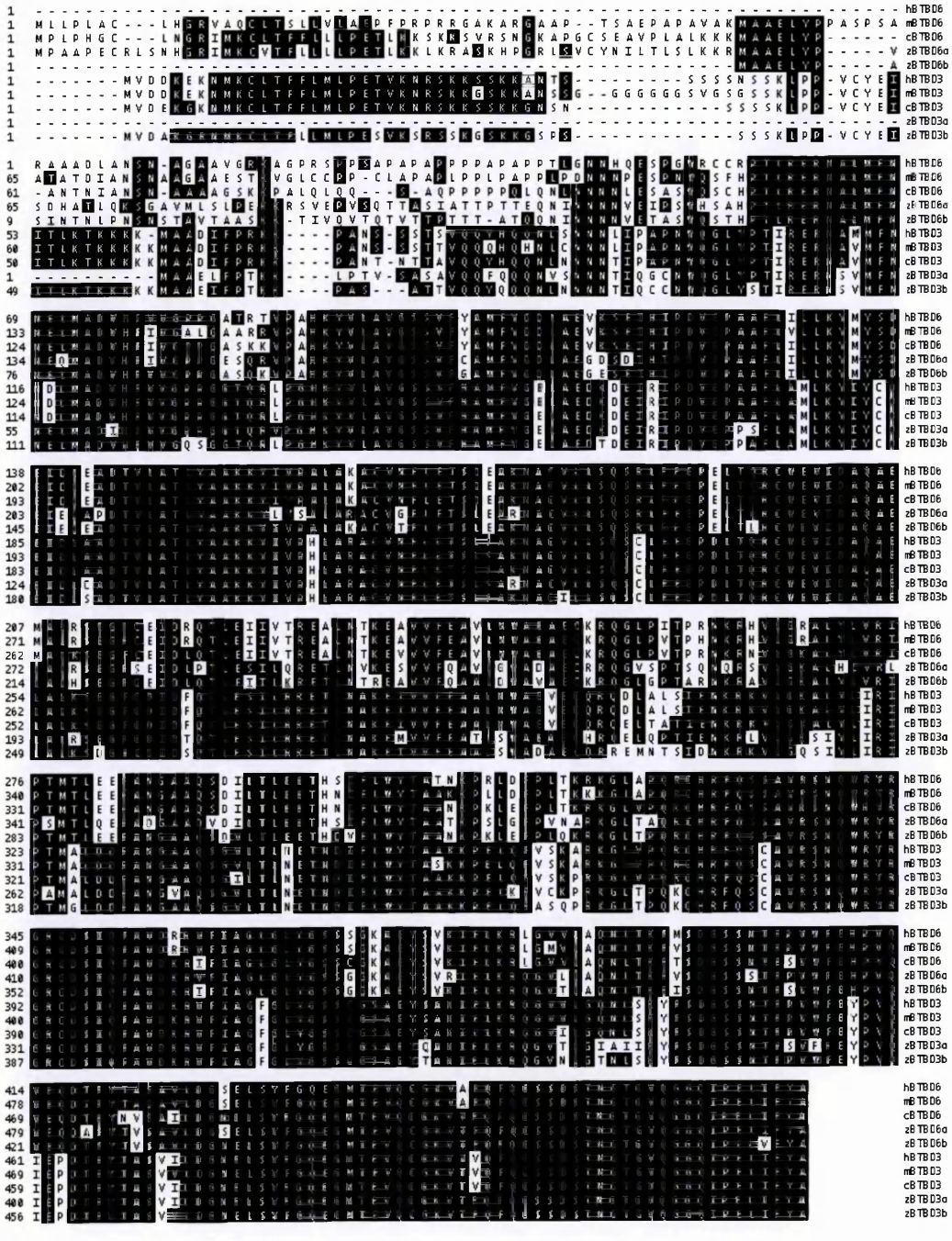
Nucleotide sequence analysis and amino acid translation of the predicted full-length chick V11 cDNA established that cV11 represents a novel gene encoding a putative protein most similar to an unknown human protein designated in the sequence databases by BTB domain-containing protein 6 (BTBD6) (Strausberg et al., 2002; Wistow et al., 2002). Based on the very high homology with human BTBD6, it is believed that V11 represents BTBD6 in chick.

Examination of the cBTBD6 protein for conserved protein sequence motifs revealed, apart from the BTB domain, the presence of two other motifs, BACK and PHR.

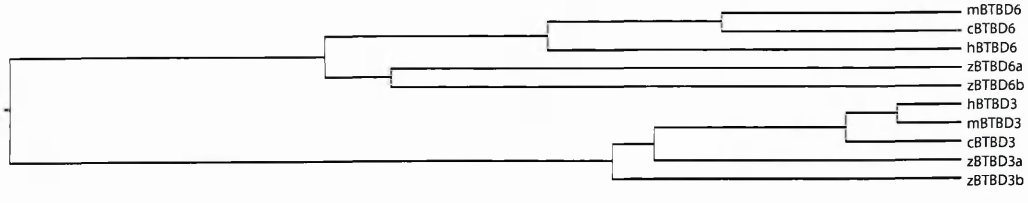
The BTB/POZ domain is highly conserved through out the species and facilitates protein-protein interaction. This motif has been found in proteins

Figure 20. Phylogenetic analysis of predicted BTBD6 and BTBD3 proteins. (A) Multiple amino acid sequence alignment of predicted BTBD6 and BTBD3 proteins in human (h), mouse (m), chick (c) and zebrafish (z). Identical proteins are denoted by the black background. Dashes represent gaps inserted for maximal alignment, and numbers of amino acid residues in each sequence are indicated on the left. The sequences were aligned using the Lasergene/DNASTAR software, CLUSTALW method. (B) Phylogenetic tree constructed on the basis of the above alignment. Distance along the horizontal axis is inversely proportional to the relatedness of the input.

A


hBTBD6
mBTBD6
cBTBD6
zBTBD6a
zBTBD6b
hBTBD3
mBTBD3
cBTBD3
zBTBD3a
zBTBD3b

B


mBTBD6
cBTBD6
hBTBD6
zBTBD6a
zBTBD6b
hBTBD3
mBTBD3
cBTBD3
zBTBD3a
zBTBD3b

involved in transcriptional regulation (Ahmad et al., 1998; Zollman et al., 1994), cytoskeletal arrangement (Kang et al., 2004; Melnick et al., 2000) and ion conductance (Kreusch et al., 1998). Within the last two years, there has been a number of reports identifying a new role of BTB-containing proteins as the substrate-specific adaptors in protein ubiquitination (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003).

The BACK domain has recently been identified as a conserved region in the BTB/kelch proteins. The role of this motif is not clearly defined, however, it has recently been reported that in the BTB/kelch proteins which mark proteins for ubiquitination, BACK domain plays a role as an interacting region (Kobayashi and Yamamoto, 2005).

The PHR motif was first identified in human PAM, *Drosophila* highwire and *C. elegans* RPM-1, proteins that do not contain BTB domain. These proteins are presynaptic regulators of synapse formation and/or growth and, interestingly, are likely to act as putative ubiquitin ligases. The PHR motif has been identified only in two other BTB-containing proteins, human BTBD1 and BTBD2 (Carim-Todd et al., 2001; Xu et al., 2002; Xu et al., 2003b). However, the function of the PHR motif is still unknown.

Thus, cBTBD6 represents a novel gene that encodes a protein characterised by the presence of three domains, BTB, BACK and PHR. BTB is a protein interaction domain and the other two domain have unknown function, however, interestingly, the presence of all three motifs is associated with the protein ubiquitination process.

BTBD6 and BTBD3 gene family

Human, mouse and zebrafish BTBD6 orthologs were cloned, as well as highly related BTBD3 genes were identified. Comparative analysis of the predicted proteins encoded by the isolated genes revealed high sequence conservation, especially within the BTB, BACK and PHR domain regions.

Phylogenetic examination of the BTBD6 and BTBD3 putative polypeptide sequences suggested that the characterised genes belong to two subgroups, BTBD6 and BTBD3, of the same family of genes. Within each subgroup there is a single copy of human, chick and mouse BTBD6 or BTBD3 genes and two copies of zebrafish orthologues. The presence of two zebrafish genes is most probably the result of a genome duplication in the zebrafish lineage (Amores et al., 1998; Meyer and Schartl, 1999; Postlethwait et al., 1998).

BTBD6 and BTBD3 represent novel genes, whose functions have not been characterised yet. Two other proteins, human BTBD1 and BTBD2, containing both BTB and PHR domains have previously been reported. The very little published data report that both BTBD1 and BTBD2 interact with Topoisomerase 1 (relaxes DNA supercoils generated during DNA replication and transcription) and co-localise with the RBCC/tripartite motif protein, TRIM5δ (potential role as a ubiquitin ligase) (Carrim-Todd et al., 2001; Xu et al.,2002; Xu et al., 2003). However no specific cellular or developmental function of BTBD1 and BTBD2 proteins has been identified.

Since BTBD6 appears to be evolutionary conserved between vertebrates, it can be reasoned that the BTBD6 function during vertebrate development is also conserved. This project was instigated in the chick system, however, when the zebrafish BTBD6 orthologue was identified, the work continued to be done in zebrafish due to its amenability to gain- and loss- function studies (Nasevicius and Ekker, 2000; Streisinger et al., 1981; Westerfield, 1993). In order to obtain initial clues in relation to the BTBD6 function, the detailed analysis of the temporal and spatial distribution of BTBD6 transcripts in chick and zebrafish was performed. The expression patterns of BTBD3 in chick and zebrafish were also obtained. The following chapter presents the results of this study.

EXPRESSION OF BTBD6 AND BTBD3 DURING DEVELOPMENT

Analysing the temporal and spatial expression pattern of a gene is a necessary step in developing hypotheses and determining the function of the gene product during development. Clone V11 (now termed *cBTBD6*), as described in the previous chapter, was identified during an in situ hybridisation based screen to isolate genes with restricted expression in the developing chick hindbrain. The initial results indicated that *cBTBD6* transcripts are expressed segmentally in rhombomeres 5 and 6 of the HH9 stage chick hindbrain. To investigate other potential sites of cBTBD6 function in embryonic development, a more detailed analysis of the *cBTBD6* expression pattern during chick embryogenesis was undertaken. The *cBTBD6* expression profile was compared with the expression sites of *cBTBD6* orthologs during zebrafish embryogenesis. To elucidate a possible functional relationship between *BTBD6* and *BTBD3* genes, which are highly related, the *BTBD3* expression profile in chick and zebrafish embryos was also determined. This chapter describes the results of these studies.

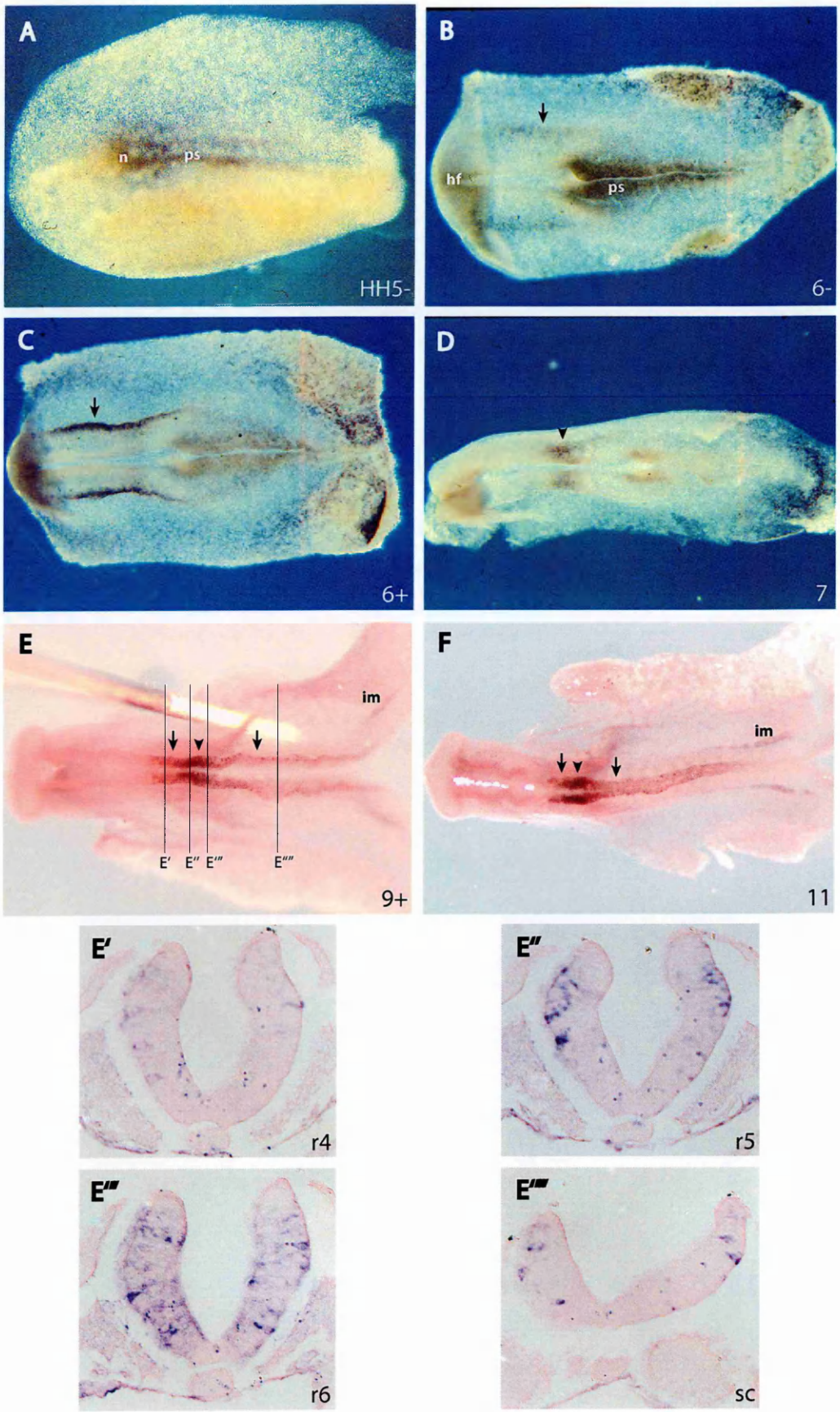
SPATIO-TEMPORAL ANALYSIS OF BTBD6 EXPRESSION PATTERN

cBTBD6 expression profile

Whole mount in situ hybridisation was performed on chick embryos ranging from stage 5 to 20 with an antisense digoxigenin-labelled RNA probe generated from the entire initial *cBTBD6* cDNA clone.

The onset of *cBTBD6* expression is first observed around the HH5 stage in the Hensen's node (n) and in the primitive streak (ps) (Fig. 21A). By the HH6- stage, expression is upregulated in the primitive streak and also detected in the ventral part of the developing head fold (hf), and to a lesser extent along the neural plate at the boundary between the neural and nonneural ectoderm (arrow; Fig. 21B). In the HH6+ stage embryo *cBTBD6* transcripts are maintained in the streak, the head fold and at elevated levels

Figure 21. Early *cBTBD6* expression pattern. Expression of *BTBD6* in chick embryos was visualised by whole mount in situ hybridisation between stages HH5- and 11. (A-F) Dorsal views of the embryos, anterior to the left. Developmental stages are indicated in the lower right corners. (E′, E″, E‴, E⁗) Transverse sections of the embryo in E cut along the line marked by the corresponding small letter. (A). Stage HH5- (early head process) embryo: transcripts are detected in the primitive streak (ps) and around Hensen's node (n). (B) Stage HH6- (head fold): expression is upregulated in the primitive streak and also detected in the ventral head fold (hf) as well as occurs in the lateral neural plate (arrow). (C) Stage HH6+: transcripts are maintained in the streak, the head fold and at elevated levels in the lateral edges of the neural tube (arrow). (D) Stage HH7 (1s): expression is just detectable in the developing hindbrain anterior to the level of the newly formed somite (arrowhead). (E) Stage HH9+ (8s): strong labelling is observed in r5 and r6 (arrowhead) which in cross sections is seen to be confined to cell clumps scattered within the neural tube (E″ and E‴, respectively); speckled staining (arrow) is also detected in the anterior neural tube and spinal cord (see smaller number of *BTBD6*-positive cells in E′ and E⁗, respectively); in addition, transcripts are present in the intermediate mesoderm (F) Stage HH11 (12s): expression is maintained in r5 and r6 and in isolated cell clumps (arrow) anterior to r5 and in the spinal cord as well as in the intermediate mesoderm (im). sc, spinal cord.

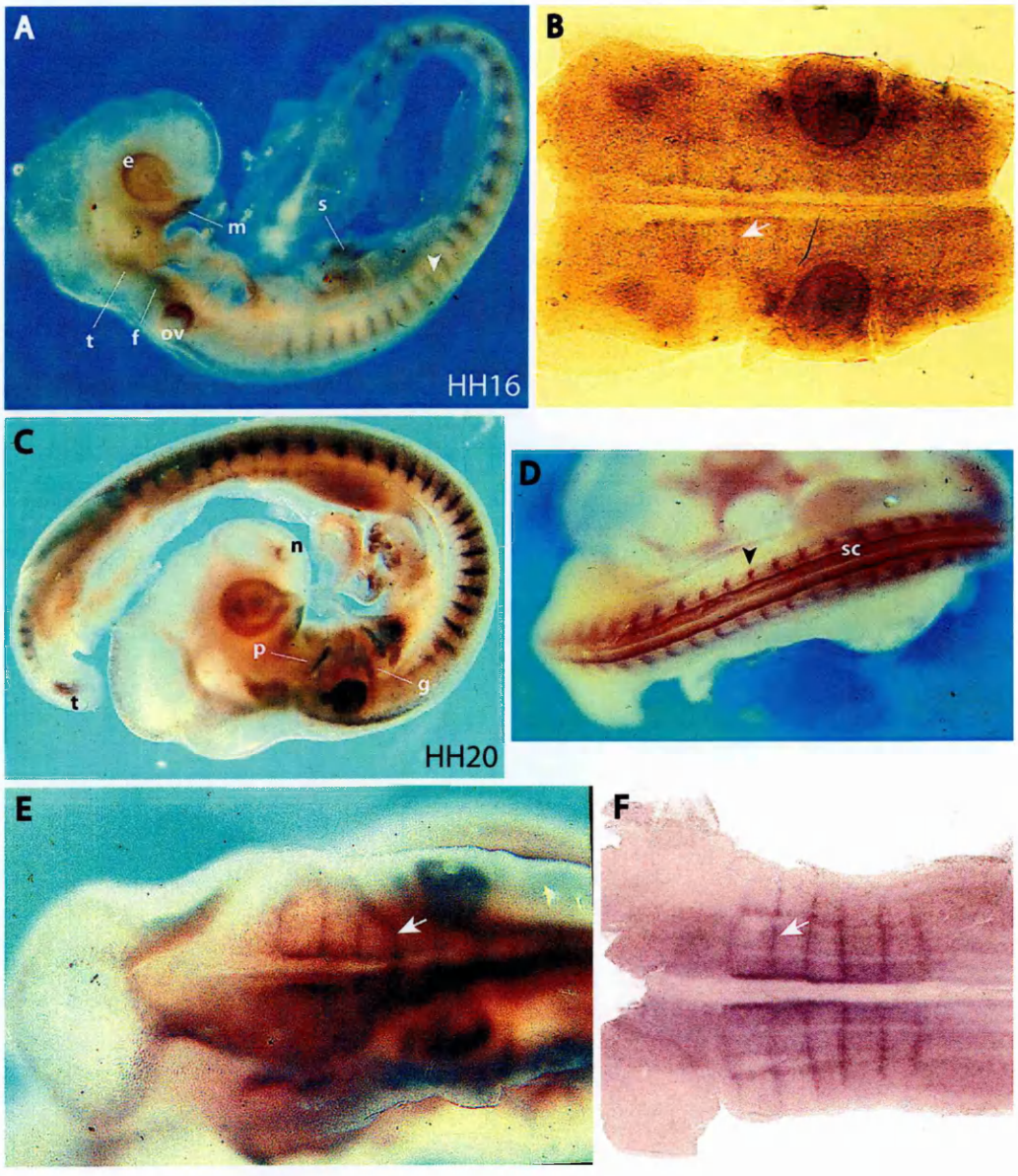

A
n
ps
HH5-
B
hf
ps
6-
C
6+
D
7
E
im
E′
E″
E‴
E⁗
9+
F
im
11
E′
r4
E″
r5
E‴
r6
E‴
sc

in the lateral neural plate (Fig. 21C). The expression in the streak is downregulated at HH7, except for the most anterior region where *cBTBD6* RNA is still observed (Fig. 21D).

cBTBD6 expression is first detected within the prospective hindbrain at the HH7 (1 somite) stage, before the commencement of morphological segmentation (Guthrie, 1996; Lumsden, 1990; Vaage, 1969), and is confined to its posterior part at the level anterior to first somite (Fig. 21D). An initially weak *cBTBD6* expression in this area becomes stronger as the embryo develops, and by the HH9+ (8s) stage is observed in the already delineated (Grapin-Botton et al., 1995) rhombomeres 5 and 6 (r5 and r6). A weaker, patchy staining is detected in the neural tube anterior to r5, and in the spinal cord (Fig. 21E). Transverse sections of r4, r5 and r6 as well as of the spinal cord revealed that the *cBTBD6* transcripts are located in cell clusters scattered within the neural tissue. In r5 and r6 the *cBTBD6*-positive cell clusters are much bigger and denser (Fig. 21E'', E''') than those present in r4 and in the spinal cord (Fig. 21E', E''''). In addition, *cBTBD6* transcripts are detected in the intermediate mesoderm (im; Fig. 21E). This pattern of *cBTBD6* expression is maintained at the HH11 (12s) stage (Fig. 21F).

In the HH16 (26-28s) stage embryos, *cBTBD6* expression is observed in a number of tissues, such as the eye (e), the otic vesicle (ov), the maxillary process (m), the trigeminal (t) and facial-acoustic (f) ganglia, the stomach (s), the intersomitic spaces (arrowhead) (Fig.22A), the spinal cord (sc, shown at HH20, Fig. 22D.) and the hindbrain rhombomere boundaries (arrow; Fig. 22B). At the HH20 (38-40s) stage, all *cBTBD6* expression domains detected at HH16 are maintained and some additional sites of *cBTBD6* signal are detected, including the nasal placode (n), the glossopharyngeal ganglion (g), the pharyngeal pouches (p) and the tail bud (t) (Fig. 22C). In the hindbrain, *cBTBD6* expression occurs in the rhombomere boundaries, the basal plate of r1 and in cells adjacent to the floor plate in r2 and r4 (Fig. 22E, F).

Figure 22. *cBTBD6* expression pattern at later stages. Expression of *cBTBD6* in chick embryos at stages HH16 (A, B) and 20 (C, D, E, F) was analysed by whole mount in situ hybridisation (A) Expression is observed in the eye (e), the otic vesicle (ov), the maxillary process (m), the trigeminal (t) and facial-acoustic ganglia (f), the stomach (s) and the intersomitic spaces (arrowhead). (B) Flat mount of the hindbrain: transcripts are restricted to the rhombomere boundaries (arrow). (C) Expression is maintained in the eye, the otic vesicle, the maxillary process, the trigeminal and facial-acoustic ganglia, the stomach and the intersomitic spaces and is also detected in the nasal placode (n), the glossopharyngeal ganglion (g), the pharyngeal pouches (p) and the tail bud (t). (D) Trunk region: labelling is observed in the spinal cord (sc) and in the intersomitic spaces (arrowhead). (E) Dorsal view and flat mount (F) of the hindbrain: transcripts are present in the rhombomere boundaries (arrow), in the basal plate of r1 and in cells adjacent to the floor plate in r2 and r4.


A
e
m
s
t
f
ov
HH16
B
C
n
p
g
t
HH20
D
sc
E
F

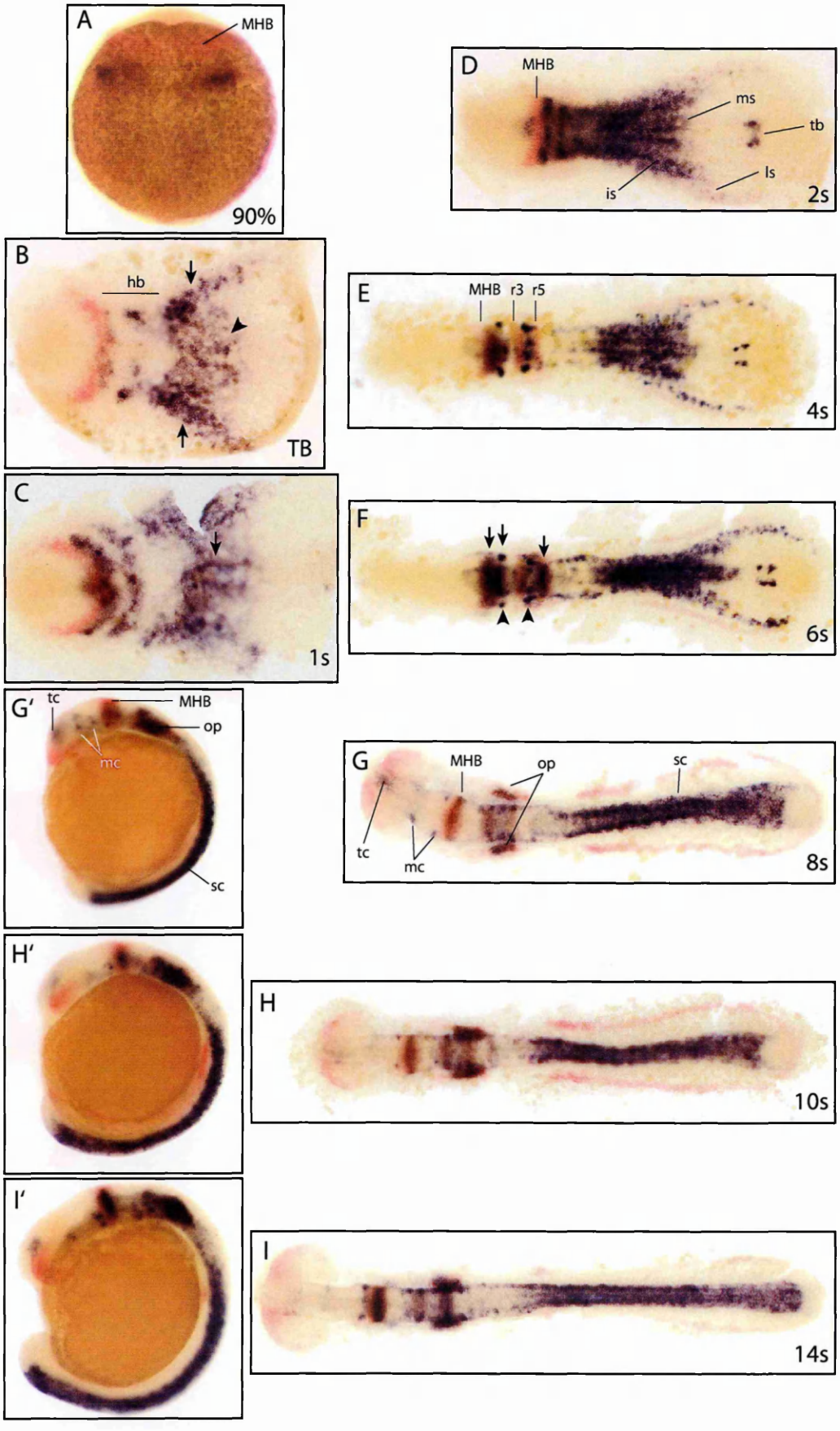
Spatial analysis of *BTBD6* orthologs during zebrafish development

As described in the previous chapter, two *cBTBD6* co-orthologs have been identified in zebrafish, *zBTBD6a* and *zBTBD6b*. In order to identify the precise localisation of the transcripts, double in situ hybridisation was carried out, in which the expression domains of *pax2a* (e.g. at midbrain-hindbrain junction and in otic placodes) and *krox-20* (in r3 and r5) were used as landmarks (Krauss et al., 1991; Oxtoby and Jowett, 1993).

zBTB6a expression profile

Expression of *zBTBD6a* is first detected at 90% epiboly in two bilateral clusters of the anterior future neurectoderm located posterior to the forming midbrain-hindbrain boundary (MHB), which is labelled with the *pax2a* RNA probe (Fig. 23A). In the early tailbud stage embryo, *zBTBD6a* expression appears in the forming neural plate: laterally, in two bilateral stripes marking the edge of emerging neural plate (arrows); medially in the large patch of contiguous cells (arrowhead); and anteriorly in the presumptive hindbrain (hb), in two transverse stripes one of which is adjacent to the midbrain-hindbrain junction (Fig. 23B). By the 1-somite stage when the neural plate is well defined, the bilateral stripes of *zBTBD6a* expression at the margins of the neural plate are wider. In the medial region of previously uniform expression, two paraxial bands of more intensely stained *zBTBD6a*-positive cells appear (arrow), while the transverse stripes in the future hindbrain, widen and extend laterally (Fig. 23C). In 2-somite embryo, the overall staining in the neural plate intensifies and expands caudally, and the pattern of transcription in the trunk neural plate is maintained, with longitudinally extending rows of cells expressing *zBTBD6a* RNA (Fig. 23D). Three rostrocaudal stripes of expression can now be identified: intermediate (is), running laterally from the level of the presumptive hindbrain in the anterior neural plate; lateral (ls), positioned a bit more lateral and posterior; and medial (ms). In the most posterior neural plate, *zBTBD6a* expression occurs in a group of cells positioned medially in the tailbud (tb). This pattern of

Figure 23. *zBTBD6a* expression pattern in zebrafish embryos from 90% epiboly to 14 somite stages. *krox-20* and *pax2a* expression shown in red are used as landmarks. Developmental stages are indicated in the lower right corner. (A) Dorsal view, anterior to the top. (B-J) Dorsal views, anterior to the left, yolks removed. (H', I', J') Lateral views, anterior to the top. (A) *zBTBD6a* expression is first detected at the 90% epiboly stage in bilateral cell clusters (arrows) posterior to midbrain-hindbrain boundary (MBH), which is marked in red by *pax2a* expression. (B) Transcripts are found in bilateral stripes marking the borders of the forming neural plate (arrows), in the medial region of the neural plate (arrowhead) and in two faint transverse stripes in the presumptive hindbrain (hb). (C) The expression expands and two paraxial rows of cells with elevated levels of transcripts emerge within the medial expression domain (arrow). (D) The overall expression of *zBTBD6a* increases, and stripes of *zBTBD6a*-positive cells with increased expression can be identified: two transverse stripes in the hindbrain and three bilateral rostrocaudal stripes in the more posterior neural plate: intermediate, lateral and medial; the most posterior neural plate is devoid of *zBTBD6a* transcripts except for the edges where lateral stripes of expression are present, and a group of cells positioned centrally in the tail bud. *zBTBD6a* expression in the posterior region remains essentially unchanged until the 6 somite stage (F, G) and persists in the developing spinal cord (sc) in the older embryos between stages 8-14 somites (H-J). The anterior expression in the region of developing hindbrain and MHB is very dynamic in 4-14 somite stages. This expression occurs in transverse stripes (e.g. arrows in G) situated at different positions along anteroposterior axis as well as in small clusters of cells (e.g. arrowheads in G), whose locations correspond to forming neurons (F-J). Other sites of *zBTBD6a* expression include: MHB (see varying degree of staining intensity in F-J) observed from the 4 somite stage, otic placodes (op), ventral clusters of cells in the midbrain (mc) and dorsal cluster of cells in telencephalon (tc), detected from the 8 somite stage (H-J).


A
MHB
90%
B
hb
TB
C
1s
D
MHB
ms
tb
is
ls
2s
E
MHB r3 r5
4s
F
6s
G′
tc
MHB
op
mc
sc
G
MHB
op
sc
tc
mc
8s
H′
H
10s
I′
I
14s

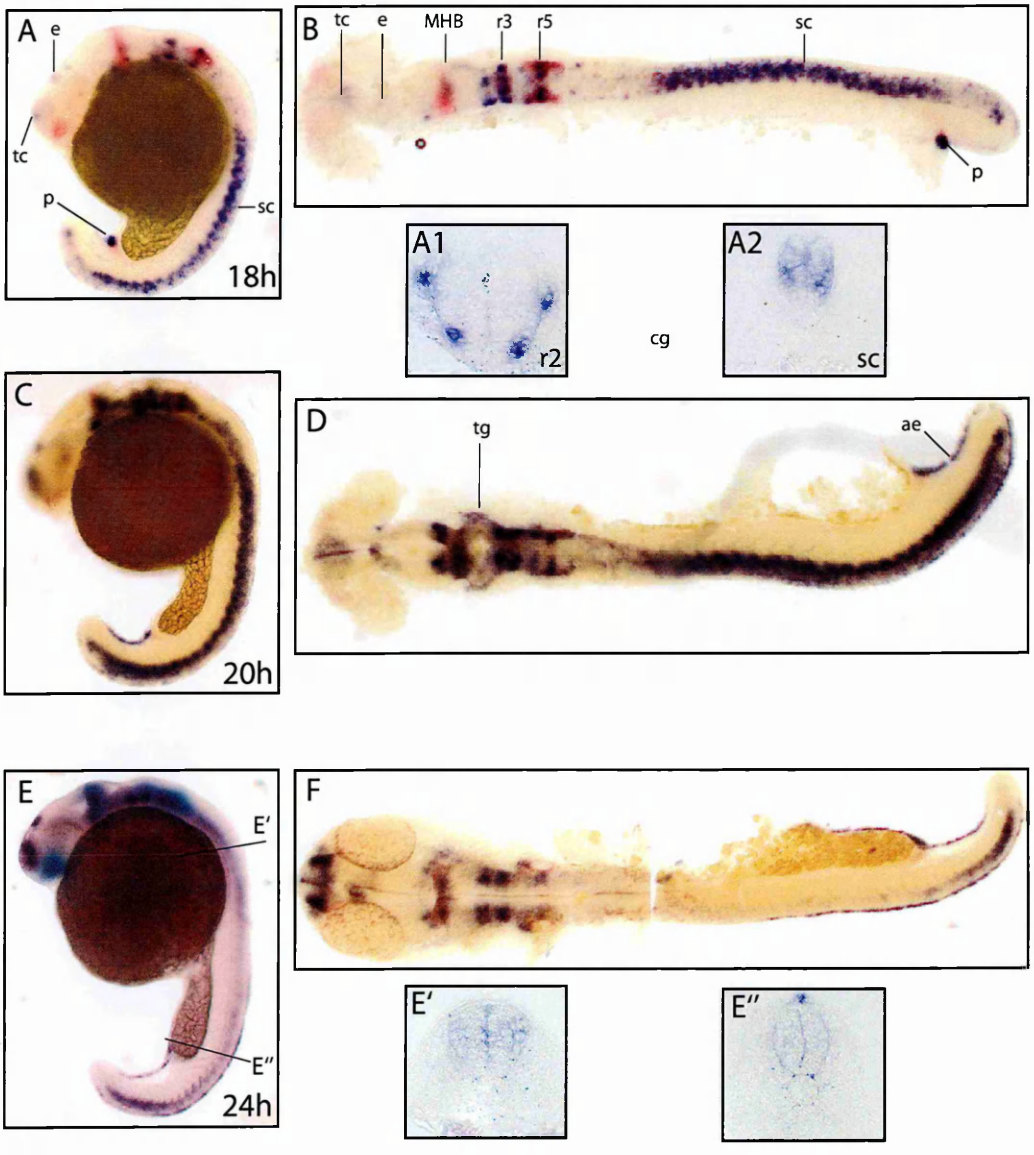
zBTBD6a expression in the posterior neural tube remains similar until 8-somite stage (Fig. 23E, F).

Similar longitudinal expression domains are observed at the tail-bud and early stages of somitogenesis for the neurogenic genes: *neurogenin1* (Blader et al., 1997), *deltaA* (Appel and Eisen, 1998; Haddon et al., 1998), and *deltaB* (Haddon et al., 1998), corresponding to the regions in which primary neurogenesis is taking place. The neuronal precursor cells are arranged in intermediate, lateral and medial longitudinal domains, where a subset of cells in each domain differentiates to form interneurons, sensory neurons and motor neurons, respectively (Inoue et al., 1994; Kim et al., 1996; Korzh et al., 1993).

In embryos between 13 (8 somite) to 20 hpf (hours post fertilisation), the posterior *zBTBD6a* expression is confined to the spinal cord (sc; Fig. 23G-I, Fig. 24A-D). Transverse sections of the spinal cord of the 19-somite (18.5 hpf) embryo reveal that *zBTBD6a* transcripts are distributed throughout the walls of the spinal cord and are at higher levels in ventrolateral regions (Fig. 24A2). The expression in the spinal cord is down regulated at around 24 hpf with the exception of the anterior and very posterior (tailbud) part, where faint staining is still observed (Fig. 24E-F, E′, E″).

The anterior *zBTBD6a* expression first observed at the tail bud stage in the presumptive hindbrain is very dynamic during the later stages of embryogenesis. In addition to transverse segmental expression between the MHB and rhombomere 6 in different developmental stages (e. g. arrows in Fig. 23F), transcripts are detected in isolated clusters of cells (e.g. arrowheads in Fig. 23F) that may correspond to forming neurons (Fig. 23B-I & Fig. 24A-F). In transverse sections of r2 at 18.5 hpf, *zBTBD6a* transcripts are detected in four cell clusters, two of them located dorsolaterally adjacent to the neural tube and the other two positioned ventrolaterally within the neural tube, suggesting that they may correspond to cranial ganglia and motor neurons, respectively (Fig. 24A1).

Figure 24. *zBTBD6a* expression at later developmental stages. Distribution of *zBTBD6a* transcripts in zebrafish embryos between stages 18 and 24 hpf was revealed by whole-mount in situ hybridisation. Developmental stages are indicated in the lower right corner. *krox-20* and *pax2a* expressions shown in red (A,B,C,D,F) and in turquoise (E), are used as landmarks. (A, C, E) Lateral views with anterior to the left; (B, D, F) Dorsal views with anterior to the left. (A1, A2) Transverse sections of r2 and the spinal cord at 18.5 hpf. (E′ ,E″) Sections of the embryo shown in E cut along the lines marked by the corresponding letters. (A, B) At 18hpf, *zBTBD6a* transcripts are highly expressed in the spinal cord (sc; see also transverse section at 18.5 hpt in [A2]) and proctodeum (p), and weakly expressed at the midbrain-hindbrain boundary (MHB), telencephalon cluster (tc) and epiphysis (e); in the hindbrain there are bilateral clusters of *zBTBD6a*-positive cells in r3 and 5 (stained in red by *kr20*), and in r2 and 4. Crossection through r2 (A1) in a slightly older embryo (18.5 hpf) shows two bilateral clusters of *zBTBD6a*-expressing cells, one located ventrolaterally adjacent to the neural tube and the other positioned dorsolaterally within the neural tube, which may correspond to the forming cranial ganglia and differentiating motor neurons, respectively. (C, D) At 20 hpf, additional sites of expression include trigeminal ganglia (tg) and apical ectoderm (ae); staining in the hindbrain is more abundant as it is stronger in the MHB. (E, F) At 24 hpf expression in the spinal cord is down regulated except for the most anterior and posterior regions where low level transcripts persist, as indicated in the transverse sections of these areas (E′ and E″).


A
e
tc
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B
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e
MHB
r3
r5
sc
p
A1
r2
cg
A2
sc
C
20h
D
tg
ae
E
E'
E''
24h
F
E'
E''

zBTBD6a-positive cells are also observed in the otic placode (op) in stages between 8 to 14 somites (Fig. 23G'-I', G-I) and in the trigeminal ganglia (tg) at 20 hpf (Fig. 24C, D). Additional sites of *zBTBD6a* expression in the central nervous system (CNS) include clusters of cells in the telencephalon (tc) and ventral midbrain (mc) from the 8-somite stage onwards (Fig. 23G'-I', G-I, Fig. 24A-F), and in the epiphysis (e) where transcripts are first observed at 18 hpf (Fig. 24A-F). Other domains of *zBTBD6a* transcription outside the nervous system are found in the proctodeum (p) and apical ectoderm (ae) of the tailbud from 18 hpf onwards (Fig. 24A-F).

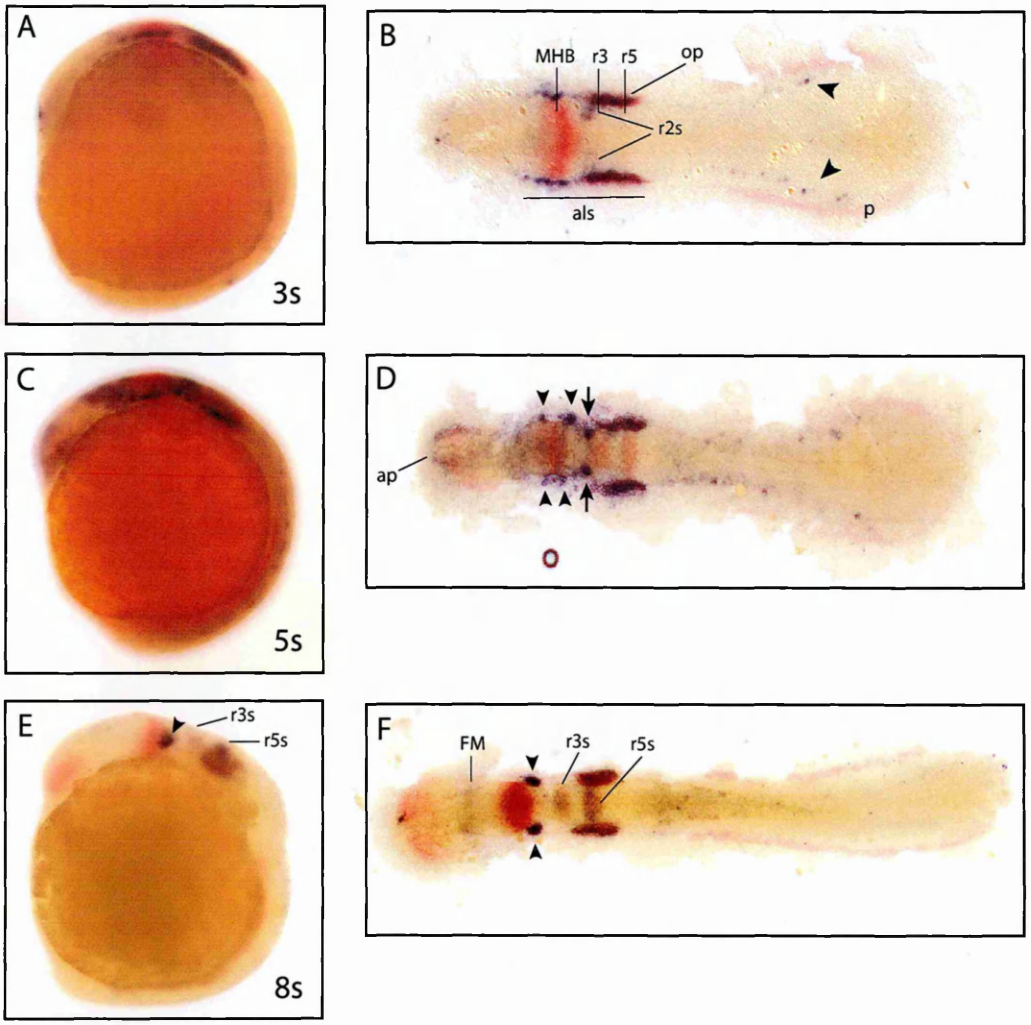
zBTB6b expression profile

Very weak *zBTBD6b* expression is initially detected at 90% epiboly in bilateral patches located at the level of the MHB and in a single band along the midline of the prospective neuroectoderm (data not shown). By the 3 somite stage, stronger expression starts to appear at the edge of the anterior neural plate in two lateral bands (als), extending along the presumptive hindbrain (r3 and 5 detected in red by *kr20* expression), the midbrain-hindbrain junction and the posterior part of the prospective midbrain (Fig. 25A, B). *zBTBD6b*-positive cells positioned at the level of the central hindbrain exhibit a higher level of expression and partially overlap with the *pax2a* staining which marks the otic placode (op).

The timing and location of the anterolateral expression appearing at the border between neural and non-neural ectoderm correspond to the overlapping domains where neural crest and placodes arise in the 3-somite embryo (Baker and Bronner-Fraser, 2001; Bronner-Fraser, 1995; Eisen and Weston, 1993; Halloran and Berndt, 2003; Huang and Saint-Jeannet, 2004). Therefore, the *zBTBD6b*-positive cells in this region may belong either to neural crest or placodal primodia or to both.

Low level expression is also detected at this stage in two stripes of cells in dorsal r2 (r2s) as well as in a bilateral row of cells in the posterior neural plate

Figure 25. Analysis of *zBTBD6b* expression in zebrafish embryos between 3- to 8-somites stages. Developmental stages are indicated in the lower right corner. *krox-20* and *pax2a* expression are indicated in red. (A, C, E) Lateral views. (B, D, F) Dorsal views of the embryos in A, C, E, respectively, anterior to the left. (A, B) *zBTBD6b* expression is detected in two anterolateral stripes (als) extending along the prospective hindbrain (see r3 & r5 marked in red), the midbrain-hindbrain boundary (MHB, in red) and the posterior presumptive midbrain. High-level transcripts in the otic placode (op) primodium overlap with *pax2a* expression indicated in red. Weak staining is also observed in a dorsal stripe in r2 (r2s) and in a bilateral row of cells in the posterior neural plate (arrowheads). (C, D) In the anterior neural plate, labelling appears in bilateral cell clusters: dorsal in rostral r2, dorsal anteriorly to MHB (arrowheads) and ventral in posterior r2 (arrow). The horseshoe-shaped band of *zBTBD6b*-positive cells in the most anterior ridge of neural plate might correspond to the anterior pituitary (ap). (E, F) The staining in the bilateral cell patches of anterior r2 becomes prominent while expression in other cell clusters recedes. Additional sites of weak expression include r3 (r3s) and r5 (r5s) and the forebrain-midbrain junction (FM). (p) pronephric anlage (stained by *pax2a* in red).


A
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8s
F
FM
r3s
r5s

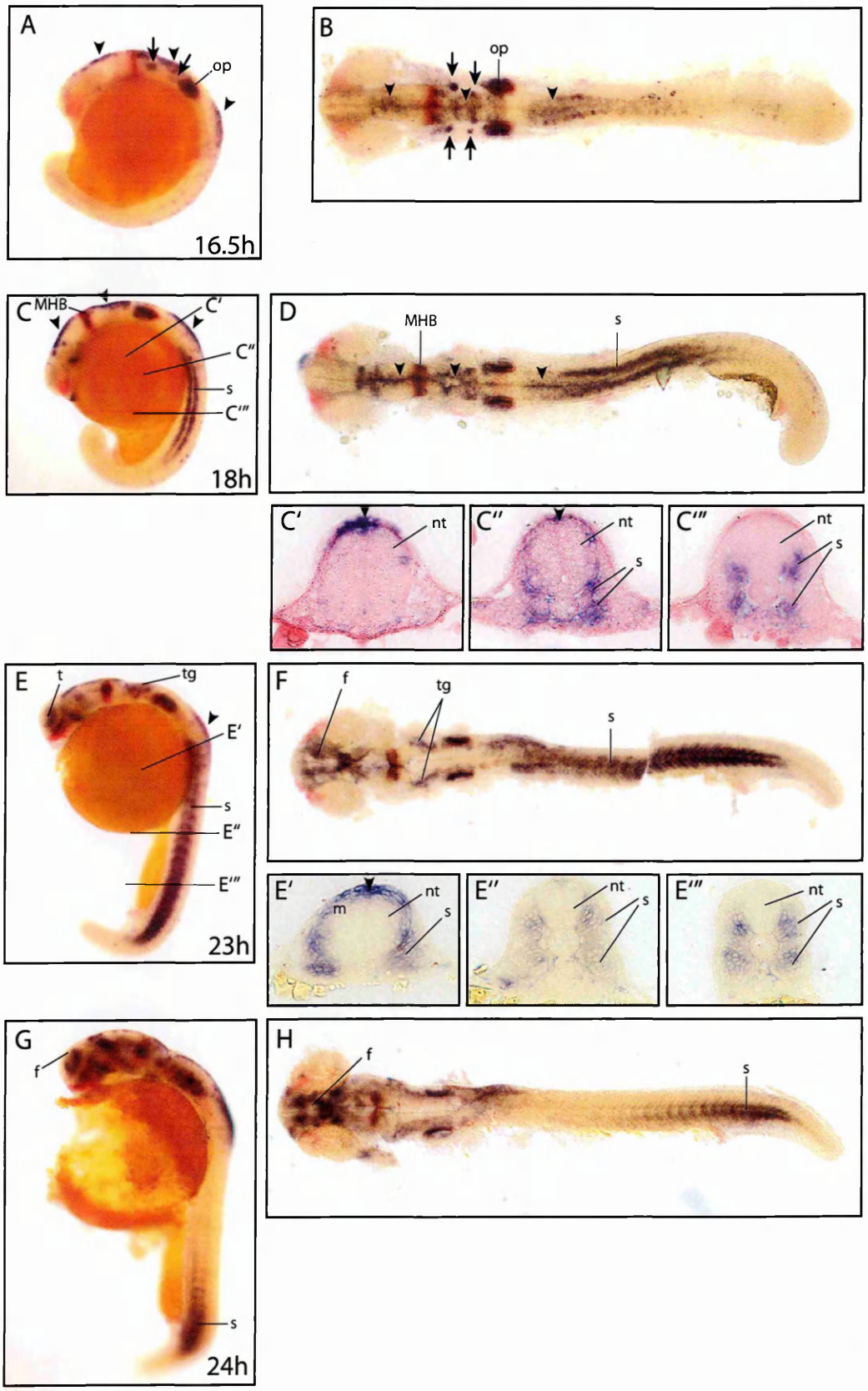
(arrowheads) (Fig. 25B). The location of the posterolateral stripes of cells in relation to the prospective pronephros, stained in red by *pax2a*, suggest that they denote differentiating Rohon-Beard (RB) sensory neurons.

zBTBD6b transcripts are maintained in the 5-somite embryo in the posteriolateral rows of cells as well as in the otic placodal primodium (Fig. 25C, D). Staining is also observed in three pairs of cell clusters; out of the two cell clusters located more lateral (arrowheads), one is located rostrally and the other caudally to the MHB, while the third ventral cluster (arrows) is positioned in r2. In addition, *zBTBD6b*-positive cells are detected in a horseshoe-shaped band in the most anterior ridge of neural plate, which is probably the anterior pituitary (ap; Fig. 25D).

At 8 somites (Fig. 25E, F), weak staining in bilateral rows of cells in the posterior neural plate is still visible, while in the anterior regions, faint expression is observed segmentally in r3 and r5 of the hindbrain and in the forebrain-midbrain junction (FM). Intense labelling is also detected in the otic placodes, and in two lateral patches of cells located posteriorly to the MHB (arrowheads). This latter site of expression might correspond to clusters of sensory neurons of trigeminal ganglia or, alternatively, to the trigeminal nerve exit point primodia.

At 16.5 hpf (15 somites), *zBTBD6b* expression has spread mediodorsally in the midbrain and central hindbrain regions and at the level of the anterior trunk (Fig. 26A, B; arrowheads). By 18 hpf (18 somites), expression in these areas has narrowed and become more restricted to the dorsal midline (Fig. 26C, D; arrowheads). In addition, *zBTBD6b* RNA is detected in the rostral somites (s). A similar pattern of expression in the roof plate and the somites is observed in the 23 hpf embryo (Fig. 26E, F), except that, at this stage, the midline expression occurs in the anterior trunk only, and all somites (s) express *zBTBD6b,* with the anterior ones expressing more weakly than the posterior ones (compare Fig. 26E'' with E'''). Transverse sections of

Figure 26. Spatial pattern of *zBTBD6b* expression in zebrafish embryos between stages 16.5 hpf and 24 hpf. Developmental stages are indicated in the lower right corner. *krox-20* and *pax2a* expression revealed in red are used as landmarks. (A, C, E, G) Lateral views. (B, D, F, H) Dorsal views of the embryos in A, C, E, G, respectively, anterior to the left. (C′, C″, C‴ & E′, E″, E‴) Sections of the embryos in C and E, respectively, cut along the line marked by the corresponding letter. (A, B) *zBTBD6b*-positive cells are found mediodorsally in the midbrain, central hindbrain and anterior trunk (arrowheads) as well as in the bilateral cell clusters in the hindbrain (arrows). The expression in the otic placode (op) is very intense and is maintained until 24 hpf. (C, D) Mediodorsal staining is maintained (arrowheads) and labelling in the anterior somites (s) appears (see the exact locations of transcripts in C′, C″ & C‴). Expression is also observed at the midbrain-hindbrain boundary (MHB). (E, F). Expressing cells are detected in the roof plate of the most anterior trunk (arrowhead) and in all somites (s); see crossections in E′, E″ & E‴. Staining is also observed in the forebrain (f) and in the trigeminal ganglia (tg). (G, H) The expression in the anterior somites is down regulated, whereas it is maintained in the posterior somites. Forebrain expression is still observed (f).


A
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16.5h
B
op
C
MHB
C'
C''
s
C'''
18h
D
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s
G
f
s
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H
f
s

the trunk at different axial levels reveal that at 18 (Fig. 26C', C'', C''') and 23 hpf (Fig. 26E', E'', E'''), *zBTBD6b*-positive cells are in the dorsal edge of the neural keel in the anterior-most trunk (arrowheads). In the more posterior region of the trunk, expression occurs along the outside of the neural tube and in the somites, while in the central trunk region transcripts are solely observed in the somites. At 24 hpf (Fig. 26G, H) the staining in the somites is maintained only in the caudal trunk.

Other sites of *zBTBD6b* expression observed between stages 16.5 and 24 hpf include the otic placode (op) (Fig. 26A-H). *zBTBD6b* RNA is also detected in the midbrain-hindbrain boundary (MHB) between stages 18 and 24 hpf (Fig. 26C-H) and in telencephalon (t) at 23 and 24 hpf (Fig. 26E-H). In addition, at 16.5 hpf, expression is observed in pairs of lateral cell clusters in the hindbrain region (Fig. 26A, B; arrows).

SPATIO-TEMPORAL ANALYSIS OF BTBD3 EXPRESSION PATTERN

cBTBD3 expression profile

In order to determine the sites of *cBTBD3* expression during avian development, three different digoxigenin-labeled antisense RNA probes were synthesised, using chick EST cDNAs as templates (Genebank Acc. No. BU444464, BU274818, BU389269). The generated probes were employed in whole mount in situ hybridisations carried out on chick embryos ranging from stage HH5 to 21, and the results obtained with each of the probes were compared. The three probes gave very similar results, and the presented data was obtained with the one that produced strongest signals.

cBTBD3 is first detected at the 1 somite stage in two bilateral stripes (arrows) posterior to the newly forming second somite (Fig. 27A, A'). Both stripes of expression are located at a distance of approximately one somite length apart from each other. With ongoing somitogenesis, these stripes of *cBTBD3* expression move in an anteroposterior direction in register with the forming

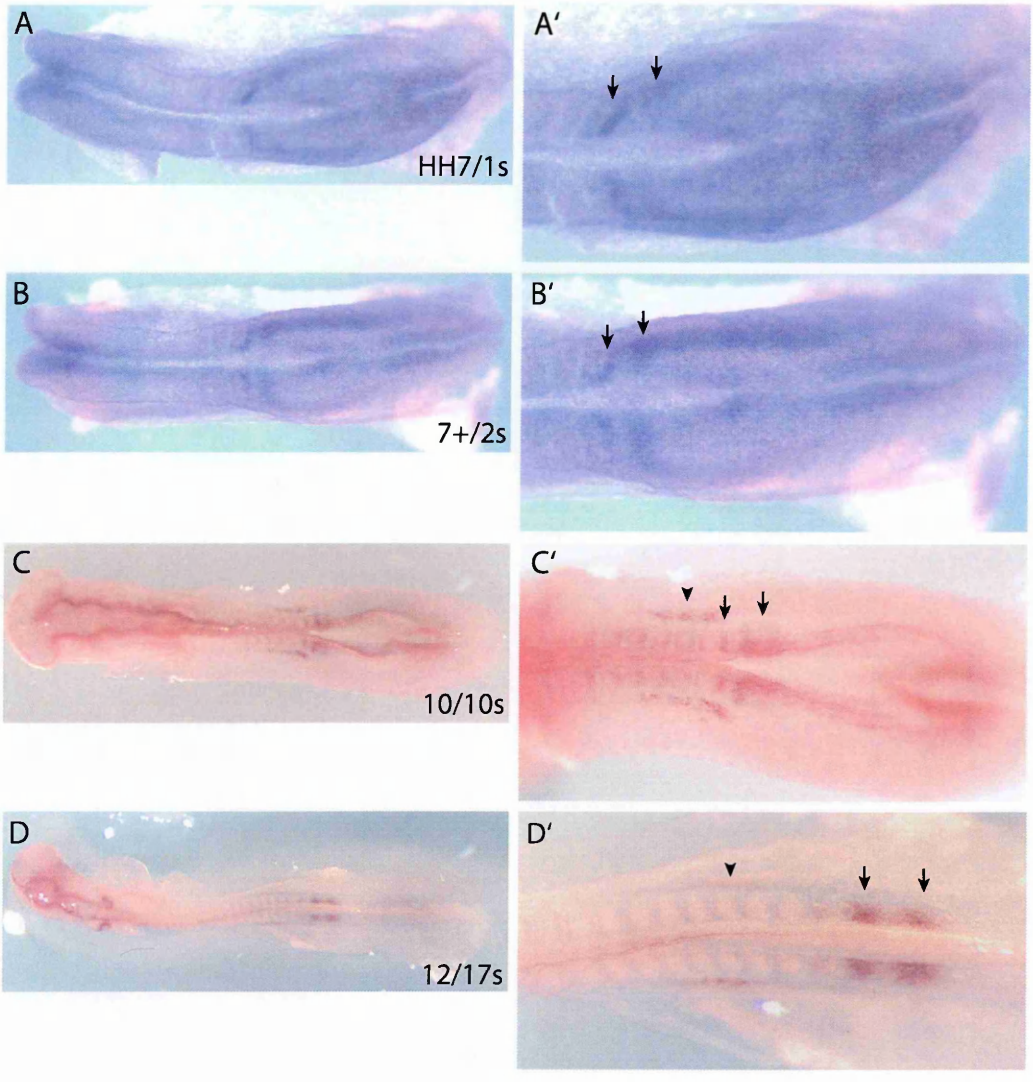


Figure 27. *cBTBD3* pattern between stages HH7 and 12. *cBTBD3* expression was revealed by in situ hybridisation. Dorsal views, anterior to the left, developmental stages are indicated in the lower right corner. (A', B', C', D') High-magnification views of the posterior part of embryos shown in A, B, C, D, respectively. In the H7 stage embryo (A, A') expression is observed as two bilateral stripes posterior to the forming somites (arrow), the posterior stripe labeling being very faint. These two stripes of expression have similar intensity in the slightly older embryo (B, B') and the posterior stripe is expanded in the embryos in C (HH10) and D (HH12). In the H10 (C, C') and H12 (D, D') stage embryos cBTBD3 transcripts are also detected in the pronephros (arrowhead in C' and D').

somites, and reach the tailbud by stage 21 (Fig. 28D). The size of these expression domains and the amount of *cBTBD3* transcripts within them, are varied. For example, the *cBTBD3* positive stripes are of the same size in some embryos (Fig. 27A, A', B, B'), whereas in some other embryos the posterior stripe is broader than the anterior one (Fig. 27C, C', D, D'; Fig. 28B, D). While in some embryos, the expression of the anterior stripe is stronger than that of the posterior one (Fig. 27A, A'), in other embryos both stripes of expression are of similar intensity (Fig. 27B, B', D, D'; Fig. 28B). However, the location of *cBTBD3* transcripts with respect to the forming somites remains the same: the anterior stripe is situated immediately next to the newly emerged somite and the posterior one, at a distance of approximately one prospective somite.

A second site of *cBTBD3* expression observed at HH10 and HH12, occurs in bilateral stripes (arrowheads) lateral to the somites and caudal to the fifth somite (Fig. 27C, C' and D, D', respectively). This staining appears to correspond to the developing pronephros and correlates with the induction of the pronephros in the intermediate mesoderm posterior to the fifth somitic level (Abdel-Malek, 1950; Kuure et al., 2000; Mauch et al., 2000; Vize et al., 1997). *cBTBD3* expression persists in the mesonephros in HH17 and HH21 embryos (Fig. 28A, B and E, respectively). In addition, at HH21 *cBTBD3* expression is observed in the developing stomach (Fig. 28F)

Spatial analysis of BTBD3 orthologs during zebrafish development

Two *cBTBD3* co-orthologs, *zBTBD3a* and *zBTBD3b*, were identified by database searches in zebrafish and I analysed their expression patterns by whole-mount in situ hybridisation.

zBTBD3a expression profile

Preliminary analysis of the *zBTB3a* expression with antisense RNA probe generated from a 2 kb EST clone (GeneBank Acc. No. AI883008) was

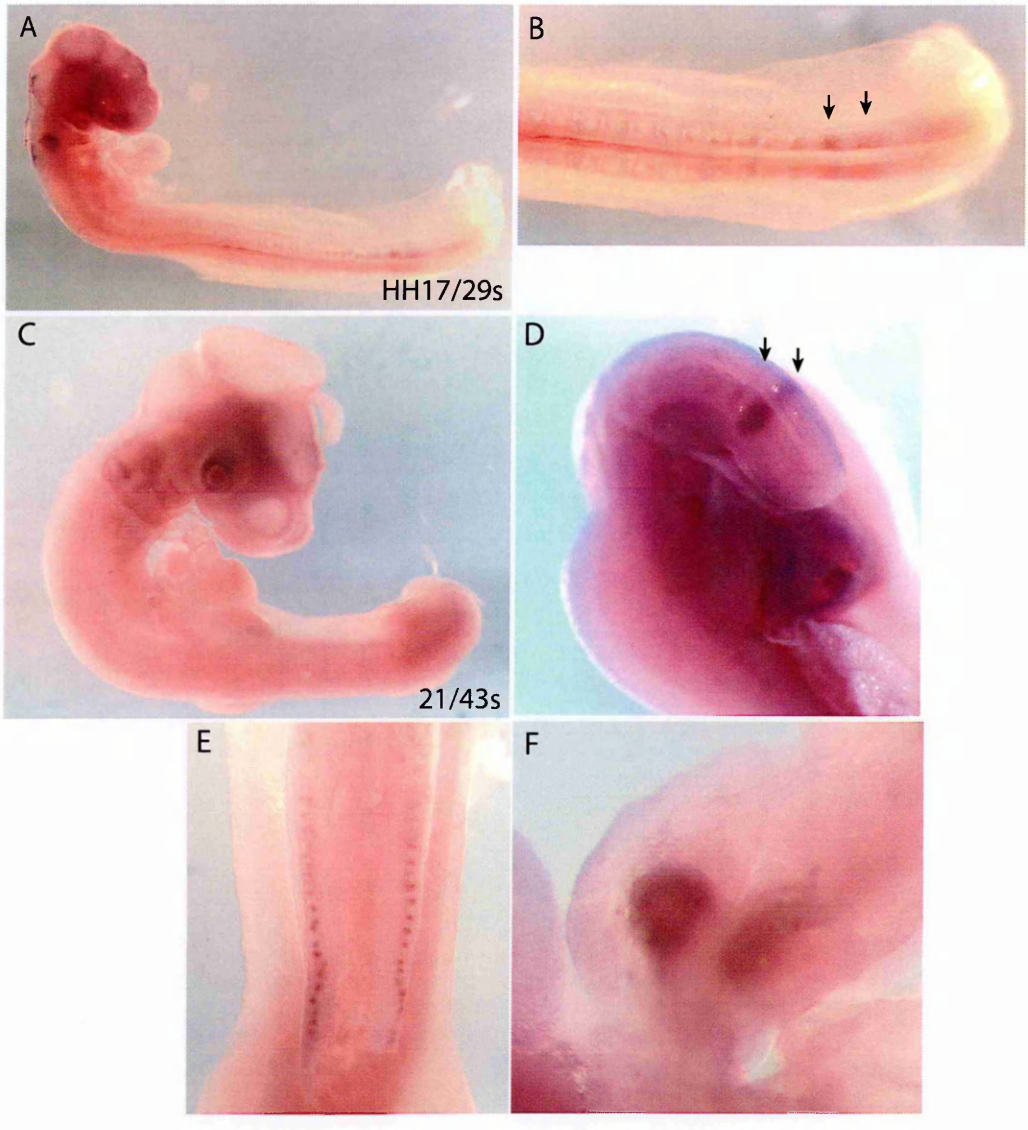


Figure 28. *cBTBD3* expression pattern between stages HH17 and 21. Whole mount in situ hybridisation was performed on chick embryos of stages HH17 (A, B) and 21 (C, D, E, F). (B) Posterior part of the embryo in A viewed at higher power magnification showing bilateral stripes of *cBTBD3*-positive cells posterior to the last formed somite (arrows). (D) Tail-bud of the embryo shown in C at high-magnification: the more posterior stripe of *cBTBD3* expression is wider than the anterior one (arrows). (E & F) Higher magnification of C showing expression in the mesonephros and developing stomach, respectively.

performed in 3-19 somite stage zebrafish embryos and showed ubiquitous transcript distribution (Fig. 29A, C-I). To exclude the possibility that the observed staining represents non-specific background rather than a specific signal, a sense RNA probe, covering the identical cDNA region was used and no labelling was detected (Fig. 29B).

zBTBD3b expression profile

To analyse *zBTBD3b* expression pattern, double in situ hybridisation with *pax2a* and *krox-20* probes was carried out.

zBTBD3b transcripts are first detected in the 2-somite stage embryo in the midline (arrow) posterior to the midbrain-hindbrain boundary (MHB) marked by *pax2a* expression (Fig. 30A). A similar distribution of transcripts is observed at the 8-somite stage (Fig. 29B&C). Later in development, around the 19 hpf (19 somite) stage, expression is detected in clusters of cells in the anterior trunk (arrowheads), and a similar localisation of transcripts is observed at 23 hpf (Fig. 30F&G). Transverse sections through the trunk of the 23 hpf embryo (Fig. 30F''&F''') showed that expression is confined to the spinal cord (sc). In the most anterior spinal cord, *zBTBD3b* transcripts are situated in bilateral ventral cell clusters (Fig. 30F'') that may correspond to motor neurons, whereas in the more posterior regions, expression is also found in more dorsal cells in the spinal cord that are likely to be interneurons and/or sensory neurons (Fig. 30F''). In addition, at 23 hpf, the *zBTBD3b* expression is detected in the otic placodes (op; see transverse section) and in the diencephalon (d) and tectal (t) ventricular zones (Fig. 30F,G).

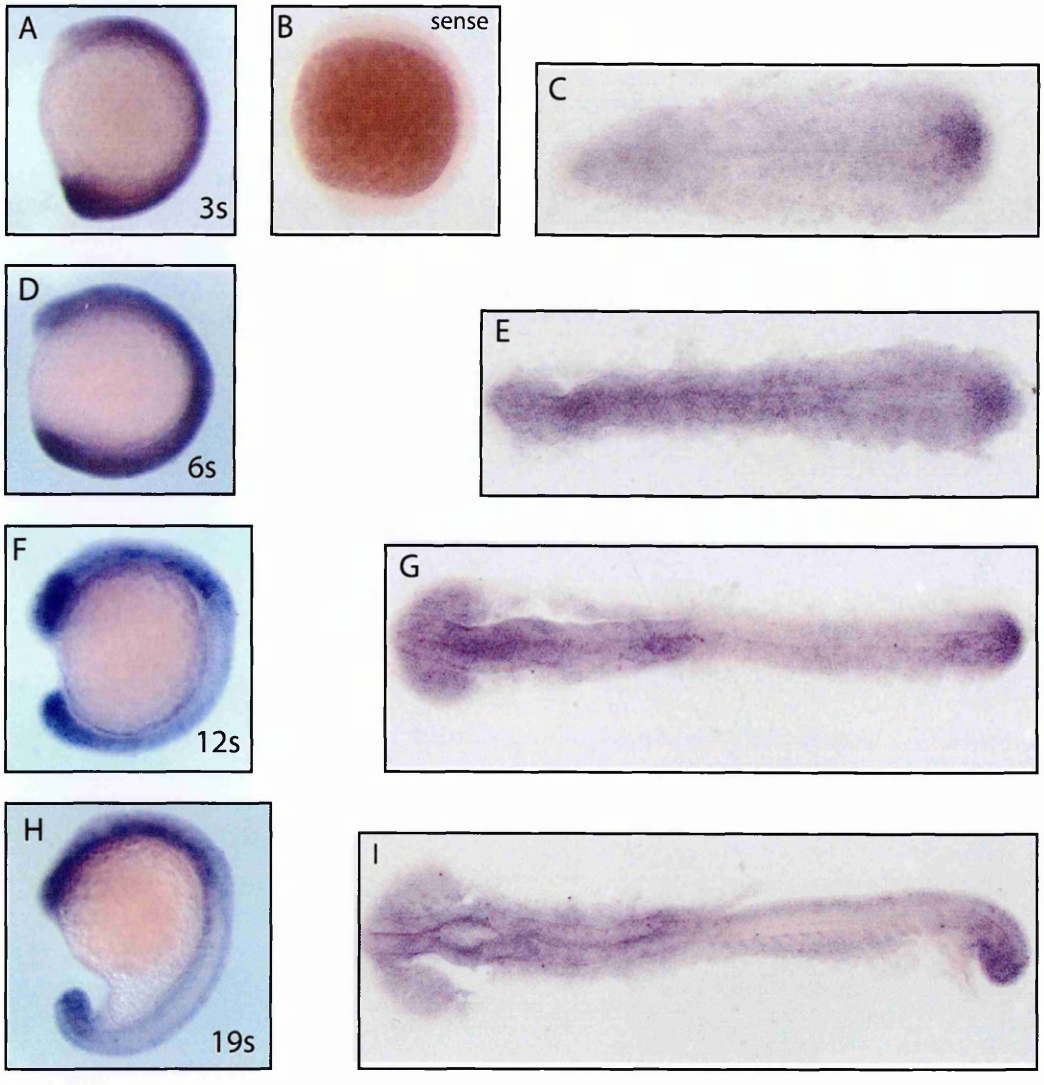
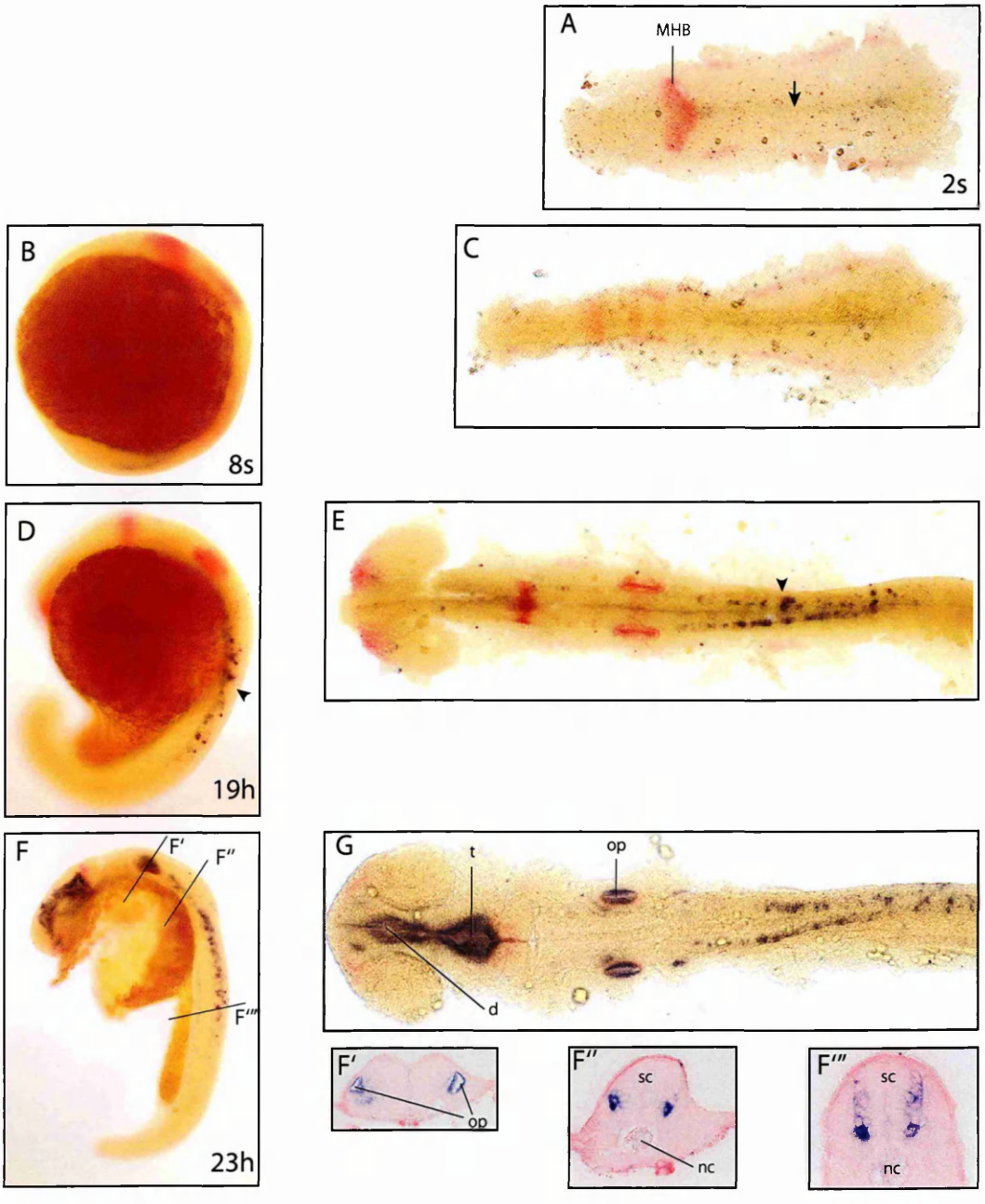


Figure 29. *zBTBD3a* expression pattern analysed by whole-mount in situ hybridisation. Developmental stages are indicated in the lower right corner. (A, B, D, F, H) Lateral views. (C, E, G, I) Dorsal views of the embryos in A, D, F, H respectively, anterior to the left. (A, C-I) Antisense RNA probe; transcripts are ubiquitously distributed. (B) Sense RNA probe, no signal detected.

Figure 30. Developmental changes in *zBTBD3b* mRNA expression. Embryonic stages are indicated in the lower right corner. *krox-20* and *pax2a* expressions revealed in red are used as landmarks. (B, D, F) Lateral views. (A, C, E, G) Dorsal views, anterior to the left, C, E and G corresponds to the embryos in B, D and F, respectively. (F',F'',F''') Sections of the embryos in F cut along the lines marked by the corresponding letters. (A) Transcripts are detected in the midline (arrow) of the neural plate posterior to midbrain-hindbrain boundary (MHB) are still present in this location in older embryo (B, C). (D, E) Clusters of zBTBD3b-positive cells (arrowheads) are found in the spinal cord. (F, G) In the anterior spinal cord (sc) expression is located in the ventral cell clusters (F'') and situated laterally in the posterior spinal cord (F'''). Transcripts are also found in the otic placodes (op, see transverse section in F'), and in the diencephalon (d) and tectal (t) ventricular zones.


A
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op
F″
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nc
F‴
sc
nc

DISCUSSION

Divergent expressions of *BTBD3* orthologs in chick and zebrafish

cBTBD3 expression is predominantly confined to domains associated with two processes in chick embryogenesis: development of the urogenital system and somitogenesis. In the urogenital system, expression is located in the developing early kidney, first in the pronephros and then in the mesonephros. In somitogenesis, a highly dynamic domain, *cBTBD3* transcripts are found in the area immediately posterior to the next prospective pair of somites. Intriguingly, this expression is reminiscent of the expression of the segmental genes *cMeso-1* and *cMeso-2*, the bHLH transcription factors, which are periodically activated in a segment-wide domain in the anterior presomitic mesoderm (PMS), preceding the morphological segmentation process (Buchberger et al., 2002; Buchberger et al., 1998; Pourquie, 2004). Somite formation is controlled by a molecular oscillator, the segmentation clock, which acts in presomitic mesoderm and appears to be driven by Notch signalling (Cooke, 1998; Pourquie, 2003). Periodic activation of Notch in the PMS acts as a signal rhythmically initiating the process of somite boundary specification and subsequent establishment of the somitic rostro-caudal polarity(Buchberger et al., 2002; Takahashi et al., 2000). Transcription factors of the Mesp/Meso family act upstream of a genetic cascade involving the Notch pathway and their periodic activation, as demonstrated in frog and mouse, requires Notch signalling (Jen et al., 1999; Moreno and Kintner, 2004; Takahashi et al., 2000). Therefore, it would be interesting to determine whether there is a regulatory relationship between *cBTBD3* and *cMeso-1* and/or *cMeso-2* and, consequently, whether cBTBD3 has a role in the process of somitogenesis.

Neither expression of the *BTBD3* co-orthologs in zebrafish was similar to the expression profile found in the chick system. *zBTBD3a* is ubiquitously expressed between the stages of 3-19 somites, while *zBTBD3b* is expressed at

different time points in notochord, otic placodes, midbrain, forebrain and in a population of cells in the spinal cord.

cBTBD6 is expressed in the developing nervous system

The earliest expression of *cBTBD6* is detected at the end of gastrulation and confined to the Hensen's node and the primitive streak. The primitive streak is first identified morphologically at the posterior margin of the epiblast as the site of ingression for endodermal and mesodermal cells. Subsequently, it undergoes progression during which it elongates rostrocaudally. When the *cBTBD6* expression is first observed, the primitive streak is fully extended, at which time the epiblast adjacent to the streak is destined to form the medial part of the neural plate (Lemaire and Kessel, 1997; Schoenwolf and Smith, 2000a; Schoenwolf and Smith, 2000b). Hensen's node is a specialised cluster of cells at the anterior tip of the primitive streak, a transient structure through which cells move during gastrulation (Boettger et al., 2001; Narasimha and Leptin, 2000). It is a source of secreted signals Chordin, Noggin and Follistatin, which induce the neural plate (Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Sasai et al., 1995; Sasai et al., 1994; Smith and Harland, 1992; Smith et al., 1993).

With the formation of the neural plate, *cBTBD6* transcripts demarcate its lateral edges, at initially low and then gradually increasing levels. In these domains and at the same time, *BMP4*, *BMP7* and the BMP target genes, *MSX1* and *DLX5*, are expressed and specify the boundary between the neural and non-neural ectoderm (McLarren et al., 2003; Streit, 2002; Streit and Stern, 1999a). These regions also express early neural markers such as *Sox3* or *Sox2* (Streit and Stern, 1999a). The border cells are unique because, unlike cells inside the future neural plate or in the neighbouring prospective epidermis, they can be diverted to either a neural or a non-neural fate by BMP and its inhibitors (Streit and Stern, 1999a).

The edges of the neural plate include also the territory fated to give rise to epidermal placodes and neural crest (Baker and Bronner-Fraser, 2001; Marchant et al., 1998; Schlosser, 2005; Streit, 2004; Tribulo et al., 2003). Thus, in the border or intermediate region between the neural plate and the epidermis, precursors of four tissues: neural, epidermal, placodal and neural crest, are intermingled (Streit, 2002). To determine which precise cell population *cBTBD6* is expressed in, a comparative analysis of *cBTBD6* expression with neural (e. g. *SOX3* (Uwanogho et al., 1995)), epidermal (e. g. *epidermal keratin;* (Sato and Yasugi, 1997)), placodal (e. g. *SIX4;* (Esteve and Bovolenta, 1999; McLarren et al., 2003)) and neural crest (*SLUG;* (Nieto et al., 1994) markers, is required.

At the onset of somitogenesis, *cBTBD6* RNA is detected in the posterior prospective hindbrain, adjacent to the newly formed somites. Within this area the first neurons of the CNS are born (McConnell and Sechrist, 1980; Sechrist and Bronner-Fraser, 1991). *cBTBD6* expression in this location is similar to that of the Notch ligand *Delta1,* which foreshadows the spatio-temporal pattern of neuronal differentiation and labels neuronal progenitor cells that have left the cell cycle (Henrique et al., 1995). Subsequently, *cBTBD6* expression is detected within the neuroepithelium of the forming hindbrain and the spinal cord in a pattern similar to *Delta1* and genes, e.g. *Ngn1* and *NeuroM,* which participate in the process of neurogenesis (Akai et al., 2005; Henrique et al., 1995; Perez et al., 1999; Roztocil et al., 1997). Detailed examination of the location of *cBTBD6* transcripts revealed that *cBTBD6* expression in the hindbrain is confined to clusters of cells in the neural tissue. Single cells and small clusters of *cBTBD6*-expressing cells are also observed anterior to r5 and in the spinal cord. This suggests that *cBTBD6* transcripts are present in a specific population of differentiating cells within the neural tube. Double in situ hybridisation with *cBTBD6,* and *Delta1, Ngn1, NeuroM* and other neuronal differentiation markers would assess whether there are overlapping expression patterns and would help to reveal the character of *cBTBD6*-positive cells.

In addition, at the later stages, *cBTBD6* transcripts are detected in the cranial sensory nerve ganglia (trigeminal, facial-acoustic and glossopharyngeal) that are derived partially from both the neural crest and epidermal placodes. No *cBTBD6* expression is however detected in the migrating neural crest.

Thus, it appears that *cBTBD6* is expression accompanies development of the CNS, both at the early stages of neurulation and establishment of the neural plate and at the later stages of neuronal differentiation. *cBTBD6* expression may also be associated with the formation of the PNS as it is detected at sites where placodes and neural crest arise and later in development, in the cranial ganglia that originate from these tissues.

The *cBTBD6* ortholog in zebrafish, *zBTBD6a*, is expressed predominantly in the forming CNS

Analysis of the spatiotemporal expression of a *cBTBD6* ortholog in zebrafish, *zBTBD6a*, revealed that *zBTBD6a* transcripts are primarily detected in the developing CNS, in the developing hindbrain, spinal cord and the anterior brain.

zBTBD6a expression is first detected at the late gastrula stage in two bilateral cell clusters in the future neuroectoderm in the anterior epiblast. Once the neural plate is well defined, *zBTBD6a*-positive cells are distributed along three longitudinal stripes in the posterior neural plate: lateral, intermediate and medial, which mark the domains of primary neurogenesis (Blader et al., 1997). A subset of cells within each of these domains will differentiate into sensory neurons, interneurons, and motor neurons, respectively (Inoue et al., 1994; Kim et al., 1996; Korzh et al., 1993). *zBTBD6a* transcription accompanies neuronal differentiation throughout the time of spinal cord formation until 24 hpf and then is downregulated. In the hindbrain, *zBTBD6a* expression is very dynamic and detected initially segmentally and then in cell clusters corresponding to forming neurons.

Therefore, it appears that *zBTBD6a,* similar to its counterpart in chick, *cBTBD6,* is expressed in the forming CNS at the sites where neuronal differentiation occurs. Other similar sites of expression include otic placodes and trigeminal ganglia.

zBTBD6b has a mostly non-overlapping expression pattern with _zBTBD6a_

Whereas *zBTBD6a* expression occurs in the neuroepithelium of the CNS, the expression of *zBTBD6b* appears to be associated with sites of neural crest and/or placodal induction and neural crest migration.

At the early neurula stage, similar to *cBTBD6, zBTBD6b* RNA is detected at the edges of the neural plate where the neural crest and placodes arise (Baker and Bronner-Fraser, 2001; Bronner-Fraser, 1995; Eisen and Weston, 1993; Halloran and Berndt, 2003; Huang and Saint-Jeannet, 2004). Double staining with premigratory neural crest markers, e.g. *foxd3* (formerly *fkd6*) (Odenthal and Nusslein-Volhard, 1998) or *sox9b* (Li et al., 2002) or one of the placodal markers, e.g. *six4.1* (Kobayashi et al., 2000b), would acertain the identity of cells labelled by *zBTBD6b.*

At later stages, *zBTBD6b* expression is observed in the mediodorsal regions of the midbrain, hindbrain and anterior trunk, and subsequently along the dorsal and lateral edges of the neural tube in the trunk. The location and timing of the appearance of *zBTBD6b* transcripts suggests that the expressing cells correspond to the neural crest, which, around the 12 somite stage (15hpf), commences migrating from the dorsal neural keel in a characteristic rostrocaudal sequence. In the trunk, neural crest cells migrate on a medial pathway between the neural tube and somite, and on a lateral pathway between ectodermal epithelium and the somite-derived dermomyotome (Eisen and Weston, 1993; Halloran and Berndt, 2003). The *zBTBD6b* signal observed in the analysed embryos may correspond to the neural crest of the medial pathway. However, the labelling within the somites is very broad which indicates that the *zBTBD6b* transcripts are also in the somitic tissue and

not solely restricted to the neural crest cells. Double in-situ hybridisation with migrating crest markers, e.g. *crestin* (Luo et al., 2001) or *snail1b* (Knecht and Bronner-Fraser, 2002), would verify the identity of the *zBTBD6b*-positive cells.

zBTBD6b expression is also detected in the otic placode primodia and maintained there until the last stage analysed (24 hpf). In addition, there is transient *zBTBD6b* expression in the trigeminal ganglia. These expression sites are common for all three *cBTBD6, zBTBD6a* and *zBTBD6b* orthologs.

Therefore, *zBTBD6a* and *zBTBD6b* have mostly non-overlapping expression profiles but share some aspects of their expression patterns with *cBTBD6*.

Gene duplications and *BTBD3* and *BTBD6* expression profiles

BTBD3 and *BTBD6* belong to a novel family of genes characterised by the presence of BTB, BACK and PHR domains. Many gene families within a species are constituted of paralogous genes derived from a common ancestral gene. These paralogous genes arise as a result of regional gene duplications or whole genome duplications (increases in ploidy) (Lundin, 1993; Ohno, 1970; Taylor and Raes, 2004). Some gene duplicates acquire a new function and others may lose their capacity to make a functional protein (Kuo et al., 2005). Whole genome duplications in ancestral vertebrates generally appear to have occurred before the separation of fish and tetrapods (including chicken). However, many gene families of zebrafish consist of more members than in tetrapods (Amores et al., 1998; Ekker et al., 1997; Holland and Garcia-Fernandez, 1996; McClintock et al., 2001). The greater number of genes in zebrafish was probably caused by additional genome-wide duplication which happened in the lineage leading to modern ray-finned fishes (including zebrafish) but not along the lineage leading to tetrapods (Hoegg et al., 2004; Postlethwait et al., 2004; Postlethwait et al., 2000; Postlethwait et al., 1998).

Phylogenetic analysis assigned *zBTBD6a* and *zBTBD6b* as being the co-orthologs of *cBTBD6,* while *zBTBD3a* and *zBTBD3b* are orthologs of *cBTBD3.* A detailed spatio-temporal analysis of the expression patterns of *cBTBD3* and zebrafish orthologs, revealed no common sites of expression, suggesting that the zebrafish *BTBD3* genes do not represent functional orthologs of *cBTBD3.* This could be explained in a number of ways: there are additional (duplicated) members of the chick *BTBD3* subfamily that are expressed similar to *zBTBD3a* and/or *zBTBD3b* during embryogenesis; *zBTBD3a* and *zBTBD3b* represent an equivalent of other duplicated chick *BTBD3* genes/gene, which were lost during an evolution of the chick lineage while they were maintained in the zebrafish; or a different BTB gene family member, a predecessor of *BTBD6* and *BTBD3,* has the role of *zBTBD3a* and/or *zBTBD3b.*

Comparison of the *cBTBD6, zBTBD6a* and *zBTBD6b* expression profiles showed that whereas zebrafish *BTBD6a* and *BTBD6b* have distinct developmental expression, they share some aspects of their expression patterns with *cBTBD6.* These data suggest that some features of the *cBTBD6* function may be divided up between these two co-orthologs, each having a more restricted function than the original one. *zBTBD6a,* similar to *cBTBD6,* is expressed in sites of neuronal differentiation, suggesting that its role might be associated with the process of neurogenesis. This aspect of potential function was chosen for further investigation in zebrafish.

FUNCTIONAL ANALYSES OF *zBTBD6a*

Analysis of the *zBTBD6a* expression profile in zebrafish embryos up to 24 hpf revealed that *zBTBD6a* transcripts are detected in the developing CNS. During zebrafish neurogenesis, two populations of neurons are successively generated, early primary, and later secondary (Blader and Strahle, 2000; Chapouton and Bally-Cuif, 2004). Primary neurogensis begins during late gastrulation and continues during embryogenesis to produce neurons required for the movements of larvae after hatching. Secondary neurogenesis takes over the primary system and occurs particularly from post-embryonic stages (2 days post fertilisation, dpf) onward, but the first secondary neurons are already born at about 16 hpf (Appel et al., 2001; Kimmel et al., 1994; Mueller and Wullimann, 2003).

zBTBD6a expression occurs in the CNS throughout the process of primary neurogenesis. It is not clear whether *zBTBD6a* expression accompanies secondary neurogenesis as it is downregulated in the spinal cord at 24hpf and later stages of development were not analysed for *zBTBD6a* expression. At the onset of primary neurogenesis, *zBTBD6a* is expressed in paraxial domains of the posterior neural plate, a region marked by proneural gene expression. In these proneuronal or neurogenic domains Notch-mediated lateral inhibition controls selection of neuronal progenitors that subsequently differentiate into primary neurons (Appel, 2000; Mumm and Kopan, 2000). The expression of *zBTBD6a* during neurogenesis and this early pattern of expression and timing suggests the possibility that *zBTBD6a* plays a role during early neuronal determination and/or differentiation. In order to investigate this possibility, *zBTBD6a* expression was first compared in detail with early and late markers of neurogenesis. The relationship between the Notch signalling pathway and the *zBTBD6a* expression was then analysed. Finally, a potential function of *zBTBD6a* in the process of primary neurogenesis was tested by gene knockdown and misexpression approaches.

COMPARATIVE ANALYSIS OF *zBTBD6a* EXPRESSION WITH SELECTED PRONEURAL AND NEURONAL MARKERS

zBTBD6a and *deltaA, B* and *D* expression

zBTBD6a expression in the neural plate was compared with the distribution of transcripts of *deltaA (dla), deltaB (dlb)* and *deltaD (dld)* (Appel and Eisen, 1998; Haddon et al., 1998). In the late gastrula and early segmentation stage, these genes are expressed in proneuronal domains in the neural plate and are involved in the process of selection of individual progenitor cells to become neurons through lateral inhibition (Appel and Eisen, 1998; Appel et al., 2001). *dla* and *dld* are expressed widely in these regions, in large groups of contiguous neural cells, exhibiting initially uniform and then varied levels of transcript accumulation that are due to lateral inhibition. All cells expressing *dla* and *dld* have the potential to become primary neurons, but only the cells with high-level expression, will differentiate. *dlb* expression is nested within the *dla* and *dld* expression domains and confined to the nascent neurons marked by strong *dla* and *dld* expression. *dlb* expression correlates with withdrawal from the cell cycle and labels early differentiating (early postmitotic) neurons.

Comparative in situ hybridisation performed at 1-2-somite (Fig. 31A, C, E, G) and 5-6-somite (Fig. 31B, D, F, H) developmental stages revealed that the *zBTBD6a* expression pattern is reminiscent of all three *delta* gene expression profiles and is most similar to *dla*. *zBTBD6a* transcripts are distributed in patches comprising of many contiguous cells in the lateral and medial regions of the neural plate; in some of these cells the expression is strong, in others weak. This is different to *dlb* that is detected in fewer scattered isolated cells instead of cell clusters in the lateral and medial domains.

This suggests that *zBTBD6a* transcripts, similar to *dla*, mark all cells within the proneuronal domains rather than only the early postmitotic neurons. Similar

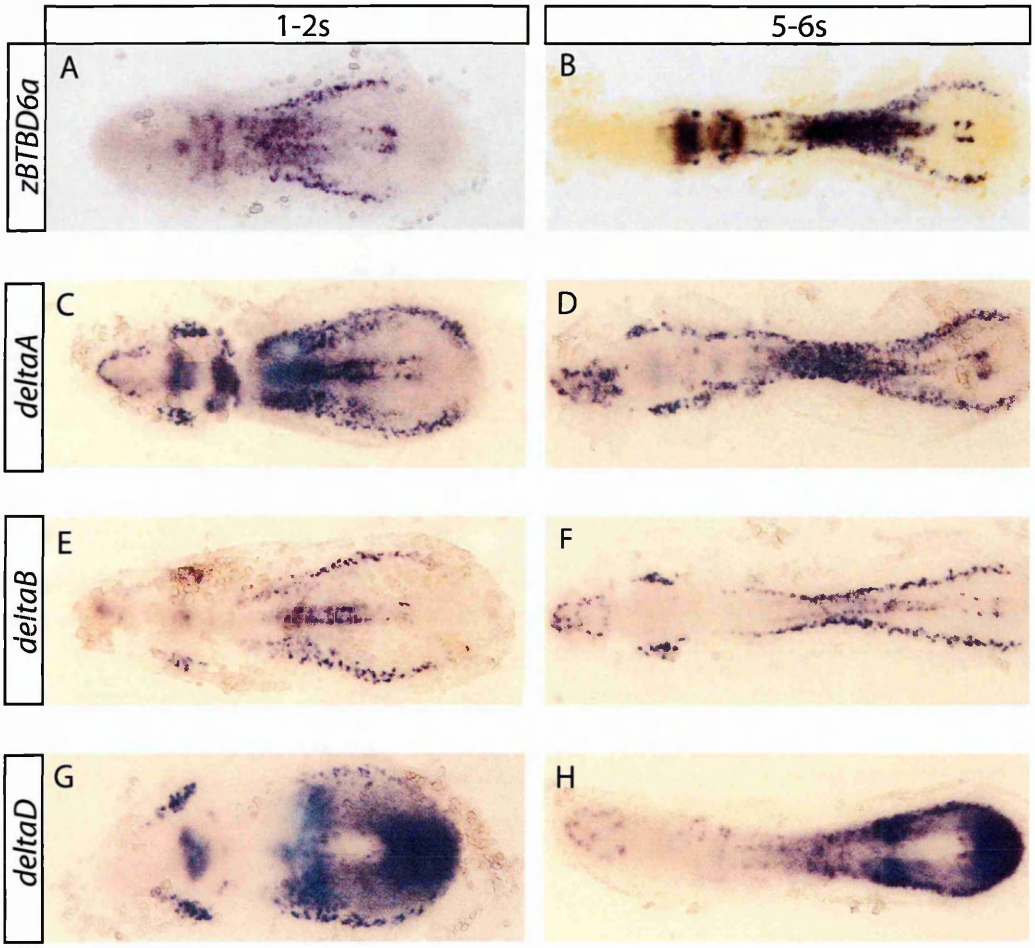


Figure 31. Comparison of the early expression patterns of *zBTBD6a* and *deltaA*, *B* and *D* genes. Dorsal views, anterior to the left. At 1-2-somite (A, C, E, G) and 5-6 somite (B, D, F, H) stages, *zBTBD6a* expression in the neural plate is reminiscent of the expression pattern of all three delta genes, and is most similar to *deltaA* (C, D).

to *dla* and *dld*, the cells with higher accumulation of *zBTBD6a* transcript are likely to correspond to the selected neuronal progenitors.

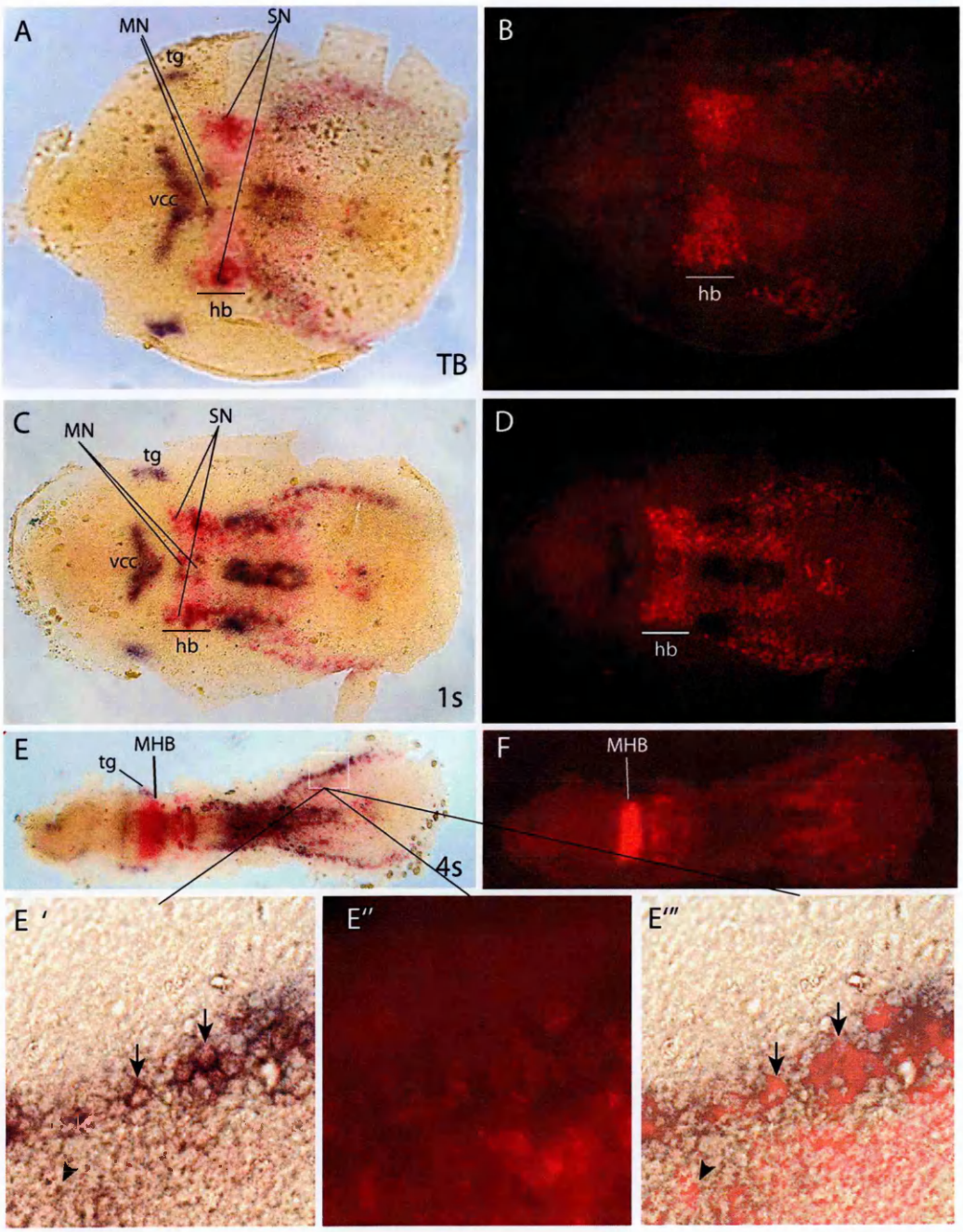
zBTBD6a and *neurog1* expression

If *zBTBD6a* is expressed in cells which have the potential to become primary neurons, one would expect that its expression is concomitant with the expression of proneural genes, e.g. *neurog1* (Blader et al., 1997; Kim et al., 1997) that marks cell populations in the neural plate, from which individual neuronal precursor cells will be singled out.

In order to compare *zBTBD6a* and *neurog1* early expression domains and establish whether both transcripts are located in the same cells, two-colour double in situ hybridisation was carried out on embryos between 80% epiboly and the 4-somite stage. This analysis revealed that *neurog1* expression, first observed at around 90% epiboly (data not shown, (Blader et al., 1997), precedes the onset of *zBTBD6a* expression, since in the youngest embryos exhibiting *neurog1* expression, no *zBTBD6a* transcripts were detected. Between the tailbud and 4-somite stages, both genes are expressed in overlapping domains in the posterior neural plate (Fig. 33A-F). *zBTBD6a* expression occurs in longitudinal stripes of cells where high levels of *neurog1* transcripts are detected (arrows) and in some adjacent areas exhibiting weak *neurog1* expression (arrowheads). Examination of the expression domains at single cell resolution confirmed that both the most highly and weakly expressing *neurog1*-positive cells in the lateral posterior neural plate express *zBTBD6a* (Fig. 32E'-E''').

In addition, these data suggest that in the hindbrain of tailbud and 1-somite stage embryos (Fig. 32A-D), *neurog1* is expressed in the forming motor neurons (MN; Fig. 31A, C) and sensory neurons (SN; Fig. 32A, C) in r2 and r4, while the *zBTBD6a* transcripts appear to be distributed throughout the hindbrain posterior to r1 (hb; Fig. 32A-D). *neurog1* is also expressed in the trigeminal ganglia (tg; Fig. 32A, C) and in midbrain ventrocaudal cluster at

Figure 32. Comparison of *neurog1* and *zBTBD6a* expression in the neural plate at early stages of zebrafish embryogenesis. *neurog1* and *zBTBD6a* are co-expressed as revealed by double in situ hybridisation for *neurog1* and *zBTBD6a*. Developmental stages are indicated in the lower right corner. Dorsal views, anterior to the left. (A, C, E) *neurog1* transcripts revealed in dark blue with BM Purple, *zBTBD6a* detected in red with Fast Red. (B, D, F) Fluorescent Fast Red staining of *zBTBD6a* viewed by epifluorescence . (E', E'', E''') bright field, fluorescence and merge of the enlarged box region in E, respectively. *zBTBD6a* expression is found in cells exhibiting high (arrow) and low (arrowhead) levels of *neurog1* transcript, as confirmed at single cell resolution (E'-E'''). In the hindbrain of tailbud and 1-somite embryos (A-D), *neurog1* is expressed in the developing motor (MN) and sensory (SN) neurons, while *zBTBD6a* expression occurs posterior to r1 (hb). *neurog1* but not zBTBD6a is expressed in the trigeminal ganglia (tg) (A, C, E), whereas in the midbrain-hindbrain boundary (MHB) of 4-somite stage embryos, *zBTBD6a* but not *neurog1* RNA is detected (E, F).


A
MN
SN
tg
vcc
hb
TB
B
hb
C
MN
SN
tg
vcc
hb
1s
D
hb
E
MHB
tg
4s
F
MHB
E'
E''
E'''

the tailbud and 1-somite stage (vcc; Fig. 32A, C), and *zBTBD6a* is not expressed in these regions, whereas at 4-somite stage only *zBTBD6a* but not *neurog1* signal is detected in the MHB (MHB; Fig. 32E,F).

In conclusion, although *zBTBD6a* expression pattern is not identical to *neurog1* in the anterior neural plate, in the posterior neural plate there is co-localisation of *zBTBD6a* and *neurog1* expression in cells within the proneuronal domains.

zBTBD6a and _islet-1_ expression

In order to determine whether *zBTBD6a* expression occurs in cells that have completed the neuronal differentiation process, double in situ hybridisation was performed with a probe for *islet1* (*isl1*), a marker of specific postmitotic terminally differentiating neurons (Fig. 33).

isl1 expression is first detected in the neural plate at the 1-2-somite stage (data not shown, (Inoue et al., 1994; Korzh et al., 1993), while *zBTBD6a* expression can already be detected at the late gastrula stage (see Fig. 33A). *isl1* transcripts label primary Rohon-Beard sensory and motor neurons differentiating in lateral and medial proneural clusters, respectively (Inoue et al., 1994; Korzh et al., 1993). Examination of 3-somite stage embryos revealed that almost all *isl1*-expressing primary neurons co-express *zBTBD6a* (arrows; Fig. 33A, B & A'-A'''). However, *zBTBD6a* expression is also found in many cells in the medial and lateral neural plate that are not expressing *isl1* (arrowheads; Fig. 33A, B), which are likely to correspond to either undifferentiated proliferating neural cells or prospective neurons at earlier stages of neurogenesis. This suggests that *zBTBD6a* expression precedes *isl1* expression and/or alternatively that *zBTBD6a* expression occurs in cells specified for other neuronal fates. In addition, a few *isl1*-expressing neurons do not contain *zBTBD6a* transcripts, suggesting that *zBTBD6a* expression is down regulated once neuronal differentiation occurs.

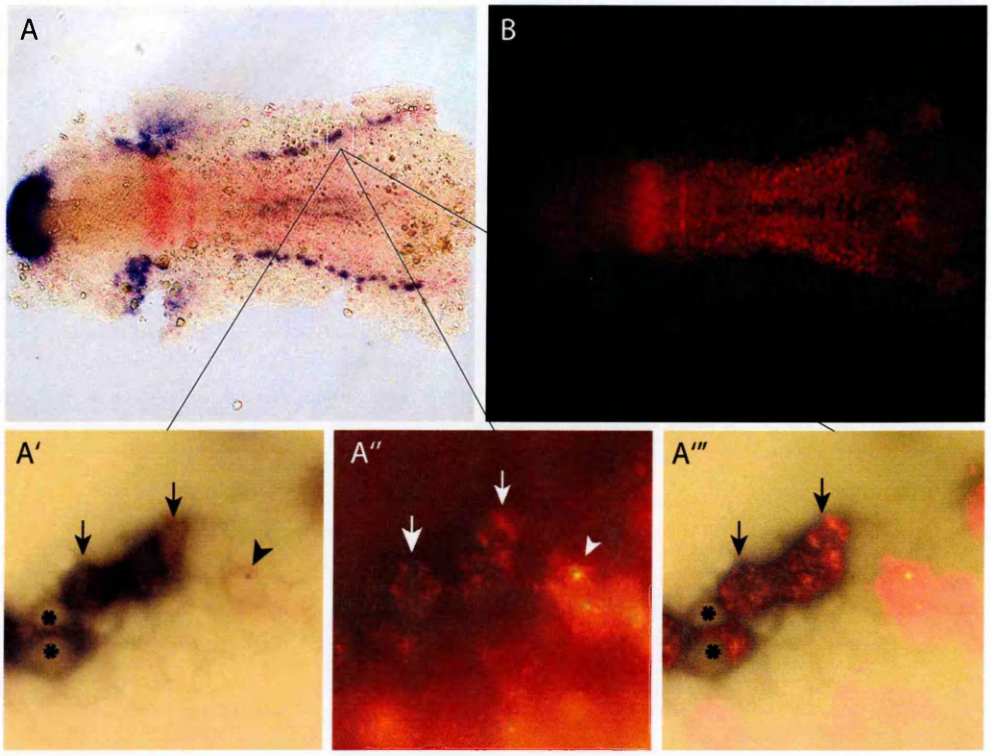


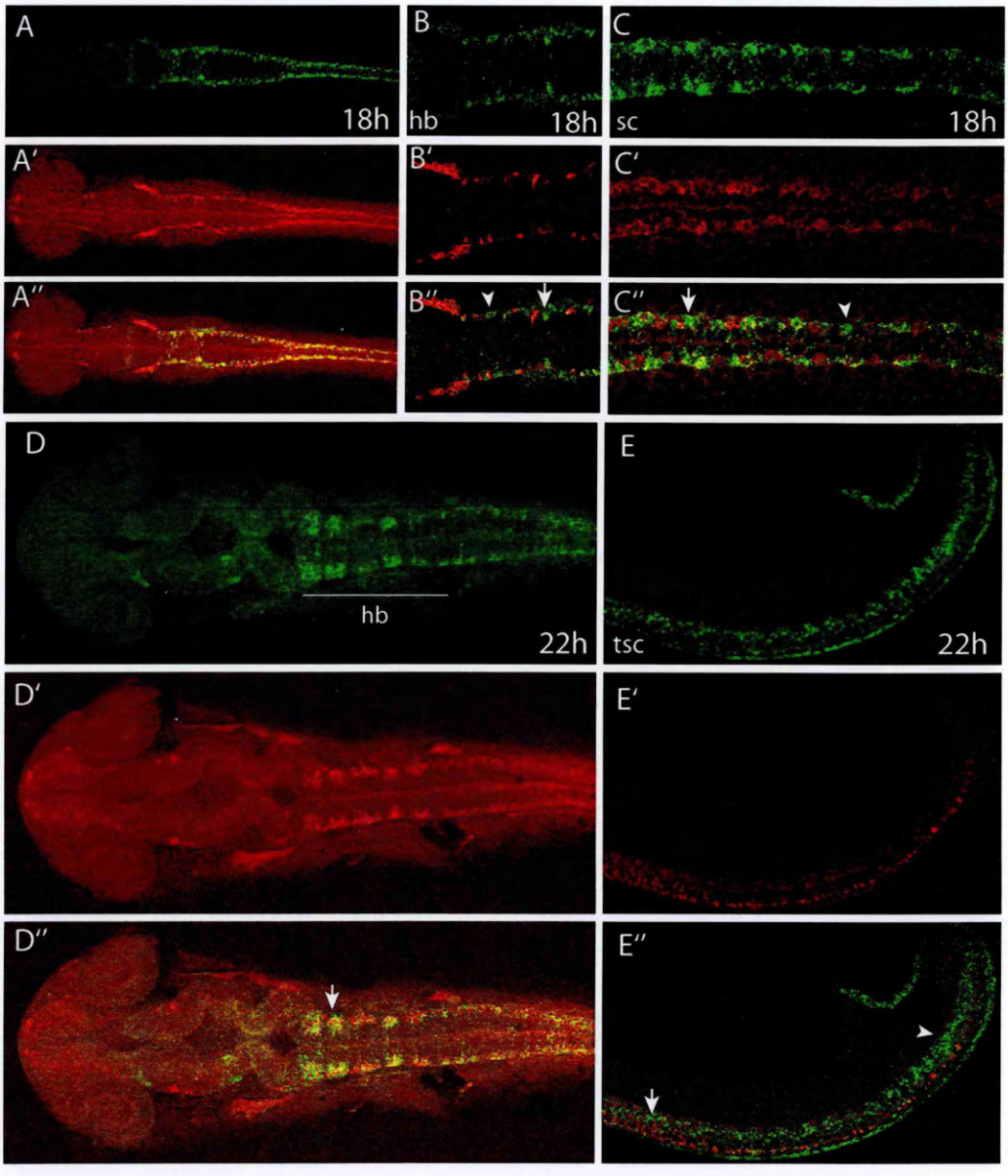
Figure 33. Expression of *zBTBD6a* and *isl1* in the posterior neural plate. *zBTBD6a* is expressed in most *isl1*-positive primary neurons as assessed by double in situ hybridisation at the 3-somite stage. Dorsal views, anterior to the left. (A) *isl1* transcripts revealed in dark blue with BM Purple, *zBTBD6a* detected in red with Fast Red. (B) Fluorescent Fast Red staining of *zBTBD6a* viewed by epifluorescence. (A', A'', A''') bright field, fluorescence and merge of both of the enlarged box region in A, respectively. Most differentiated primary neurons labelled by *isl1* co-express *zBTBD6a*, as shown in the lateral sensory neurons (arrows; A'-A'''). *zBTBD6a* expression is found in many more cells in the neural plate outside the *isl1* expression domain (arrowheads; A, B, A''). A few *isl1*-positive cells that do not express *zBTBD6a* are indicated by an asterix (A', A''').

zBTBD6a and *ELAV-like3/HuC* expression

The *ELAV-like3* (*elavl3*)/*HuC* gene encodes a neuron-specific RNA binding protein that is expressed in all post-mitotic, differentiating neurons at all stages of neuronal development (Kim et al., 1996; Park et al., 2000a). To determine whether *zBTBD6a* expression occurs at later stages of neuronal cell differentiation, a comparative analysis of the *zBTBD6a* and *elavl3* expression profiles at 18 and 22 hpf was undertaken. To this end, *zBTBD6a* transcripts were detected by in situ hybridisation in *elavl3/HuC-GFP* transgenic embryos (Park et al., 2000b), in which green fluorescent protein (GFP), was revealed by immunochemistry. *zBTBD6a* and *elavl3/HuC* expression were detected with different fluorescent signals and the images were taken using the same focal plane.

At 18 and 22 hpf, *zBTBD6a* RNA is found in a subpopulation of differentiating neurons marked by the presence of *elavl3* protein (arrows; Fig. 34A'', B'', C'', D'') in the hindbrain (hb) and the spinal cord (sc). *zBTBD6a* expression is also observed in some neural cells in which *elavl3* protein is absent (arrowheads; Fig. 34B'', C'', E''). Neurogenesis in the spinal cord progresses in an anterior to posterior sequence (Appel et al., 1995). Accordingly, in the caudal spinal cord (tsc) of the 22 hpf embryo, *elavl3* protein is distributed in a decreasing number of cells more caudally. Conversely, *zBTBD6a* transcripts are found in an increasing number of cells more caudally, suggesting that *zBTBD6a* marks neuronal progenitors at earlier stages than *elavl3* expression. Not all differentiating neurons marked by *elavl3* protein express *zBTBD6a* confirming the result with *isl1*, which suggests that neurons downregulate *zBTBD6a* as they differentiate.

Figure 34. Comparison of *zBTBD6a* and *elavl3/HuC* expression. Partially overlapping expression of *zBTBD6a* and *elavl3/HuC* was detected in the hindbrain (hb) and the spinal cord (sp) of 18 (A-A'', B-B'', C-C'') and 22 hpf embryos (D-D'', E-E''). *zBTBD6a* mRNA, shown in green (A, B, C, D, E), was detected by whole mount in situ hybridisation in *elavl3/HuC-GFP* zebrafish transgenic embryos, using TSA fluorescent substrate. Subsequently, in the same embryos, *elavl3/HuC* protein, labelled in red (A', B', C', D', E'), was revealed with anti-GFP antibody. (A'', B'', C'', D'', E'') are merges of the corresponding single staining images. All images are of the same single focal plane. *zBTBD6a* transcripts are found in the subpopulation of neurons where *elavl3/HuC* protein is detected (arrows), marked in yellow, and in other cells devoid of *elavl3/HuC* protein (arrowheads). Note that in the caudal spinal cord (tsc; E-E'), *zBTBD6a* signal is located in an increasing number of cells towards the tail tip, while the number of neurons expressing *elavl3/HuC* protein is declining.


A
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B
hb
18h
C
sc
18h
A′
B′
C′
A″
B″
C″
D
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22h
E
tsc
22h
D′
E′
D″
E″

REGULATION OF *zBTBD6a* EXPRESSION

zBTBD6a is under the control of Notch signalling

Early *zBTBD6a* expression is punctate in longitudinal domains in the neural plate. This distribution of transcripts is characteristic of neurogenic genes subjected to Notch-mediated lateral inhibition (Blader et al., 1997; Haddon et al., 1998; Takke et al., 1999). This suggests the possibility that *zBTBD6a* may also be Notch dependent.

zBTBD6a expression is suppressed by Notch-mediated signalling

Notch activation occurs upon binding to Delta or Serrate ligand by a proteolytic cleavage that releases an intracellular fragment of Notch that acts as a co-factor for Suppressor of Hairless [Su(H)], which then can drive expression of target genes (Bray and Furriols, 2001; Mumm and Kopan, 2000; Struhl and Adachi, 1998). Major Notch targets are Enhancer of Split [E(spl)] bHLH proteins that act as repressors of the proneural genes that drive neurogenesis (Artavanis-Tsakonas et al., 1999; Fisher and Caudy, 1998).

To investigate whether *zBTBD6a* expression is modulated by the Notch pathway, a constitutively active [Su(H)-Ank] form of Suppressor of Hairless 1, was expressed (Wettstein et al., 1997). A strong reduction of *zBTBD6a* expression was observed at the 4-5-somite stage in the injected embryos compared with uninjected control embryos (Fig. 35).

This result suggests that the *zBTBD6a* expression is down-regulated by Su(H)-dependent Notch signalling. It cannot be excluded that this effect of Notch activation on *zBTBD6a* expression is indirect, especially that the misexpression was performed in 1-cell stage embryos, which can potentially perturb earlier developmental processes. However, as *zBTBD6a* appears to be a marker of neurogenesis, it is likely that *zBTBD6a* expression is affected

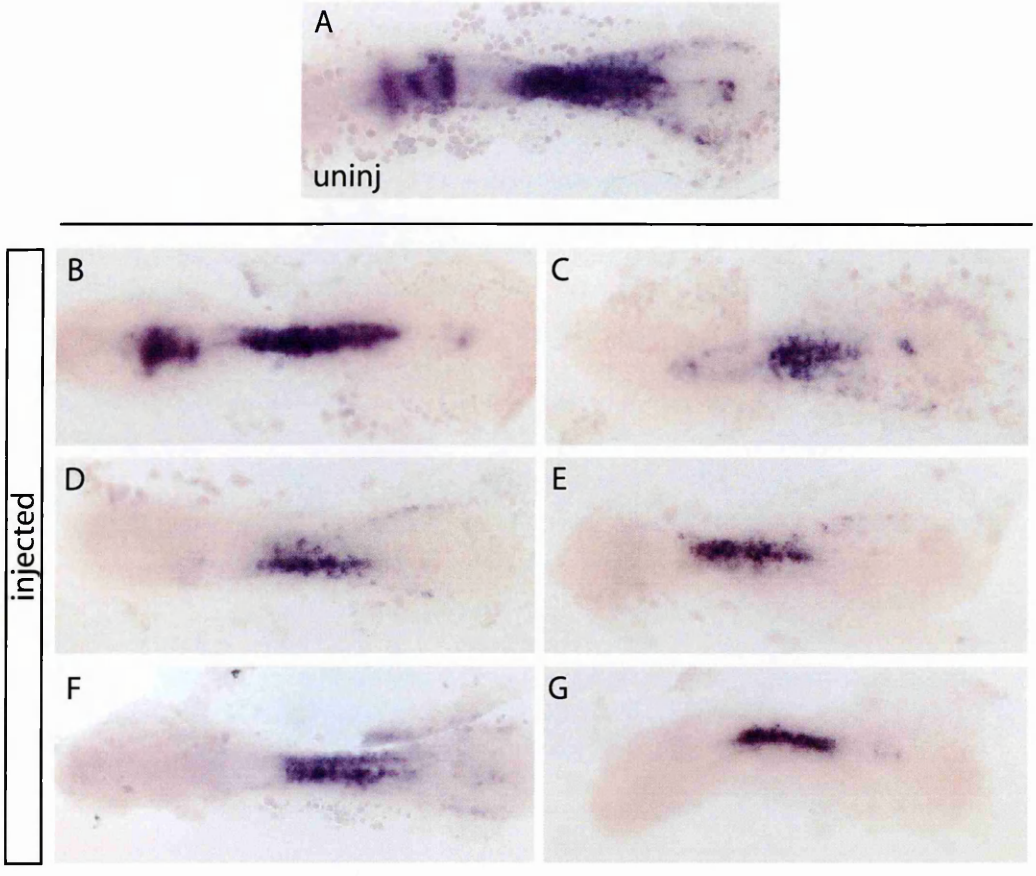


Figure 35. *zBTBD6a* expression is under control of the Notch pathway. (B-G) Embryos injected at one-cell stage with RNA encoding a constitutively active form of the Notch effector Suppressor-of-Hairless [Su(H)] and analysed at the 4-5-somite stage for *zBTBD6a* expression by whole-mount in situ hybridisation. *zBTBD6a* expression is reduced (14/16 embryos) compared to uninjected control embryo(A).

by Notch signalling in a similar manner as an expression of all other neuronal markers: downregulated upon Notch activation and upregulated when the Notch signalling is blocked. There is some retention of *zBTBD6a* expression in the middle of the neural plate, which may suggests that spinal cord neurogenesis is less sensitive to increased Notch signalling than in the hindbrain.

zBTBD6a expression is suppressed through the [Su(H)] target genes

To verify that the observed repression of *zBTBD6a* expression is caused by Notch-mediated activation of transcriptional repressors, *zBTBD6a* expression was analysed in embryos with a knockdown of *her4*, a member of the E(spl) family (Takke et al., 1999). *her4* is a direct target of Notch signalling expressed in the proneuronal domains in the neural plate where it inhibits neuronal differentiation. Prevention of the synthesis of Her4 protein by injecting antisense morpholino oligonucletides (MO) (Ekker, 2000; Ekker and Larson, 2001; Erickson, 1993; Nasevicius and Ekker, 2000; Pasini et al., 2004) resulted in up-regulation of *zBTBD6a* expression, especially in the neural keel region, as observed in 4-5-somite stage embryos (Fig. 36). *zBTBD6a* expression in the open neural plate region seems to be unaffected and this could be explained by the presence of other Her proteins (eg. Her2, Her12; Bae, 2005) activated by Notch that are still functional and prevent up-regulation of *zBTBD6a* expression in this area. This result indicates that *zBTBD6a* expression is modulated by Notch signalling through activation of at least the *her4* target gene.

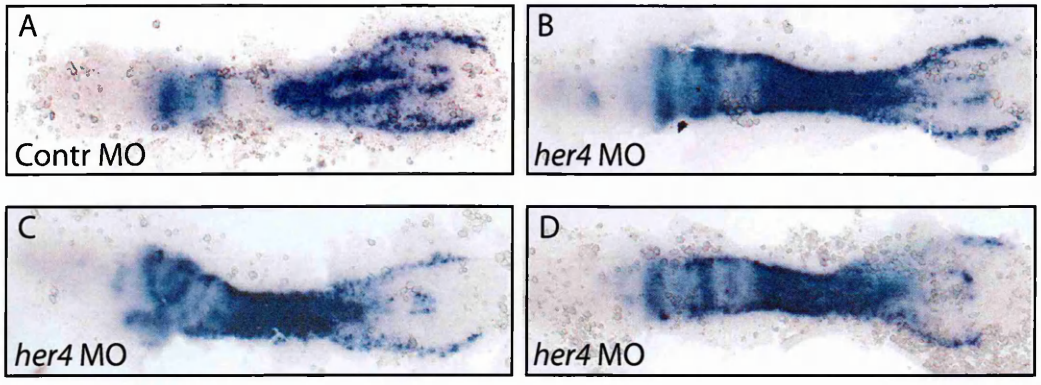


Figure 36. Effect of the *her4* knockdown on *zBTBD6a* expression. Loss of Her4 induces increased expression of *zBTBD6a* within the neural plate (21/26 embryos). Control (A) or *her4* MO-injected (B, C, D) embryos probed for the expression of *zBTBD6a* gene at the 5-somite stage.

zBTBD6a expression is up-regulated in the Notch defective embryos, *mib*

To further probe the significance of the above observations, *zBTBD6a* expression was analysed in embryos with loss of Notch signalling. To this end a neurogenesis mutant *mind bomb* (*mib*) (Jiang et al., 1996), was used, which displays a strong reduction in Notch signalling due to a mutation in the gene encoding a ubiquitin ligase for Delta (Itoh et al., 2003). In the *mib* mutant, reduced lateral inhibition permits excessive numbers of cells to become neurons and depletes the population of progenitors needed for neurogenesis in the CNS to continue.

In 3-somite stage *mib* embryos, when compared with wild type controls, *zBTBD6a* expression is increased and becomes homogenous within the neurogenic domains (Fig. 37A, B). Similarly, at 24 hpf, compared with controls, *mib* mutants exhibit elevated levels of *zBTBD6a* expression in the hindbrain, the forebrain and the spinal cord (Fig. 37C-F). This result together with the outcome of the previous experiments indicates that *zBTBD6a* is under the negative control of Su(H)-dependent Notch signalling.

Neurog1 controls *zBTBD6a* expression

neurog1 is a proneural gene that is negatively regulated by the Notch signalling. Since *zBTBD6a* expression is also suppressed upon Notch activation, the next question to address was whether this suppression is due to the loss of Neurog1 activity.

Morpholino-mediated *neurog1* inactivation abolishes *zBTBD6a* expression

First, *zBTBD6a* expression was examined when *neurog1* translation was blocked by specific MO oligonucleotodes injected into the yolk of 1-4-cell embryos. Whole mount in situ hybridisation revealed a major reduction or complete absence of *zBTBD6a* expression in *neurog1* MO-injected embryos at

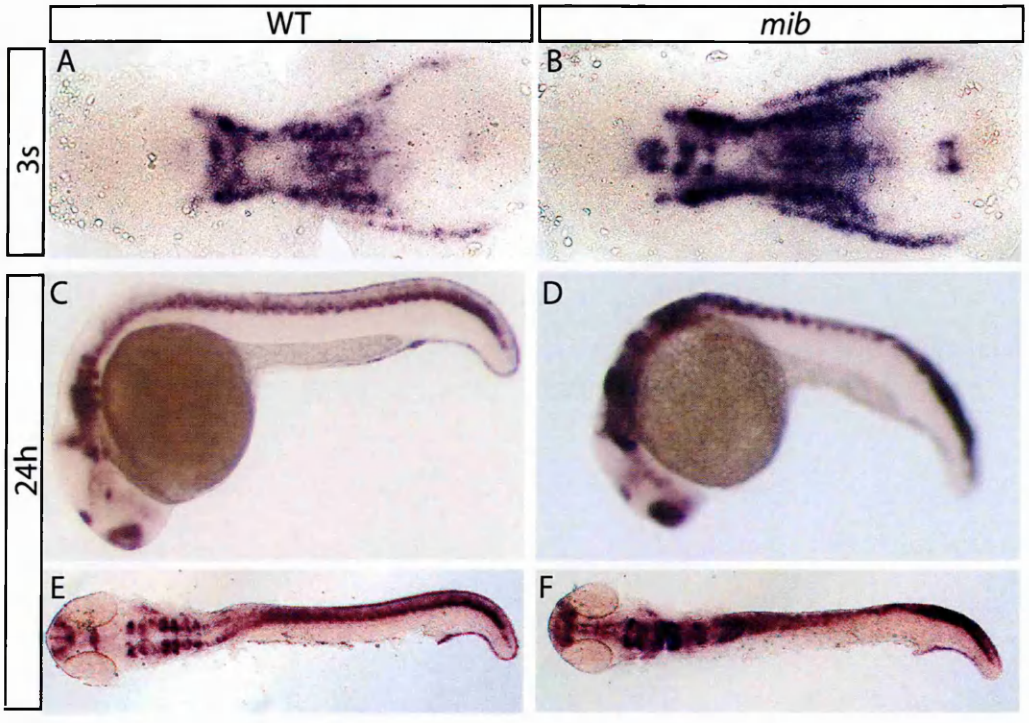


Figure 37. Expression of *zBTBD6a* is elevated in the neurogenic *mindbomb* (*mib*) zebrafish mutant. (A, C, E) Wild-type siblings (B, D, F) *mib* mutant embryos, showing an increase of *zBTBD6a*-expressing cells.

the 3-somite stage (Fig. 38; compare A to B-I)), indicating that Neurog1 is required for the expression of *zBTBD6a*.

Misexpression of *neurog1* induces ectopic *zBTBD6a* expression

To examine whether Neurog1 is capable of inducing *zBTBD6a* expression, misexpression was carried out by injection of *neurog1* RNA into zebrafish embryos. Overexpression of Neurog1 leads to the ectopic formation of *isl1*-expressing cells in the ectoderm outside the neural plate (Blader et al., 1997; Takke et al., 1999). To check the efficiency of the *neurog1* RNA construct, a control experiment was carried out. Embryos were injected into 1-cell with *neurog1* RNA and analysed for *isl1* expression at the 3-somite stage. I found that the injected embryos have ectopic *isl1*- positive cells in the yolk ectoderm (Fig. 39). Additionally, the neural plate of the injected embryos appears to be slightly expanded, and in some embryos there is an accumulation of *isl1*- positive cells at the edges of the neural plate, both anteriorly and laterally (arrows; Fig. 39B & D), while the normal *isl1* expression pattern within the neural plate is distorted and/or reduced.

Injection of *neurog1* RNA into 1-cell embryos induced ectopic expression of *zBTBD6a* within the neural plate at sites where *zBTBD6a* transcripts are not normally present (Fig. 40 and 41). As observed in the previous experiment, the neural plate is slightly enlarged. *zBTBD6a* expression occurs throughout entire neural plate except for the very posterior part. Ectopic *zBTBD6a* expression is punctate suggesting that it might be subjected to lateral inhibition.

Overexpression of Neurog1 in 1-cell stage embryos may have an indirect effect on *zBTBD6a* expression. However, taking into consideration that *zBTBD6a* and *neurog1* expression overlaps in the posterior neural plate and that *neurog1* knockdown results in abrogation of *zBTBD6a* expression, it is most likely that Neurog1 activates *zBTBD6a* transcription in the neural plate.

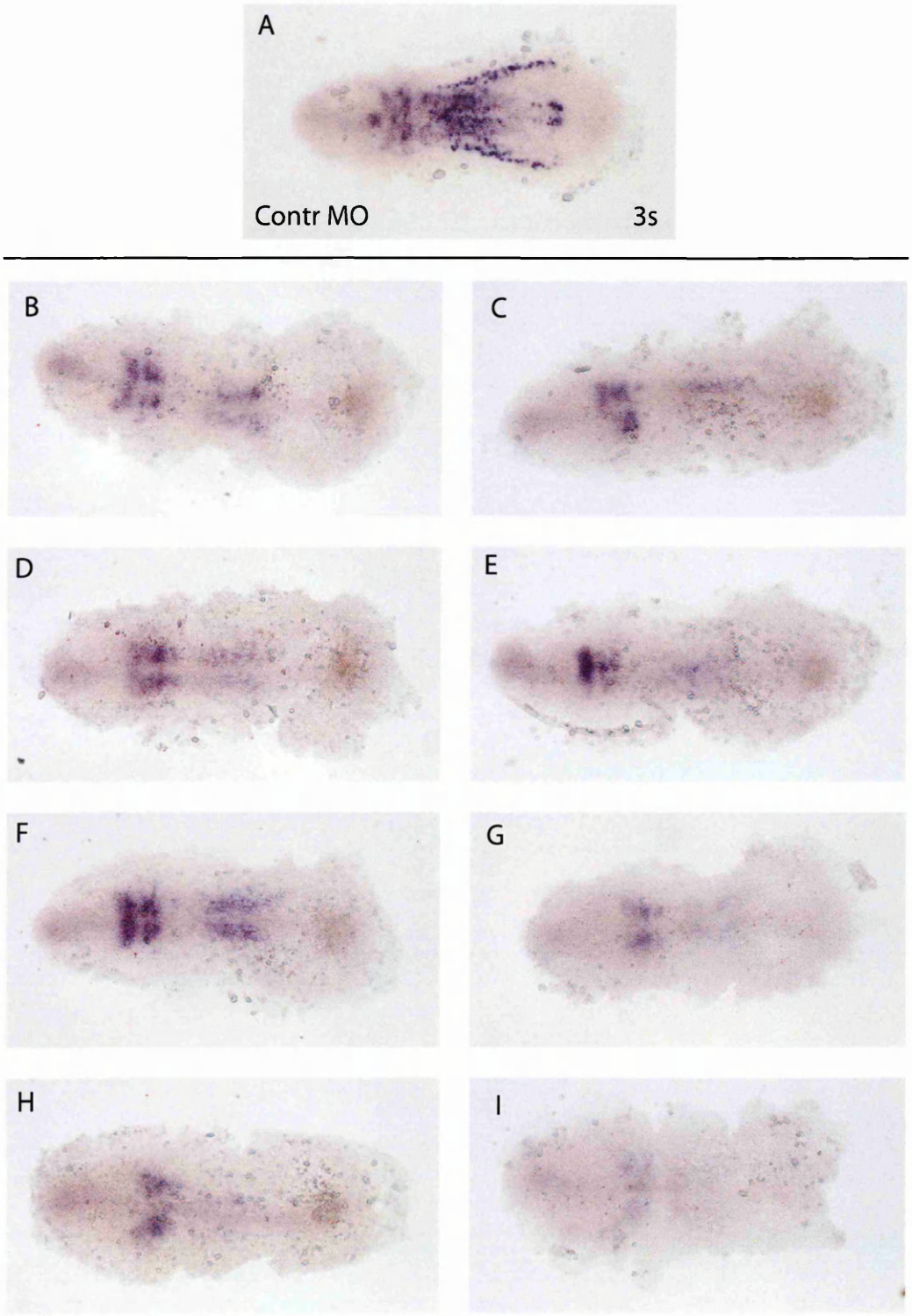


Figure 38. Effect of loss of Neurog1 on *zBTBD6a* expression. Knock down of *neurog1* is accompanied by loss of *zBTBD6a* transcripts (31/31 embryos). Embryos injected at the 1-4 cell stage with either control (A) or *neurog1* (B-I) morpholino and analysed at the 3-somite stage for *zBTBD6a* expression by in situ hybridisation.

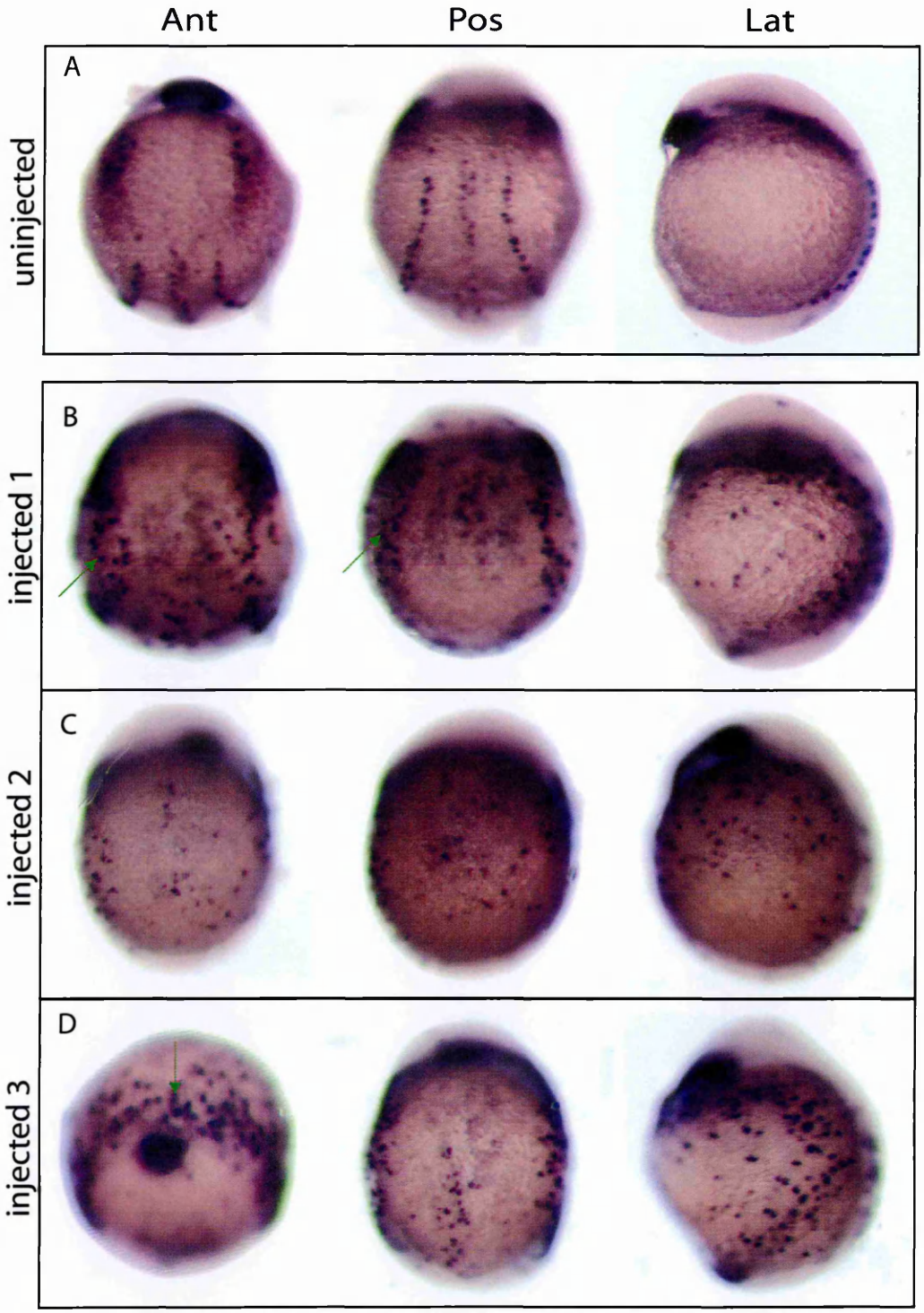


Figure 39. Effect of Neurog1 overexpression on *isl1* expression. Misexpression of *neurog1* induces ectopic *isl1* expressing cells in the non-neural ectoderm of the yolk sac and causes disruption and/or reduction of the normal *isl1* expression pattern within the neural plate (41/41 embryos). 3-somite stage embryos processed through in situ hybridisation with *isl1* RNA probe. (A) Anterior (Ant), posterior (Pos) and lateral (Lat) views of an uninjected control. (B, C, D) Examples of three embryos injected with *neurog1* RNA at the 1-cell stage. Note the accumulation of the *isl1*-positive cells at the edges of the neural plate (arrow, B, D).

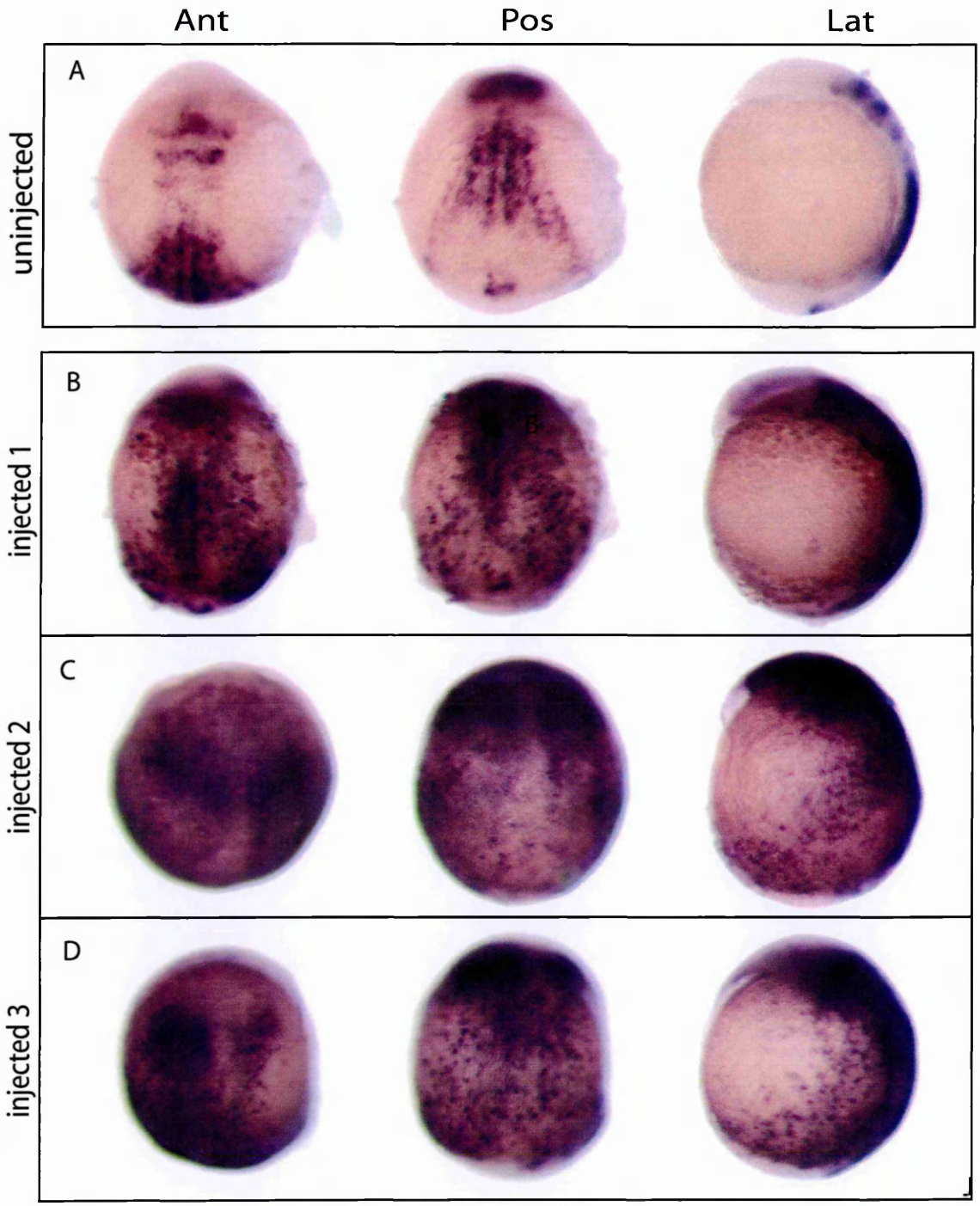


Figure 40. Effect of *neurog1* misexpression on *zBTBD6a* expression. Overexepression of Neurog1 induces ectopic expression of *zBTBD6a* within the neural plate (28/28 embryos). 3-somite stage embryos subjected to in situ hybridisation with *zBTBD6a* RNA probe. (A) Anterior (Ant), posterior (Pos) and lateral (Lat) views of an uninjected control. (B, C, D) Examples of three embryos injected with *neurog1* RNA at 1-cell stage.

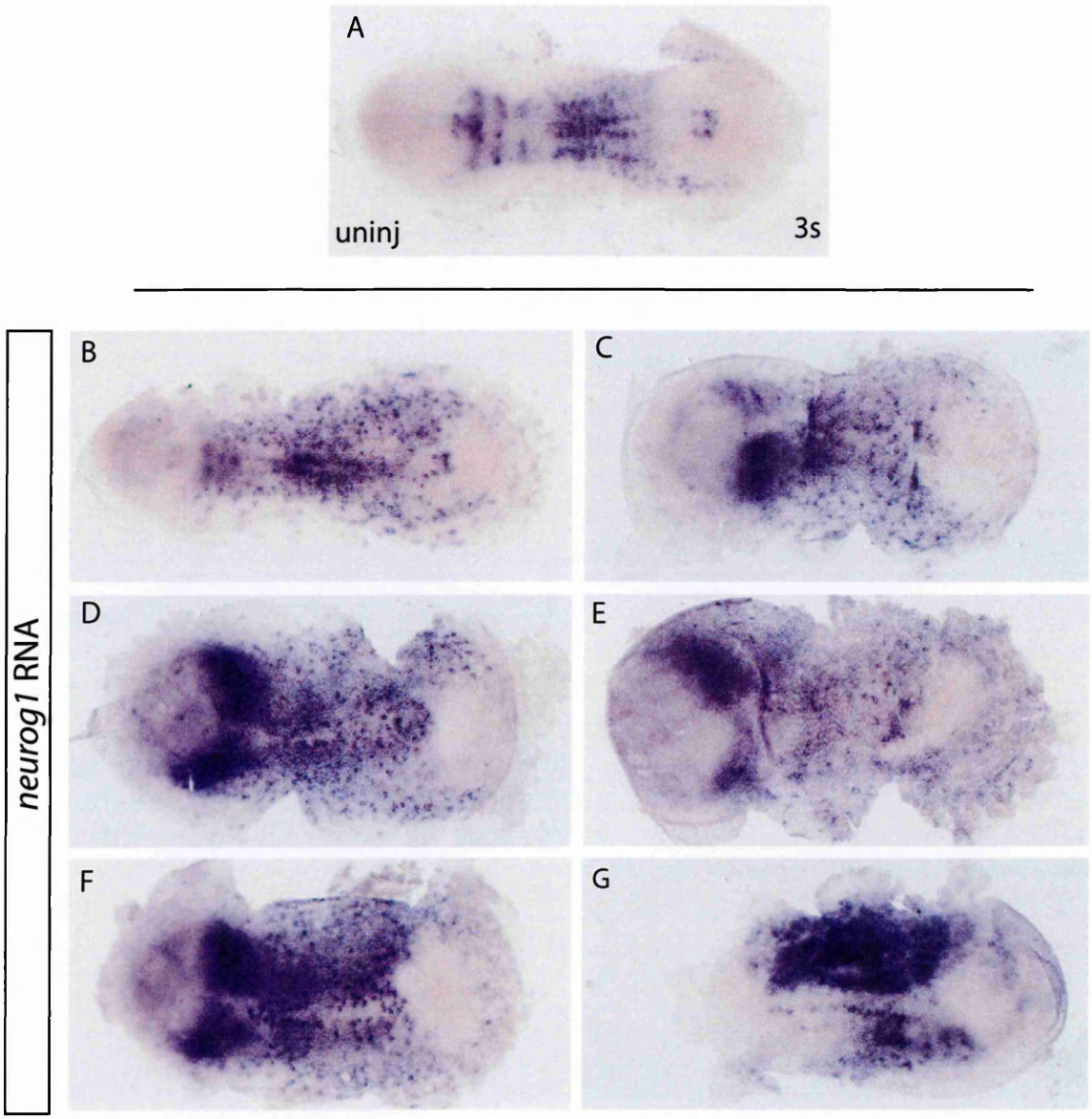


Figure 41. Dorsal views of embryos injected with *neurog1* RNA. Flat mount preparations of 3-somite stage ebryos, uninjected (A) and injected at 1-cell stage with *neurog1* RNA (B-G). Expression of *zBTBD6a* was detected by in situ hybridisation. Note the spotty appearance of ectopic expression of *zBTBD6a* located throughout the whole neural plate except for the most posterior part.

This activation could be direct, or indirect executed through another factor acting downstream of Neurog1.

FUNCTIONAL DISSECTION OF zBTBD6a

zBTBD6a is expressed in neuroepithelial cells that have the potential to become neurons as well as in differentiating neurons during primary neurogenesis. At early stages of primary neurogenesis, *zBTBD6a* expression is confined to neurogenic territories in the neural plate where early neurons are born. This expression is dependent on Notch signalling and activated by Neurog1. Therefore, taking all these data into consideration, there is a possibility that zBTBD6a function may be involved in the process of neurogenesis.

Neurogenesis is a progressive process that leads to the production of differentiated neurons (Bertrand et al., 2002; Blader and Strahle, 2000; Brunet and Ghysen, 1999). During primary neurogenesis, *neurog1* expressed in the proneuronal domains of the neural plate promotes neuronal differentiation through the activation of a cascade of genes that control successive steps of this process (Blader et al., 1997; Ma et al., 1996). The expression of these genes underlies the consecutive stages of neuronal differentiation.

In order to elucidate whether *zBTBD6a* plays a role in primary neurogenesis and, if this is the case, at what stage zBTBD6a activity is required in this process, morpholino-mediated *zBTBD6a* knockdown and *zBTBD6a* misexpression were used.

I first analysed the effects of *zBTBD6a* knockdown on the expression of late and then of progressively earlier markers of neurogenesis (Fig. 42). These include: late marker, *isl1,* expressed in specific differentiated neurons (Inoue et al., 1994; Korzh et al., 1993); intermediate markers, *neurod4, neurod,* and *dlb,* that are expressed in the early postmitotic neurons and label the

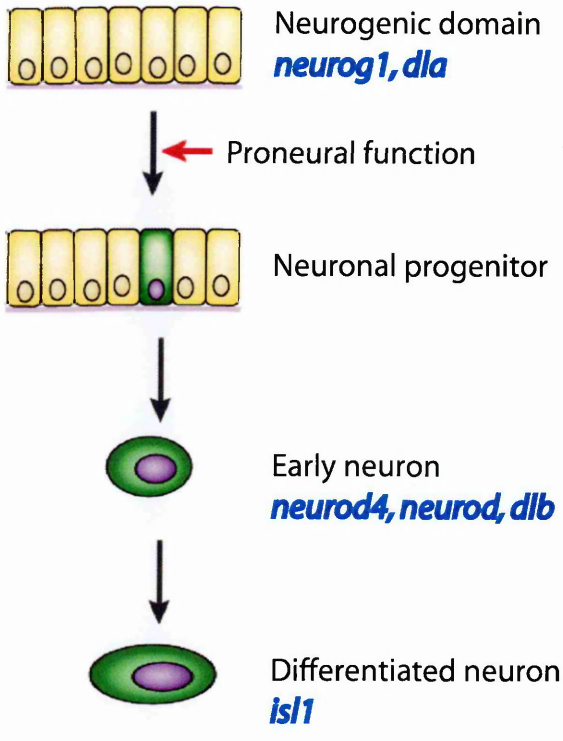


Figure 42. Elucidating the position of zBTBDa activity in the molecular pathway leading to neuronal differentiation. This involved analysis of the effects of zBTBD6a knock down on the expression of selected markers, indicated in blue, underlying successive steps of neurogenesis. *neurog1* and *dla* are the earliest markers of the neuroepithelial cells of the neurogenic regions from which neuronal progenitors arise. Postmitotic early neurons are labelled by *neurod4*, *neurod* and *dlb*, while specific terminally differentiated neurons are marked by *isl1*.

transition between the proliferating and differentiation stage (Korzh et al., 1998; Park et al., 2003; Wang et al., 2003); and early markers of neuroepithelial stem cells, *neurog1* and *dla*, taking part in neuronal selection/determination (Blader et al., 1997; Haddon et al., 1998; Kim et al., 1997; Korzh et al., 1998).

Effect of *zBTBD6a* knockdowns on neurogenesis

zBTBD6a knockdown reduces the number of differentiated primary neurons labelled by *isl1*

To test whether *zBTBD6a* is required for neurogenesis, antisense MO oligonucleotides against the *zBTBD6a* ATG region, were injected into the yolk of 1-4-cell stage embryos to specifically block translation of *zBTBD6a* mRNA. MO standard control oligonucleotides that should have no target and no significant biological activity were injected as a control. The specific and control morpholino-injected embryos were analysed at early somitogenesis stages for the presence of differentiating neurons, as identified by in situ hybridisation with the *isl1* RNA probe. When compared with controls, morphants exhibited a reduction in the number of primary neurons at the 2-3-somite stage (Fig. 43A-G). The severity of this phenotype varied from embryo to embryo, and ranged from differentiated neurons being almost completely absent (Fig. 43F, G) to a slight decrease in numbers of medially positioned motorneurons and laterally located sensory neurons (Fig. 43B, C).

The neuronal expression of *isl1* is first detected at around the 1-2-somite stage in a small number of differentiated neurons and increases gradually as a larger number of progenitor cells undergo differentiation. To test whether decreased neurogenesis occurs at late stages, the analysis was also performed in older embryos of 5-6 somites (Fig. 43H-N). Similar results were obtained, but the effects of the *zBTBD6a* inactivation were milder, with

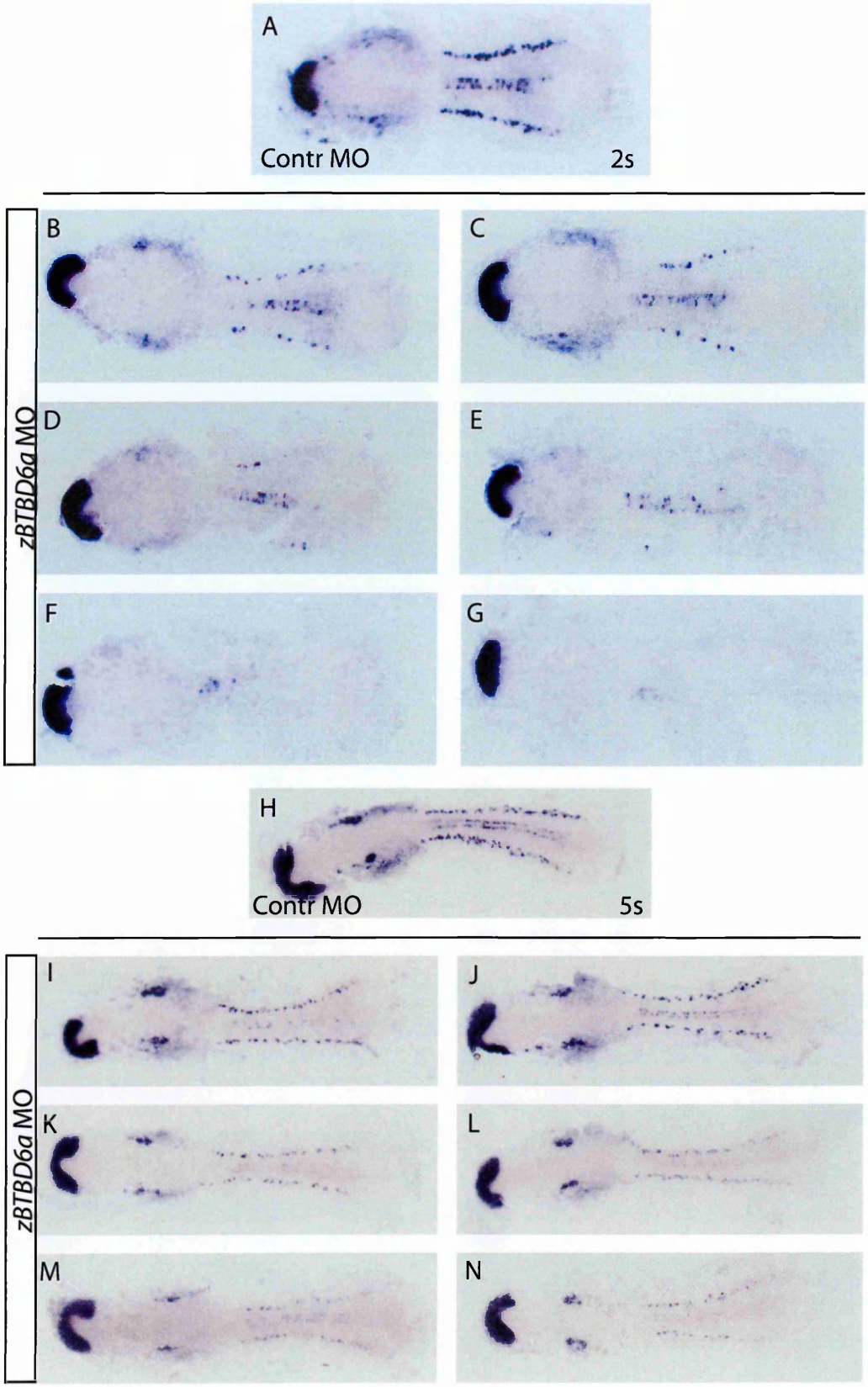


Figure 43. Effect of *zBTBD6a* knockdown on *isl1* expression.The number of cells expressing the neuronal differentiation marker *isl1* is decreased in *zBTBD6a* MO-injected embryos at the, 2-3 somite (B-G; 45/51 embryos) and 5-6 somite (I-N; 20/28 embryos) stages. (B-G) and (I-N) are examples of injected embryos with varying phenotypes. (A & H) 2- and 5-somite stage embryos, respectively, injected with the control morpholino.

most morphants having a reduction in the number of neurons rather than being completely absent.

In a number of *zBTBD6a* MO-injected embryos sensory neurons appeared to be more affected than motor neurons at 2-3 somite stage, while at 5-6 somite stage there was a more severe reduction of motor neurons than sensory neurons. This could suggest a differential effect of zBTBD6a loss on neuronal populations, however no clear quantitative evidence is available at present to confirm it.

Loss of zBTBD6a results in a decrease of *neurod* expressing cells

To ascertain whether zBTBD6a activity is required at earlier stages of neuronal differentation, the effect of loss of zBTBD6a on *neurod* expression was examined (Fig. 44A-L). In 2-3-somite *zBTBD6a* MO embryos, the number of *neurod*-expressing cells is strongly diminished (Fig. 44B-E), compared with the control (Fig. 44A). Similary, at the 5-6-somite stage, a reduction of *neurod*-positive cells in the morphants is observed but to a lesser degree (Fig. 44; compare F with G-L).

Loss of zBTBD6a causes a decrease of *deltaB* (*dlb*)-expressing cells

To test whether the *zBTBD6a* knockdown affects *dlb* expression, *zBTBD6a* MO-injected embryos, were analysed at the 2-3- and 5-6-somite stages (Fig. 45). At both developmental stages, the morphants showed mild reduction of *dlb* expression compared with the control embryos, which was due to the decline of a number of *dlb*-positive cells rather than to the overall levels of *dlb* expression being decreased (see higher magnification; Fig. 45F, G). In addition, at 5-6-somite stage *zBTBD6a* MO-injected embryos exhibited a slight reduction of *dlb* expression in the forebrain region, where *zBTBD6a* is not normally expressed. This could suggest an indirect effect of zBTBD6a loss on *dlb* expression in the forebrain.

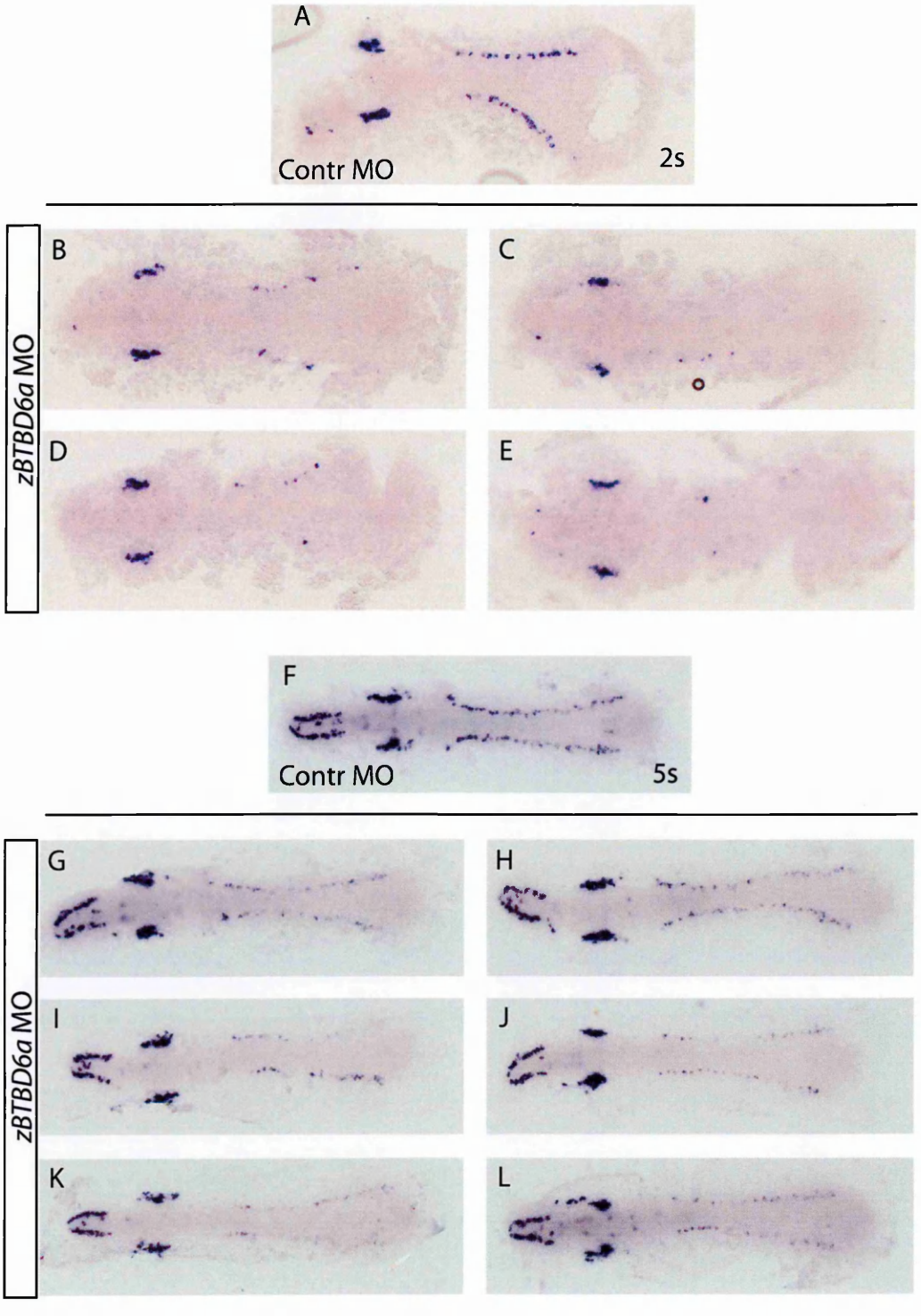


Figure 44. Effect of loss of zBTBD6a on *neurod* expression. *zBTBD6a* knock-down results in reduction of *neurod*-expressing cells in 2-3-somite (B-E; 21/23 embryos) and 5-6-somite (G-L; 26/31) stage embryos. (A & F) 2- and 5-somite stage embryos, respectively, injected with the control morpholino.

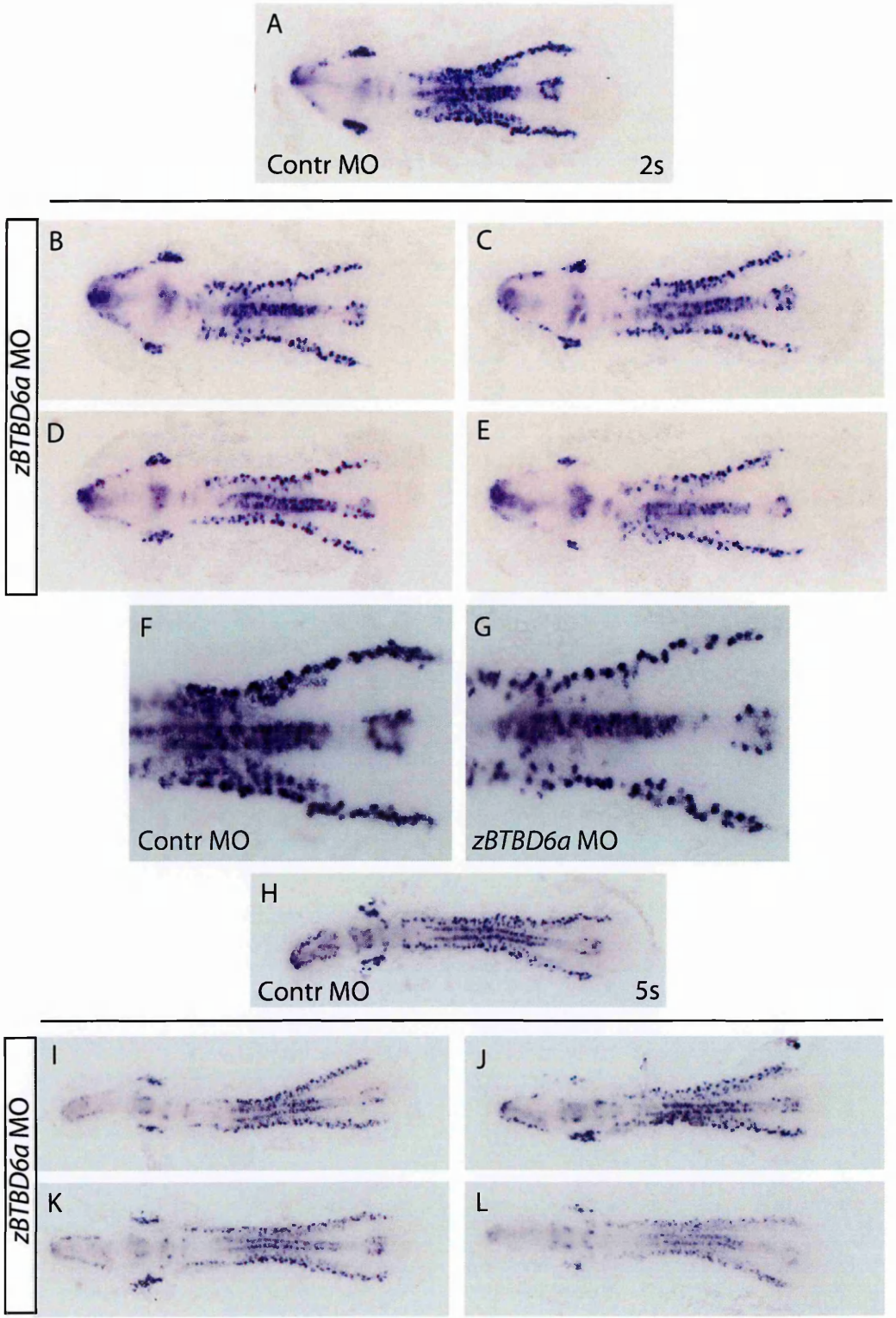


Figure 45. Effect of zBTBD6a knockdown on *deltaB* expression. Loss of *zBTBD6a* results in decreased *deltaB* expression in 2-3-somite (A-G; 15/18) and 5-6-somite (H-L; 20/25) stage embryos. (A &H) 2- and 5-somite stage embryos, respectively, injected with the control morpholino. (F & G) Higher magnification of the posterior neural plate of the control and *zBTBD6a* MO-injected embryos, respectively, showing that the number of *deltaB*-expressing cells is decreased in the morphant embryos.

Blocking of *zBTBD6a* translation results in reduction of *neurod4* expression

I next tested whether *neurod4* expression is dependent on zBTBD6a (Fig. 46A-L). The *zBTBD6a* morphants were analysed at the 2-3- (Fig. 46A-G) and 5-6- (Fig. 46H-L) somite stages. This analysis revealed a mild reduction of *neurod4* expression at the 5-6 somite stage, which was more pronounced in the 2-3-somite embryos. The observed decrease of *neurod4* signal was due to both a decrease in the number of *neurod4*-positive cells and a reduction of the expression levels, as shown in the higher magnification views of the posterior neural plate (Fig. 46F, G).

zBTBD6a* knockdown does not affect expression of *neurog1* and *dla

Experiments testing the effect of MO-induced *zBTBD6a* inactivation on the expression of genes involved in selection/determination of neuronal progenitors, *neurog1* (Fig. 47) and *dla* (data not shown), revealed no change to their expression patterns.

Taken together, the data obtained in the *zBTBD6a* knockdown experiments showed that *zBTBD6a* is required for neurogenesis to progress at the normal rate and extend. Loss of zBTBD6a does not affect expression of the earliest neuronal markers involved in neuronal determination/selection, *neurog1* and *dla* and, but negatively affects expression of early postmitotic markers, *neurod4*, *neurod* and *dlb* and consequently the neuronal differentiation marker, *isl1*. These results indicate that *zBTBD6a* is involved in the process of neuronal differentiation subsequent to the selection of neuronal progenitors.

However, to further validate the above results, rescue experiments in which zBTBD6a is overexpressed in the *zBTBD6a* MO-injected embryos should also be performed. Rescue of the phenotype resulting in normal neurogenesis would confirm a specific effect of the MO oligonucleotides on blocking *zBTBD6a* translation and *zBTBD6a* role in neuronal differentiation.

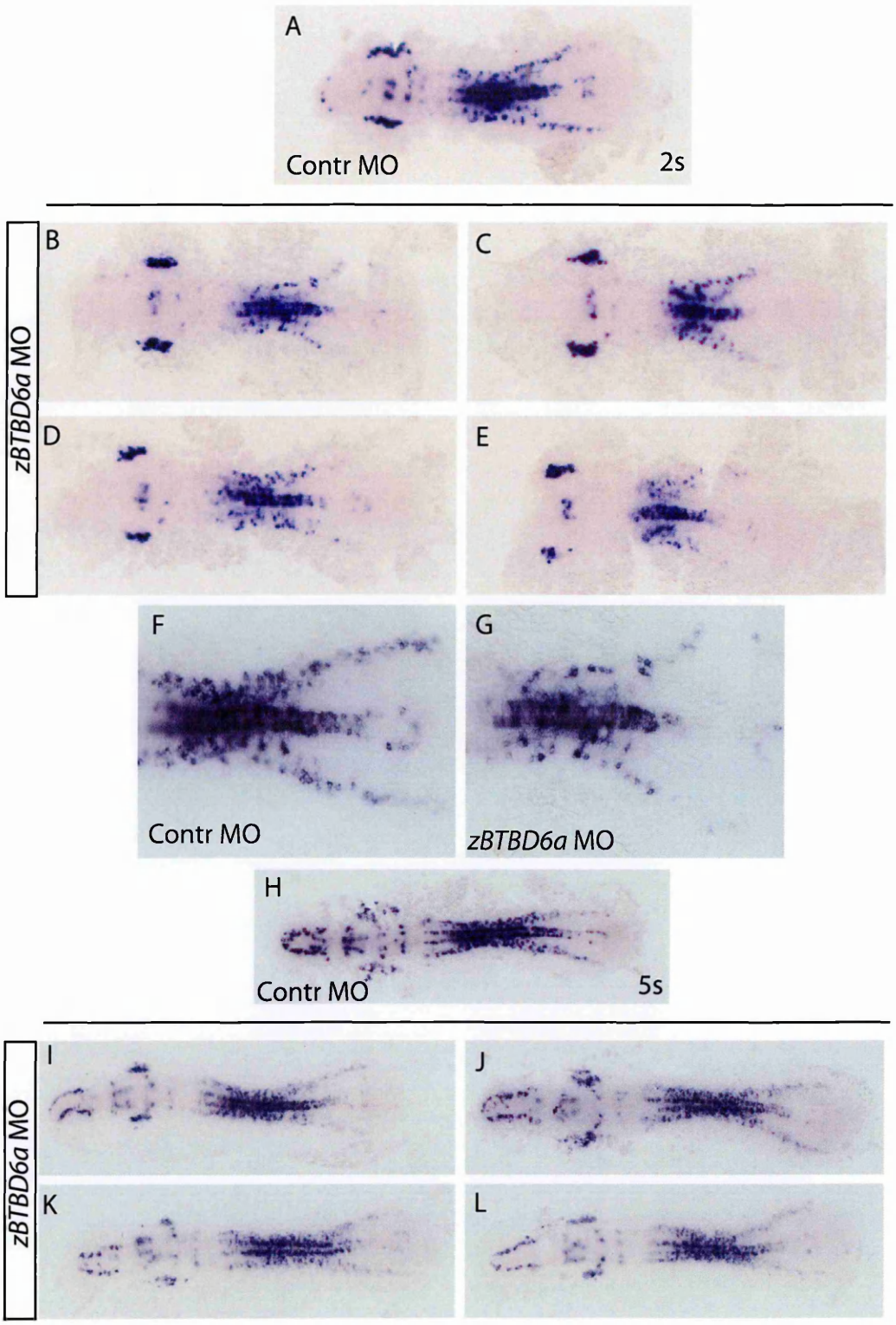


Figure 46. Effect of loss of zBTBD6a on *neurod4* expression. Expression of *neurod4* is reduced in *zBTBD6a* MO-injected embryos at both the 2-3-somite (B-G; 22/25) and 5-6-somite (I-L; 17/20) stages. (A &H) 2- and 5-somite stage embryos, respectively, injected with the control morpholino. (F & G) Higher magnification of the posterior neural plate of the control and *zBTBD6a* MO-injected embryos, respectively, showing that the level of *neurod4* expression and the number of neurod4-expressing cells are decreased in the morphant embryos.

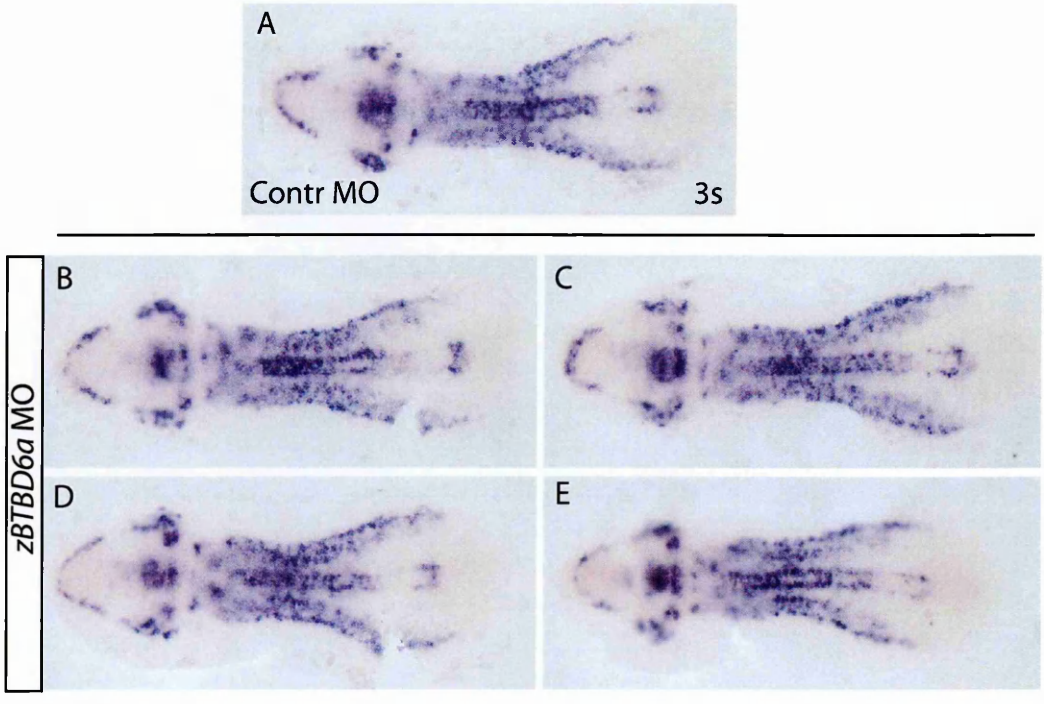


Figure 47. Effect of *zBTBD6a* knockdown on *neurog1* expression. No change to *neurog1* expression was observed in 2-3-somite *zBTBD6a* MO-injected embryos (B-E; 28/31) compared with the control morpholino-injected embryos (A).

Alternatively, splice-blocking MO oligonucleotides that span the exon/intron junction and inhibit pre-mRNA splicing could be used to inactivate *zBTBD6a* (Draper et al., 2001).

Second splice variant of *zBTBD6a* transcript

zBTBD6a knockdown causes a reduction in differentiated primary neurons, but the resulted phenotype is mild. One possible explanation for this is the presence of another molecule that functions redundantly with zBTBD6a. While this project was well under way, further DNA sequence database searches revealed the presence of a second splice variant of *zBTBD6a* transcript. According to the computer analysis provided by the Ensembl zebrafish sequence database, it is predicted that the cDNA of the first described *zBTBD6a* splice variant, now called *zBTBD6a1,* contains 4 exons, 1-4 (Fig. 48A). Based on the sequencing results of the corresponding ESTs, GenBank Acc. Nos BG308275 and XP682998, exon 1 includes a 5′ end UTR and the initiation translation codon located approximately half way through the exon. The exact beginning of this exon is not known as no gene structure analysis was performed and the data obtained from Ensembl is currently incomplete in this regard.

The other *zBTBD6a* splice variant, designated *zBTBD6a2,* contains 5 exons, 0-4 (Fig. 48B), with last three exons (2-4) being identical to *zBTBD6a1,* and exon 1 being shorter at the 5′ end but the remaining part is identical to its counterpart in *zBTBD6a1*. The additional exon 0 contains a 5′ UTR and a potential translation initiation codon located at the beginning of a truncated exon 1; this was confirmed by sequencing of the corresponding two *zBTBD6a2* EST clones (GenBank Acc. Nos. BM025225 and BM023959). Since the *zBTBD6a2* initiation ATG is in frame with the *zBTBD6a1* ATG, the amino acid sequence of a putative protein encoded by the *zBTBD6a2* isoform, is identical to zBTBD6a1, but shorter at the N-terminus by 56 amino acids (Fig. 48C). All three structural motifs - BTB, BACK and PHR - are present in the predicted zBTBD6a2 polypeptide.

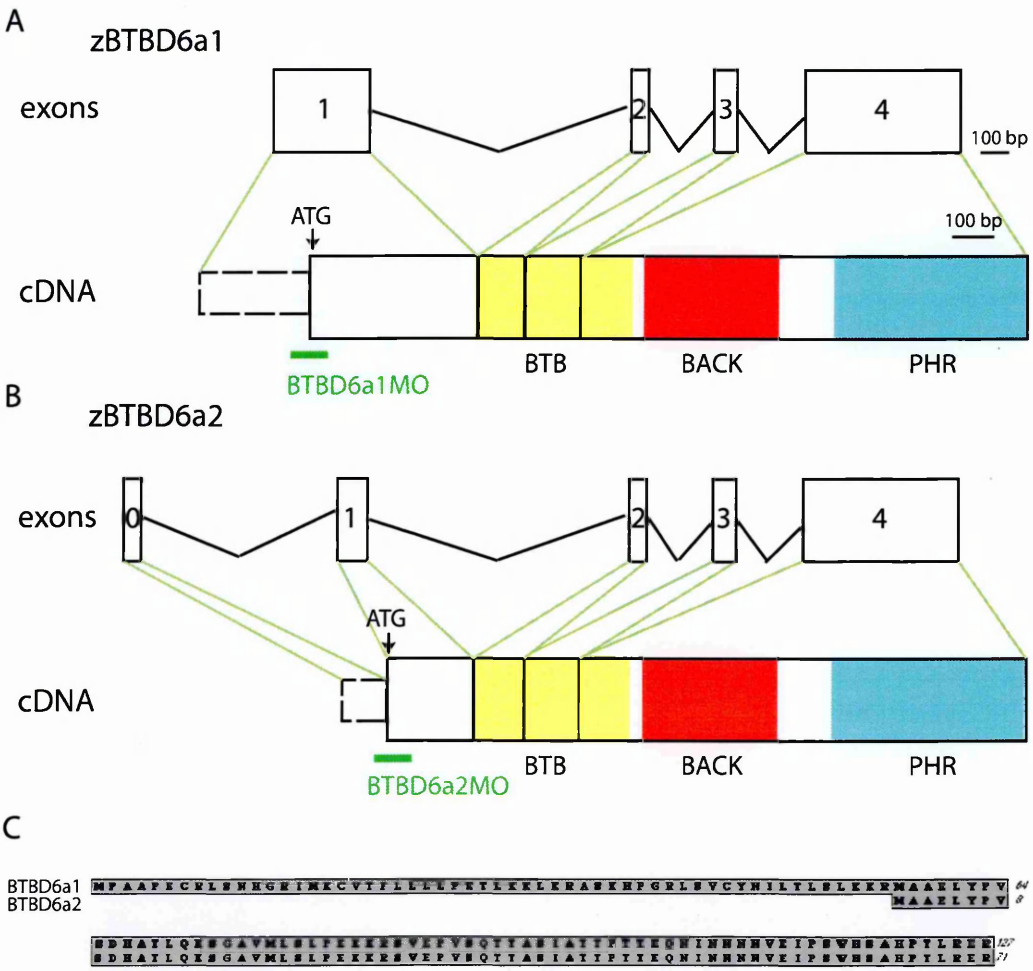


Figure 48. Two splice variants of *zBTBD6a* transcript. (A, B) Intron-exon organisation of *zBTBD6a1* and *zBTBD6a2*, respectively. Exons are indicated as boxes, and intronic sequences as thin lines. The dashed line boxes illustrate the 5′ UTR. The initiation of translation codon ATG is indicated with arrows. The positions of the encoded protein domains (BTB, BACK and PHR) with respect to the exon boundaries, are indicated. The two morpholino oligonucleotides, designed against zBTBD6a1 and zBTBD6a2, are indicated in green. (C) An alignment of the N-terminus of zBTBD6a1 and zBTBD6a2a predicted proteins. As the result of alternative splicing, the first 56 amino acids present in the zBTBD6a1 protein are missing in the zBTBD6a2 variant.

The MO oligonucleotides used in the previous experiments target only one of the *zBTBD6a* isomers, potentially leaving the product of the other one functional (Fig. 48A). If proteins encoded by *zBTBD6a* splice variants both act during zebrafish neurogenesis that could explain why the obtained phenotypes were mild.

Expression of zBTBD6a splice variants during zebrafish development

The spatio-temporal expression profile of *zBTBD6a* described in the previous chapter was obtained by in situ hybridisation utilising the RNA probe represented by BG308275 EST (the whole 2358 bp long *zBTBD6a* cDNA clone). Since the nucleotide sequences of both *zBTBD6a1* and *zBTBD6a2* isomeres are identical over the 1924 bp long 3′ end region, the probe detects the sum of both *zBTBD6a* splice variants. To discriminate between these two splice variants, RNA probes complementary to the nucleotide sequences unique for each of the *zBTBD6a* isomeres were generated and used for in situ hybridisation. These sequences encompass the 360 bp long 5′ end region of exon 1 specific for *zBTBD6a1,* and 100 bp long exon 0 specific for *zBTBD6a*2a. The sensitivity of both RNA probes in detecting specific signal was poor, most probably due to the probes being short. The in situ hybridisation performed with the *zBTBD6a1* RNA probe provided barely satisfactory results, but no reliable signal was obtained with the probe specific for *zBTBD6a2*. Nevertheless, the expression pattern acquired for the *zBTBD6a1* splice variant appears to be similar to the *zBTBD6a* expression profile described in the previous chapter (data not shown).

To examine whether both splice variants are expressed at the time of early neurogenesis, analysis of their temporal expression profiles was performed using RT-PCR. The PCR reaction was carried out on cDNA from pools of embryos ranging between 1000 (1K)-cell and 9-somites with two different pairs of primers specific for each *zBTBD6a* isomer. The results with one set of primers producing 334 bp and 100 bp bands specific for *zBTBD6a1* and *zBTBD6a2,* respectively, are presented (Fig. 49); similar results were obtained

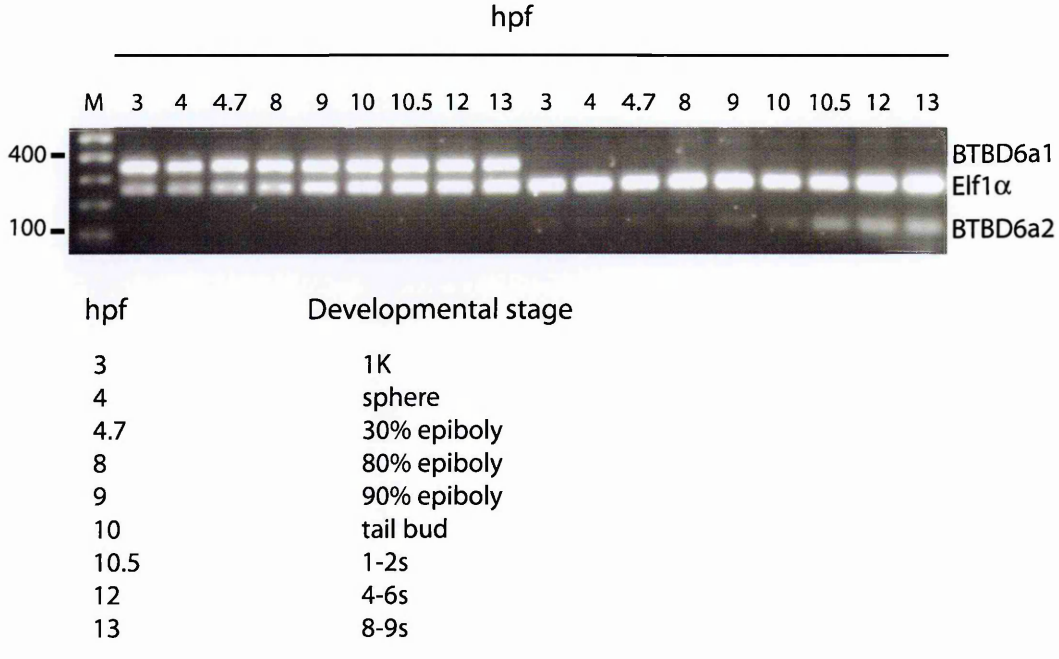


Figure 49. Analysis of the expression levels of the splice variants *zBTBD6a1* and *zBTBD6a2* during early stages of zebrafish development. Total RNA extracted from pools of individuals, of the indicated developmental stages, were subjected to RT-PCR. Reverse transcription was carried out with random hexamers, and pairs of primers were used for amplification of the two splice variants. Amplification of Elongation factor 1α (Elf1α) served as a control for cDNA synthesis and loading. Lane M contains a 100-bp ladder molecular size marker. hpf, hours post-fertilisation.

with the other primer pair (data not shown). These results show that while *zBTBD6a1* is detectable throughout the whole period of development examined, *zBTBD6a2* transcripts appear at low levels at 80% epiboly and increase in level until the 9-somite stage.

These data indicate that both genes are transcribed when early neurogenesis is taking place and in the time window analysed in this study. In addition, there is a possibility that *zBTBD6a1* is maternally expressed. *zBTBD6a1* transcripts are already detected at 1K-cell stage, the time point in development when the midblastula transition (MBT) commences, marking the time of activation of zygotic genome (Dosch et al., 2004; Kane and Kimmel, 1993; Newport and Kirschner, 1982; Pelegri, 2003). In zebrafish embryos, this transition occurs gradually and throughout a period of approximately 2 hours, therefore it is likely that the *zBTBD6a1* transcript detected at 1K-cell stage, is of maternal origin. Analysis of earlier developmental stages would clarify this issue.

Effect of zBTBD6a2 ablation on primary neuronal differentiation

The oligonucleotides used in the previously described knockdown experiments target the translation initiation of *zBTBD6a1* mRNA, but the translation of *zBTBD6a2* would remain intact (Fig. 48A). To elucidate the effects of *zBTBD6a2* inactivation on neuronal differentiation, an antisense MO against the *zBTBD6a2* ATG region was designed (Fig. 48B) and injected into embryos. The outcome was analysed by in situ hybridisation to detect *isl1* in 3-4-somite stage embryos.

Initial experiments carried out with the same dose of MO oligonucleotides as for *zBTBD6a1* knockdowns (6.5ng), produced developmental arrest of the injected embryos. The use of a lower dose (3.2ng) resulted in 90% of morphants arrested at the 80-90% epiboly stage. Further reducing the amount of MO oligonucleotides to 1.6ng was required to obtain viable embryos. The phenotype observed in embryos injected with 1.6ng of

zBTBD6a2 MO included reduction (Fig. 50C) or total loss (Fig. D, E) of primary neurons, marked by *isl1* expression (Quantification in Table 1). Occasionally, an increase of *isl1* expression in trigeminal ganglia was also observed (arrow, Fig. 50B; Table 1). There were also some morphological alterations accompanying the effects on the primary neurogenesis. About half (23% of the total number; see Table 1) of the embryos with total absence of differentiated neurons, were shorter along the AP axis (Fig. 50D). In some embryos (15%) the neural plate appeared slightly expanded (Fig. 50C). These data indicate that *zBTBD6a2* is required for primary neuronal differentiation to occur.

Synergistic effects of zBTBD6a1MO and zBTBD6a2MO on primary neurogenesis

To determine whether *zBTBD6a1* and *zBTBD6a2* have overlapping roles in the process of primary neurogenesis, injections of individual or combined oligonucleotides against both splice variants were performed and the embryos were analysed using *isl1* as a marker. The MO doses were selected based on the results of the pilot experiments, which when injected separately, elicited very mild phenotype, but when co-injected, still did not cause any toxic effect.

Injection of 3.2ng of zBTBD6a1MO (6.5ng caused an obvious phenotype) elicited a slight decrease in the number of *isl1*-expressing cells in 13% of embryos and the remaining embryos did not display any phenotype (Fig. 51B; Table 1). Embryos injected with 0.8ng of zBTBD6a2MO (1.6ng elicited severe phenotype; Table 1) showed a weak phenotype, in which 49% have a mild reduction in the number of *isl1*-positive cells, 7% have a total loss of *isl1* expression and 12% have increased *isl1* expression in the trigeminal ganglia (Fig. 51; Table 1). In contrast, when these amounts of zBTBD6a1MO and zBTBD6a2MO were injected together, the morphants showed a much more severe depletion or complete loss of differentiated neurons. These amounted

Figure 50. Effect of the *zBTBD6a2* ablation on zebrafish primary neurogenesis. Embryos injected with antisense morpholino oligonucleotides against *zBTBD6a2* were subjected to in situ hybridisation for *isl1*. (A) Anterior (Ant), posterior (Pos) and lateral (Lat) views of a 3-somite stage embryo injected with the control morpholino. (B, C, D, E) Corresponding anterior, posterior and lateral views of four 3-4 somite stage morphant embryos showing a range of phenotypes resulting from the knockdown of *zBTBD6a2*.

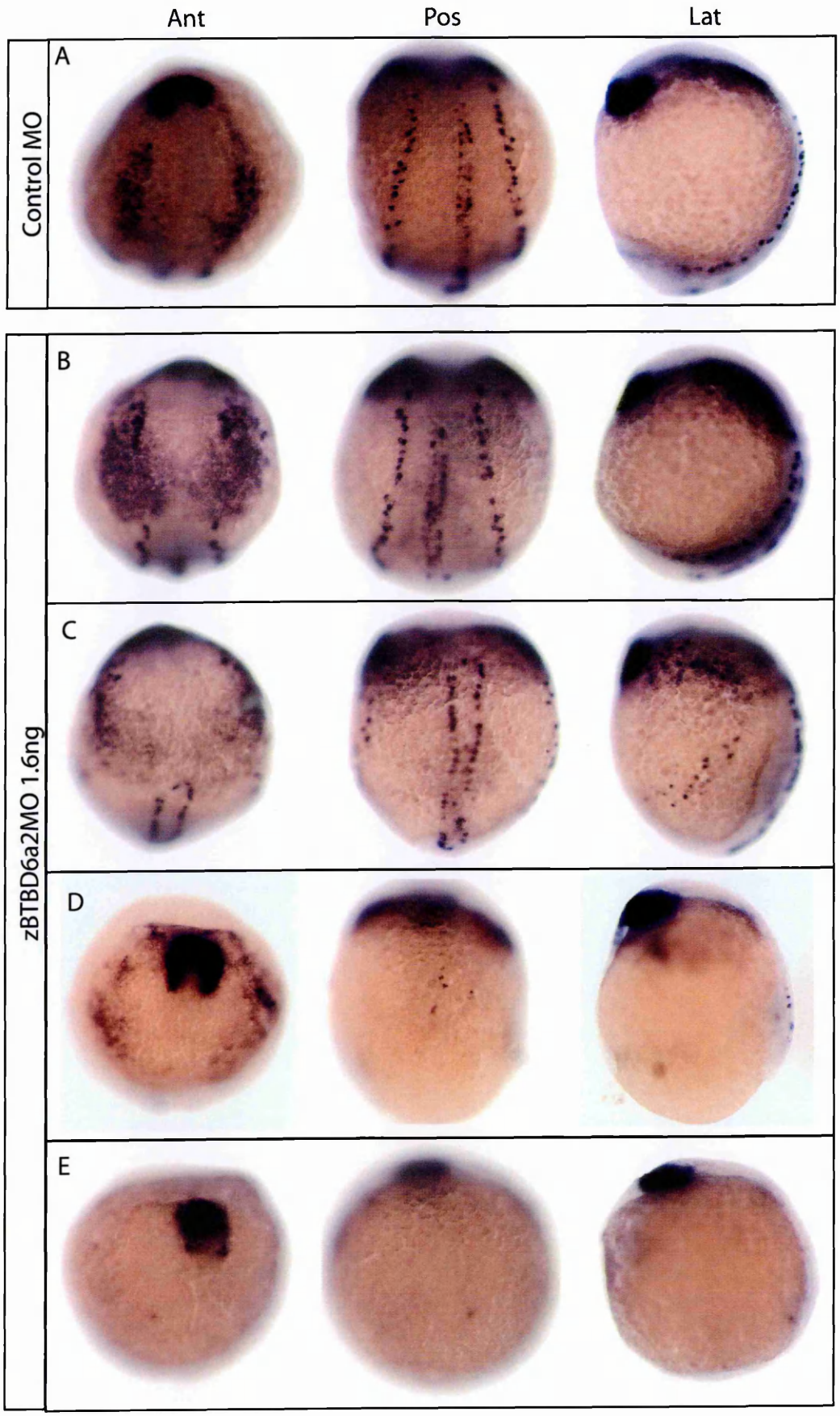
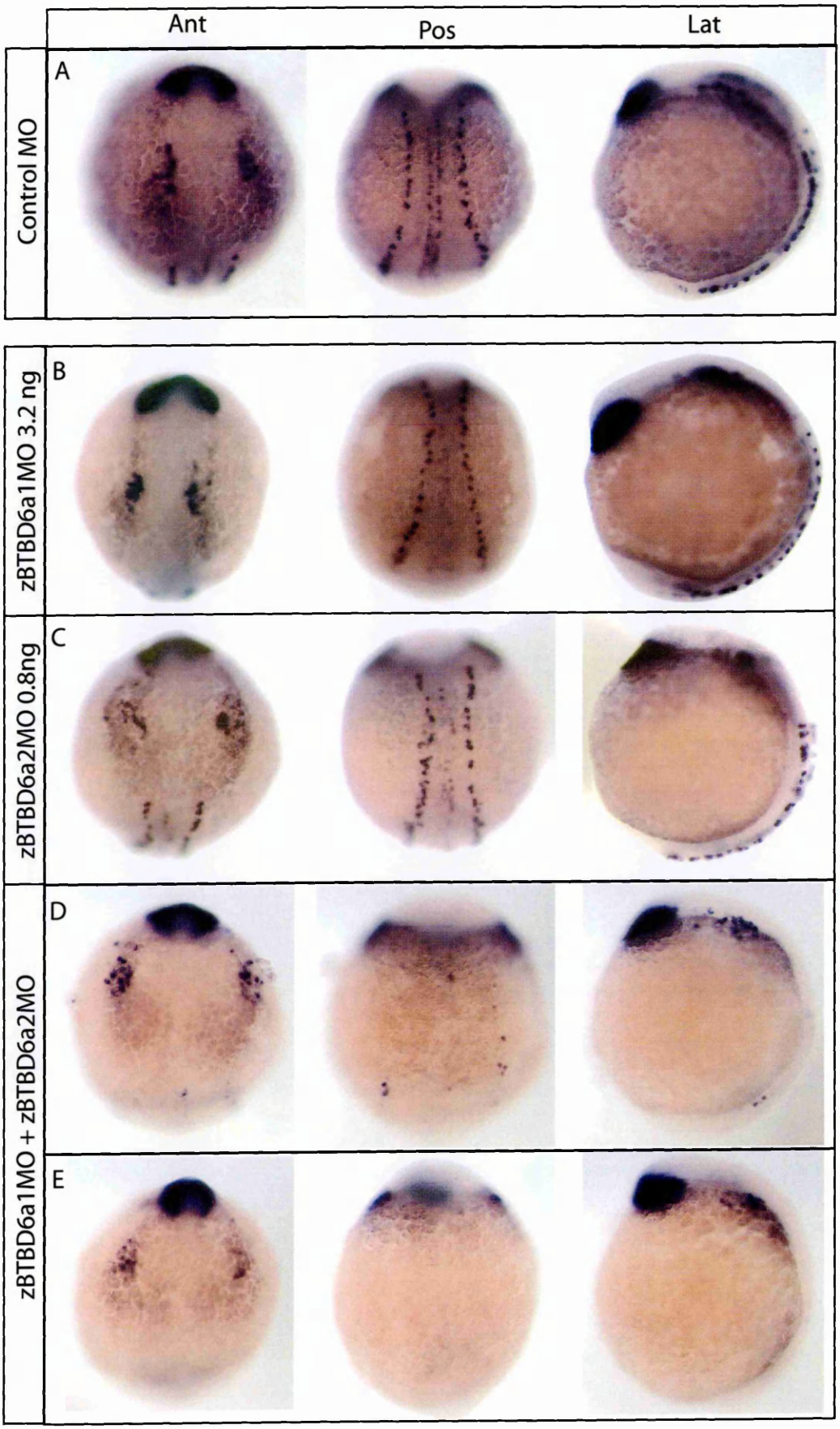

Ant
Pos
Lat
Control MO
A
zBTBD6a2MO 1.6ng
B
C
D
E

Figure 51. Cumulative effect of *zBTBD6a1* and *zBTBD6a2* knockdowns on zebrafish primary neurogenesis. Morpholino-injected embryos were subjected to in situ hybridisation for *isl1*. (A, A', A'') Anterior (Ant), posterior (Pos) and lateral (Lat) views of a 4-somite stage embryo injected with the control morpholino. (B-B'') 4-somite stage embryo injected with 3.2ng of BTBD6a1MO. (C-C'') Embryo injected with 0.8ng of BTBD6a2MO. (D-D'', E-E'') Examples of two morphant embryos resulting from the combined injection of both morpholinos against *zBTBD6a1* and *zBTBD6a2*.

	No. (%) of embryos with:			
MO injection	no *isl-1*epression in the n.plate	reduced *isl-1* expr. in the n. pl.	increased *isl-1* expr. in tgm g.	wild type *isl-1* expression
BTBD6a1 (3.2ng, *n*=45)	0	6 (13)	0	39 (87)
BTBD6a2 (1.6ng, *n*=50)	23 (46)	17 (34)	12 (24)	10 (20)
BTBD6a2 (0.8ng, *n*=41)	3 (7)	20 (49)	5 (12)	13 (32)
BTBD6a1+2 (3.2ng+0.8ng, *n*=60)	53 (88)	7 (12)	0	0

n indicates the total number of embryos injected

Table 1. Quantification of the effect of morpholino-mediated knockdown of *zBTBD6a1* and *zBTBD6a2* on neuronal differentiation.


Ant
Pos
Lat
Control MO
A
zBTBD6a1MO 3.2 ng
B
zBTBD6a2MO 0.8ng
C
zBTBD6a1MO + zBTBD6a2MO
D
E

to 12% (Fig. 51D) and 88% (Fig. 51E) of morphants with strongly reduced or completely absent *isl1*-positive cells, respectively (Table 1).

These results show that the loss of both zBTBD6a1 and zBTBD6a2 has a cumulative effect in preventing primary neurogenesis. This suggests that the proteins encoded by the *zBTBD6a* splice variants have overlapping functions in promoting primary neuronal differentiation.

Ectopic zBTBD6a does not elicit increased neurogenesis

In order to test whether misexpression of *zBTBD6a* would elicit an increase in neuronal differentiation, zBTBD6a1 was overexpressed in zebrafish embryos and the outcome was analysed by in situ hybridisation for *isl1*. *zBTBD6a1*-Myc-tagged RNA was injected together with β-galactosidase RNA, as a marker for the presence of injected RNA. The injections were performed into 1 blastomere of 2-cell embryos, and the embryos with unilateral X-gal staining permitting comparison with the other control side were selected for further analysis. The generation of zBTBD6a protein in the injected embryos was confirmed by Western analysis (Fig. 52B). Misexpression of *zBTBD6a* did not produce any change in *isl1* expression on the injected side of the embryos, indicating that zBTBD6a overexpression is not sufficient to increase neuronal differentiation (Fig. 53).

The misexpression and subsequently described experiments involve only the *zBTBD6a1* isoform, as at the time they were carried out, the *zBTBD6a2* isoform had not yet been identified.

Ectopic expression of zBTBD6a deletion mutants elicits defects similar to those of *zBTBD6a* knockdowns

zBTBD6a encodes a protein characterised by the presence of three domains, BTB, BACK and PHR. As will be discussed later, these motifs suggest that BTBD6a may act as an adaptor protein that assembles a protein complex.

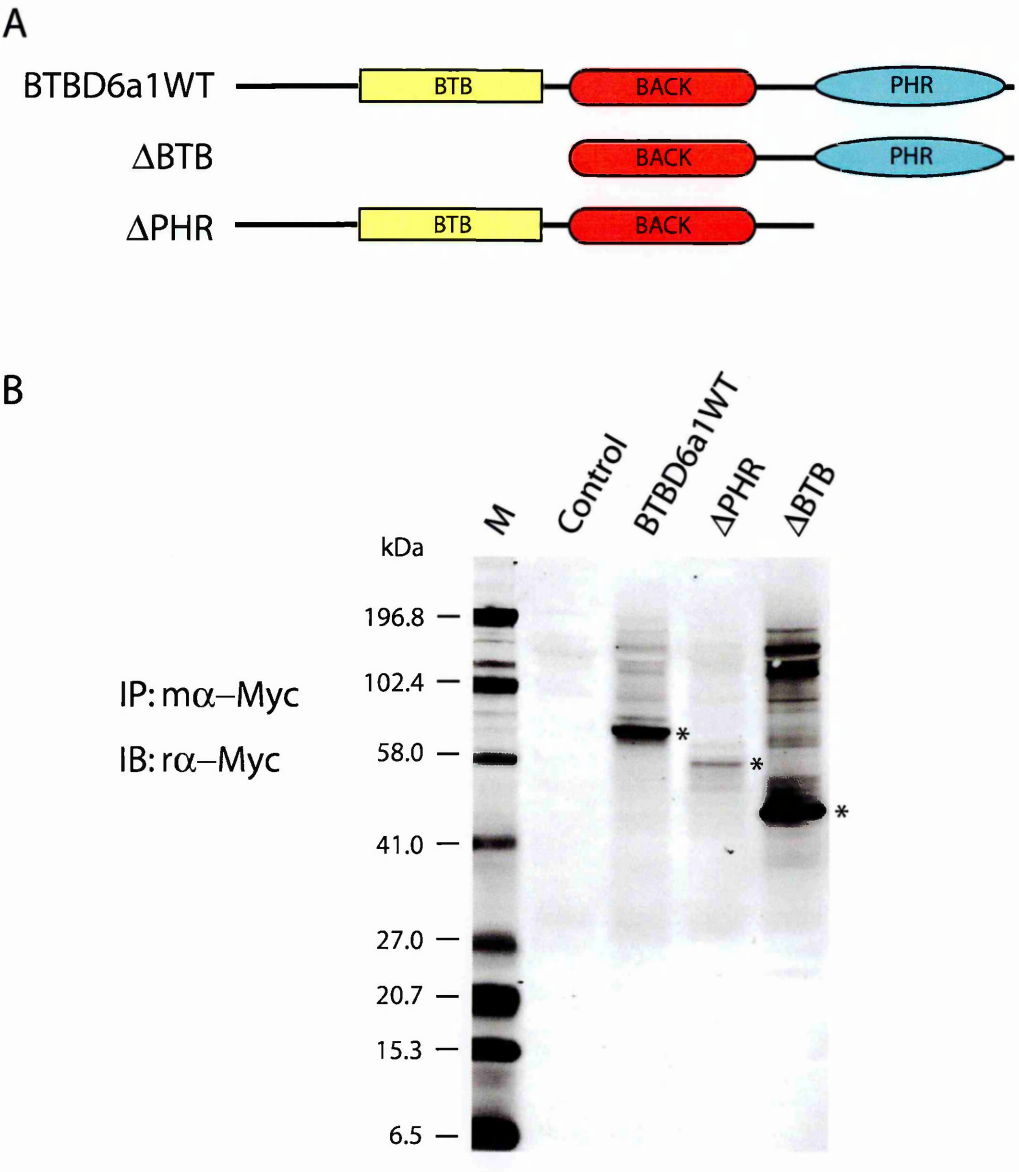


Figure 52. Construction and expression of wild type (WT) and mutant BTBD6a proteins. (A) Schematic illustration of constructs used for the generation of BTBD6a recombinant proteins in zebrafish embryos and HEK293 cells. (B) One-cell stage zebrafish embryos were injected with capped RNA encoding Myc-tagged WT or mutant BTBD6a, cultured until the 3-somite stage, lysed and the fusion protein detected by immunoprecipitation(IP) and Western blot analysis. The molecular weights of the BTBD6aWT, ΔPHR and ΔBTB recombinant proteins correspond to their predicted sizes based on conceptual translations of the open reading frames (asterix). IB, immunobloting; *a*-Myc, anti-Myc antibody.

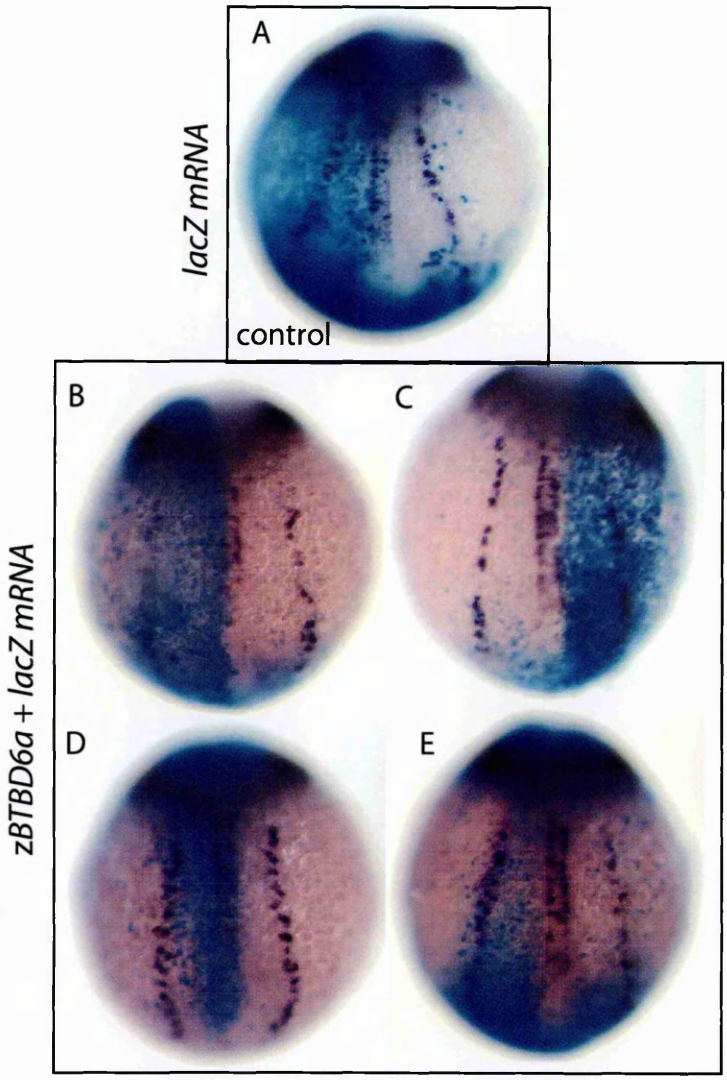


Figure 53. Effect of *zBTBD6a* misexpression on primary neurogenesis. Overexpression of the full-length zBTBD6a does not affect *isl1* expression (39/39 embryos). Embryos at the 2-cell stage were injected into one blastomere, either with *lacZ* RNA alone (A) or co-injected with *zBTBD6a WT* and *lacZ* RNA (B-E) and stained at the 3-4-somite stage for β-gal activity (turquoise) before in situ hybridisation with *isl1* antisense probe (dark blue).

This predicts that its function will be impaired by deletion of domains, and this may have a dominant negative effect. To gain insight into the requirements of these domains for *zBTBD6a* function, two deletion variants of the *zBTBD6a1* cDNA, ΔBTB and ΔPHR, lacking sequences encoding for BTB and PHR motifs, respectively, were constructed (Fig. 52A). The expression of mutant proteins in the injected embryos was confirmed by Western blot analysis (Fig. 52B). The mutant RNAs were co-injected with β-galactosidase RNA into 1-blastomere of 2-cell embryo and the effects on neuronal differentiation was monitored at 3-4-somite stage by *isl1* expression. The misexpression of either ΔBTB (Fig. 54) or ΔPHR (Fig. 55) has similar effects. Both result in a reduction in the number of *isl1*-expressing cells and/or a slight expansion of the neural plate on the injected side of the embryos. The severity of this phenotype depends on how far laterally the truncated zBTBD6a protein is expressed in relation to the neural plate, as marked by X-gal staining. In embryos where the mutant zBTBD6a protein extends more laterally, the decrease in the number of *isl1*-positive cells is more profound (arrow; Fig. 54D-G & Fig. 55E, F, H).

These defects are similar to those obtained in the experiments with MO-mediated zBTBD6a inactivation, indicating that removal of any of the domains blocks zBTBD6a activity. Therefore, the presence of both BTB and PHR domains is indispensable for zBTBD6a function in neuronal differentiation.

Subcellular localisation of zBTBD6a1

To shed some light on the potential molecular mode of zBTBD6a function, the subcellular localisation of the full-length and truncated forms of zBTBD6a protein was examined. To this end, stable HEK293 cell lines expressing Myc-tagged, wild-type (BTBD6a1WT), PHR domain-deleted (ΔPHR) and BTB domain-deleted (ΔBTB), zBTBD6a1 proteins, were established. The expression of the recombinant proteins was confirmed by Western blotting (Fig. 56A). Immunocytochemical staining with anti-Myc antibodies showed

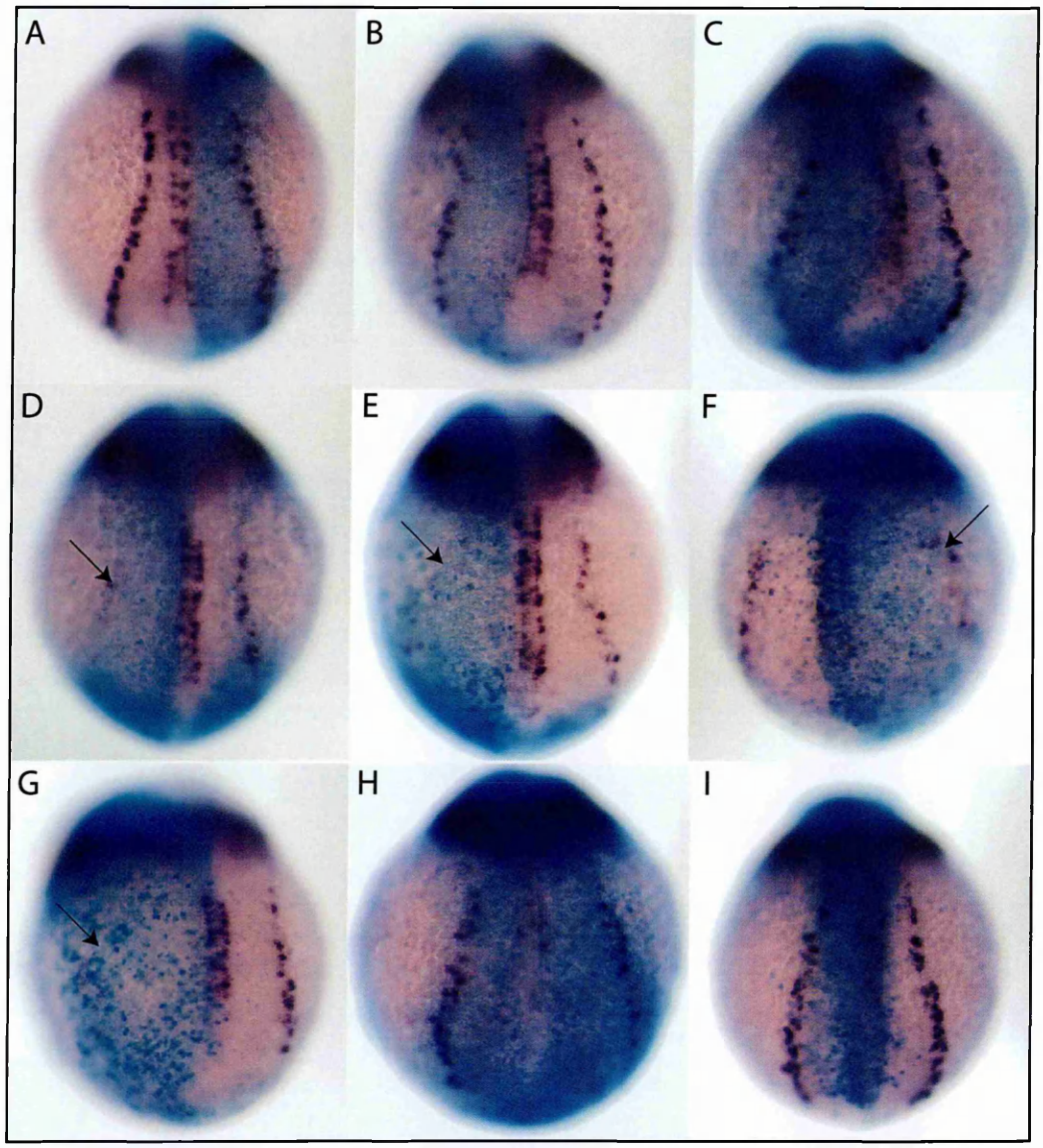


Figure 54. Effect of ΔBTB overexpression on primary neurogenesis. ΔBTB RNA was injected, together with *lacZ* RNA as a marker, into one blastomere of 2-cell embryos. Dorsal views of 3-4-somite stage embryos, with *isl1* expression in dark blue, and *lacZ* marker in turquoise; anterior to the top. The number of differentiated neurons, labelled by *isl1* is reduced on the injected side (59%; 24/41 embryos, A-I). This reduction is more severe where the overexpressed protein extends more laterally in the neural plate (arrow; D-G). A slight expansion of the neural plate on the affected side is also observed (60%; 25/41 embryos).

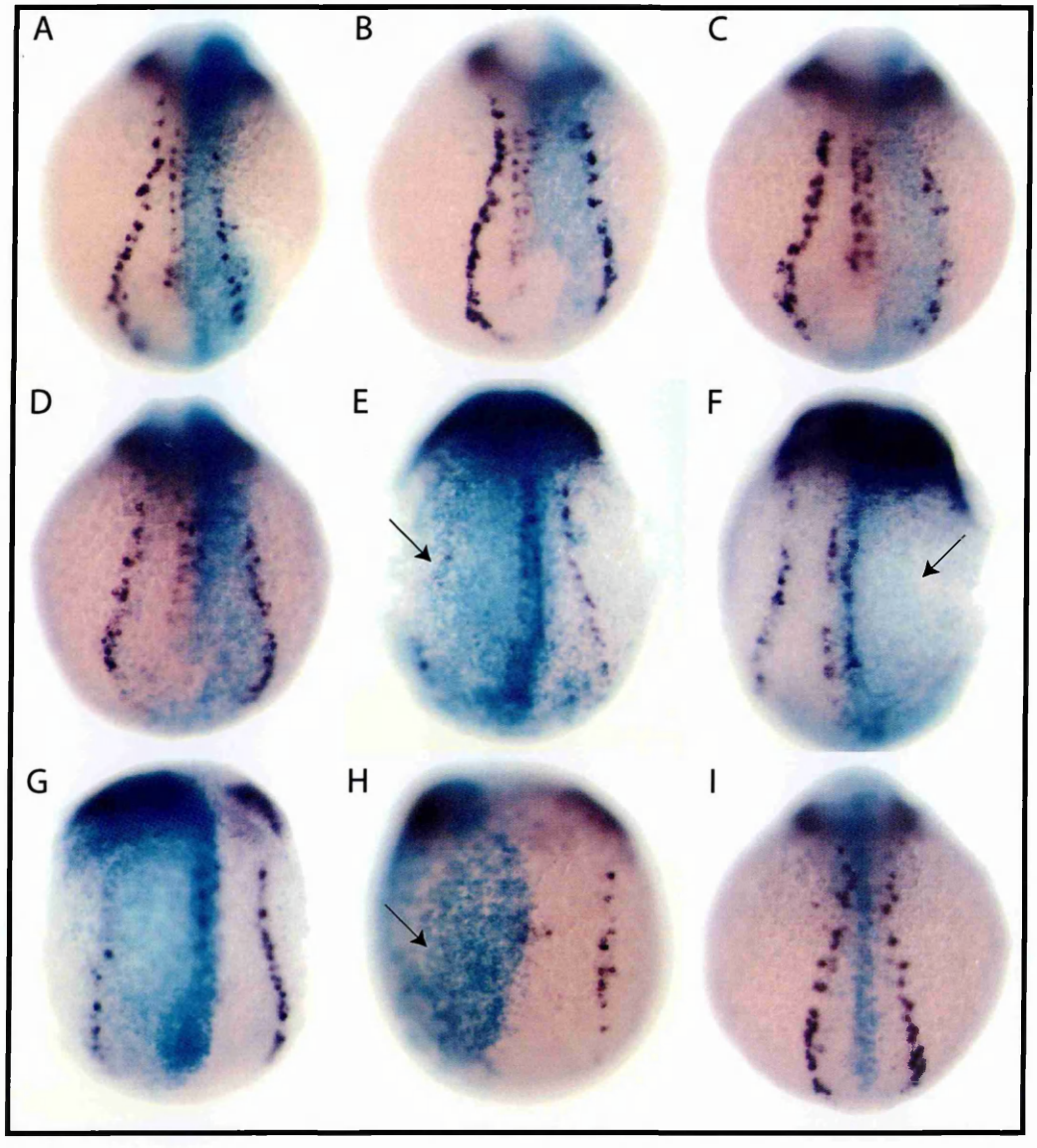
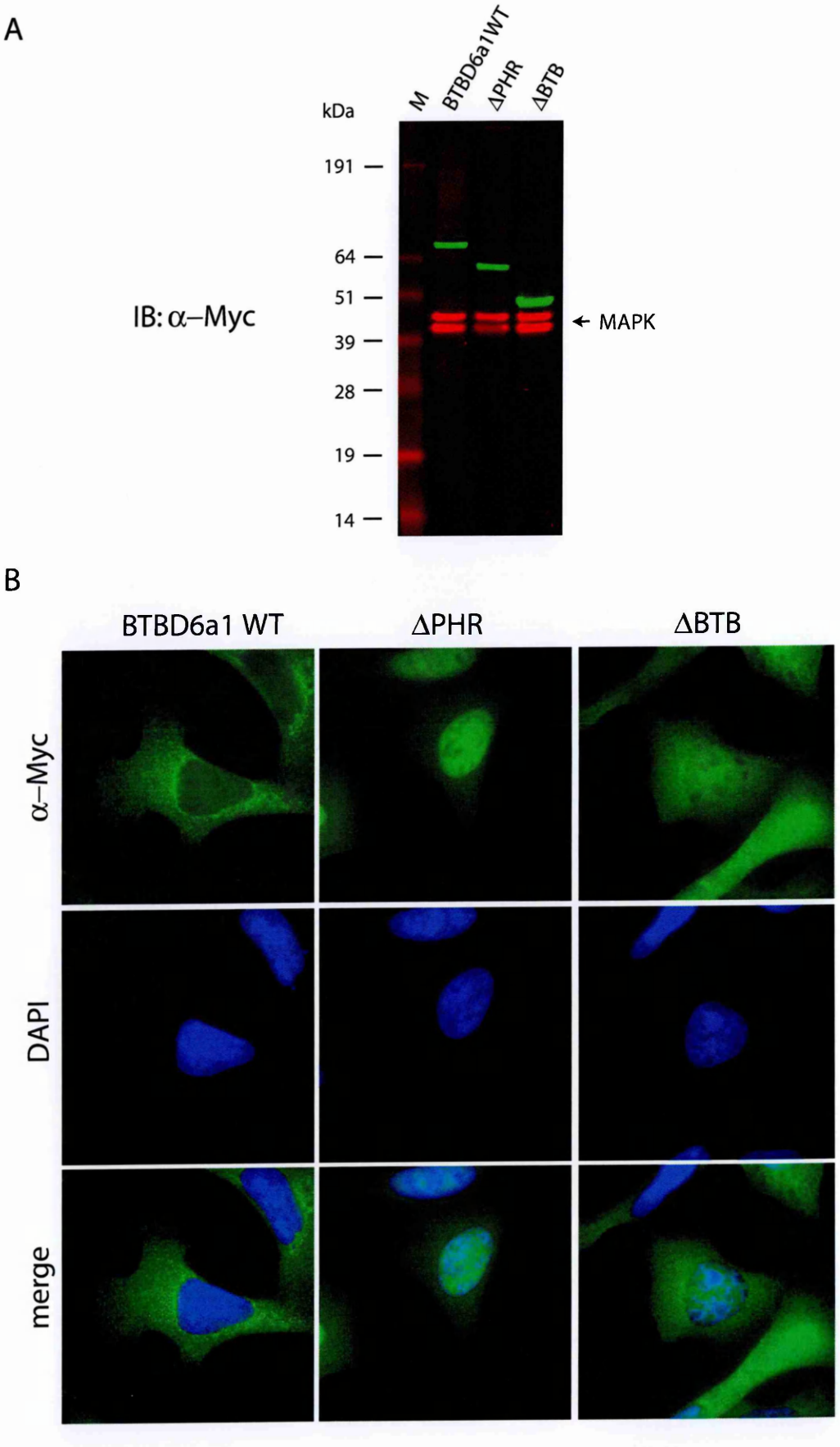


Figure 55. Effect of ΔPHR overexpression on primary neurogenesis. (A-I) Dorsal views of 3-4-somite stage embryos injected into one out of two blastomeres with ΔPHR RNA, together with *lacZ* RNA as a marker, and stained for β-galactosidase (turquoise) and *isl1* (dark blue); anterior to the top. The number of primary neurons marked by *isl1* expression is diminished on the injected side (63%; 17/27 embryos) and more severely decreased where the overexpressed protein extends more laterally in the neural plate (arrow; D-G). A slight expansion of the neural plate on the affected side is also observed (89%; 24/27 embryos).

Figure 56. Subcellular localisation of normal and truncated zBTBD6a1 recombinant proteins. (A) Detection of Myc-tagged wild-type (BTBD6a1WT), BTB domain-depleted (ΔBTB) and PHR domain-depleted (ΔPHR), BTBD6a1 proteins expressed in HEK293 stable cell lines. Total cell lysates were resolved by SDS-PAGE, followed by immunoblotting (IB) with anti-Myc (α-Myc) antibodies. MAPK in red is a loading control, while the relevant zBTBD6a1 proteins are labelled in green. (B) Subcellular localisation of BTBD6a1 WT and mutant proteins was detected immunohistochemically with anti-Myc antibodies (first row). Nuclei are shown with DAPI staining in the second row and merged views are shown in the third row.


A
M
BTBD6a1WT
ΔPHR
ΔBTB
kDa
191
64
51
39
28
19
14
IB: α–Myc
MAPK
B
BTBD6a1 WT
ΔPHR
ΔBTB
α–Myc
DAPI
merge

that BTBD6a1WT protein was localised in the cytoplasm, ΔPHR protein was found only in the nucleus, whereas ΔBTB protein was detected in both cytoplasm and nucleus (Fig. 56B, first row). The cytoplasmic expression of both BTBD6a1WT and ΔBTB proteins has a granulated appearance.

These data show that the subcellular localisation of the full-length zBTBD6a protein confined normally to cytoplasm, is altered with the removal of the BTB and PHR domains. These changes may reflect zBTBD6a function at the molecular level and may depend on other proteins that zBTBD6a associates with.

BTBD6a1 associates with Cul3 in vivo

Recent reports showed that a subset of BTB proteins are involved in the ubiquitination process and function as an adaptor for the Cul3-type E3 complex (Furukawa et al., 2003; Geyer et al., 2003; Kobayashi et al., 2004; Pintard et al., 2003; Xu et al., 2003a). To determine whether this is the case for zBTBD6a, the potential interaction of zBTBD6a1 with Cul3 in vivo was tested by immunoprecipitation analysis. Stable HEK293 cell lines expressing Myc-tagged full-length, BTBD6a1WT, and the truncated forms, ΔPHR and ΔBTB, were used for this purpose. Endogenous Cul3 was immunoprecipitated with an anti-Cul3 antibody and immunoblot analysis for zBTBD6a was performed with an anti-Myc antibody (Fig. 57). This analysis showed that BTBD6a1WT and to a much lesser degree ΔBTB were co-immunoprecipitated with Cul3, indicating that both, the wild-type and BTB-depleted zBTBD6a1 proteins, associate with Cul3.

These results indicate that zBTBD6a1 specifically interacts with Cul3 and this interaction is dependent on the presence of the PHR domain. The presence of BTB domain may be dispensable as the ΔBTB mutant co-immunoprecipitated, albeit to a low extend. Therefore, zBTBD6a1 may be involved in ubiquitination functioning as a substrate-specific adaptor for the Cul3-type E3 ubiquitin ligase.

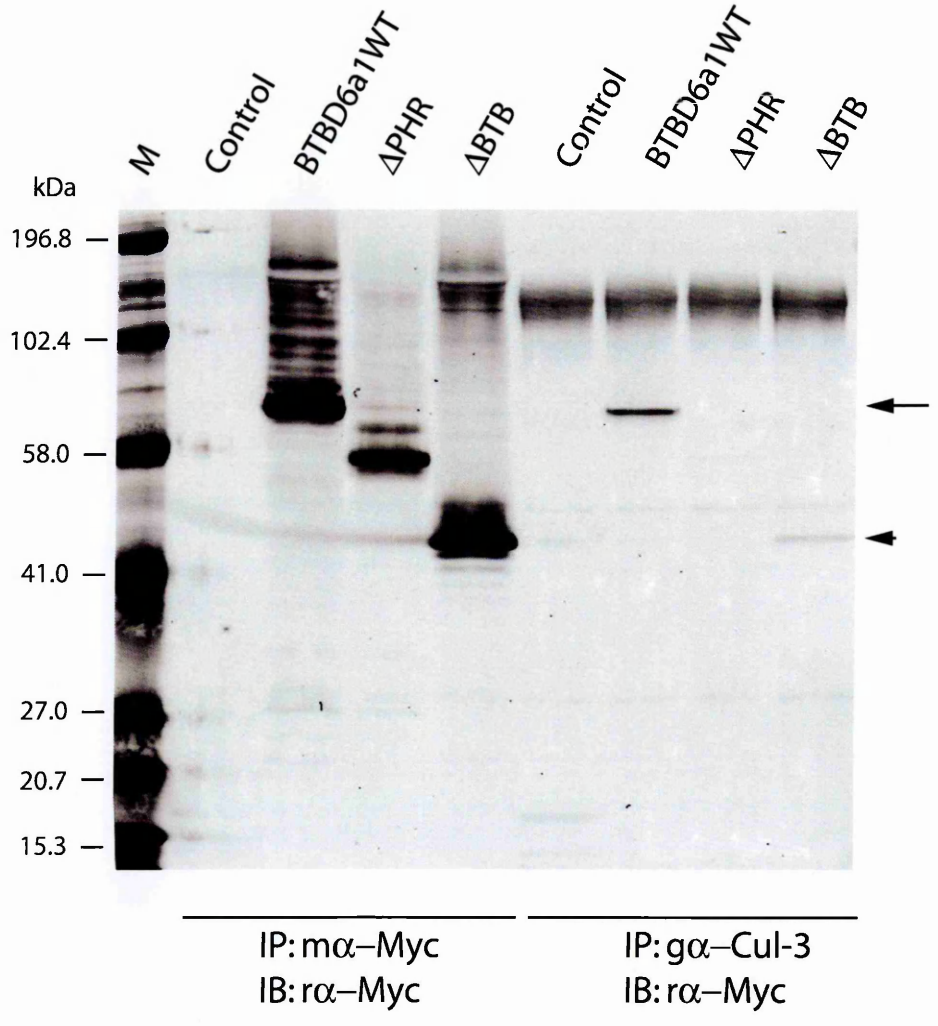


Figure 57. zBTBD6a associates with Cul-3. Ectopically expressed wild-type zBTBD6a1, but not PHR domain-deleted zBTBD6a1, interacts with endogenous Cul-3 in HEK293 cells. HEK293 cells, not transfected (Control) or expressing Myc-tagged wild-type (BTBD6a1WT), BTB domain-deleted (ΔBTB) or PHR domain-deleted (ΔPHR) zBTBD6a1 proteins, were lysed and the lysates were immunoprecipitated with anti-Myc (α-Myc, left panel) or anti-Cul-3 (α-Cul-3, right panel) antibodies. Generation of the correctly sized wild-type and truncated BTBD6a1 proteins (left panel) and complex formation between ectopically expressed proteins and endogenous Cul-3 (right panel), was checked by immunoblotting with anti-Myc antibodies. Cul-3 binds BTBD6a1WT (arrow) and to a lesser extent ΔBTB protein (arrowhead). g, goat; m, mouse; r, rabbit.

DISCUSSION

Based on the data presented in this section and on published literature, the following model for *zBTBD6a* role in zebrafish neurogenesis is proposed (Fig. 58). zBTBD6a acts downstream of *neurog1* and promotes neurogenesis by enabling degradation/deactivation of a factor that represses neurogenesis. This factor could be an Id, Sox or other protein that antagonises a transcription factor that promotes neuronal differentiation, such as *neurod4* or *neurod*. As will be discussed later, an alternative possibility is that zBTBD6a activates a transcription factor that promotes neurogenesis.

zBTBD6a1 expression and regulation

My studies have shown that *zBTBD6a* is expressed in the developing CNS. At early neurula stages, *zBTBD6a* transcripts are detected in the prospective hindbrain and in longitudinal domains in the posterior neural plate. This posterior *zBTBD6a* expression is similar to the expression pattern of the Notch ligand, *dla*, and overlaps with expression of the proneural gene, *neurog1*. Expression of *dla* and *neurog1* in the posterior neural plate prefigures primary sensory neurons, interneurons and motor neurons (Blader et al., 1997; Chapouton and Bally-Cuif, 2004; Haddon et al., 1998). Therefore, *zBTBD6a* expression marks the posterior neurogenic/proneuronal regions where primary neurogenesis takes place. The proneuronal domains are separated by inter-proneuronal domains which do not undergo neurogenesis while it is occurring in the proneuronal domains. It has recently been proposed that the position of proneuronal and inter-proneuronal regions is controlled by Hairy- and Enhancer of split E(spl)-related (Her) genes *her3* and *her9* that function as prepattern genes (Bae et al., 2005). These genes are expressed in the inter-proneuronal stripes where they actively inhibit neurogenesis. Combined knockdown of Her3 and Her9 induces expansion of the proneuronal domains of *neurog1*, *dla*, *neurod4* and *elav3* expression into the inter-proneuronal domains. *neurog1* and *zBTBD6a* are co-expressed in the posterior neural plate, and *neurog1*

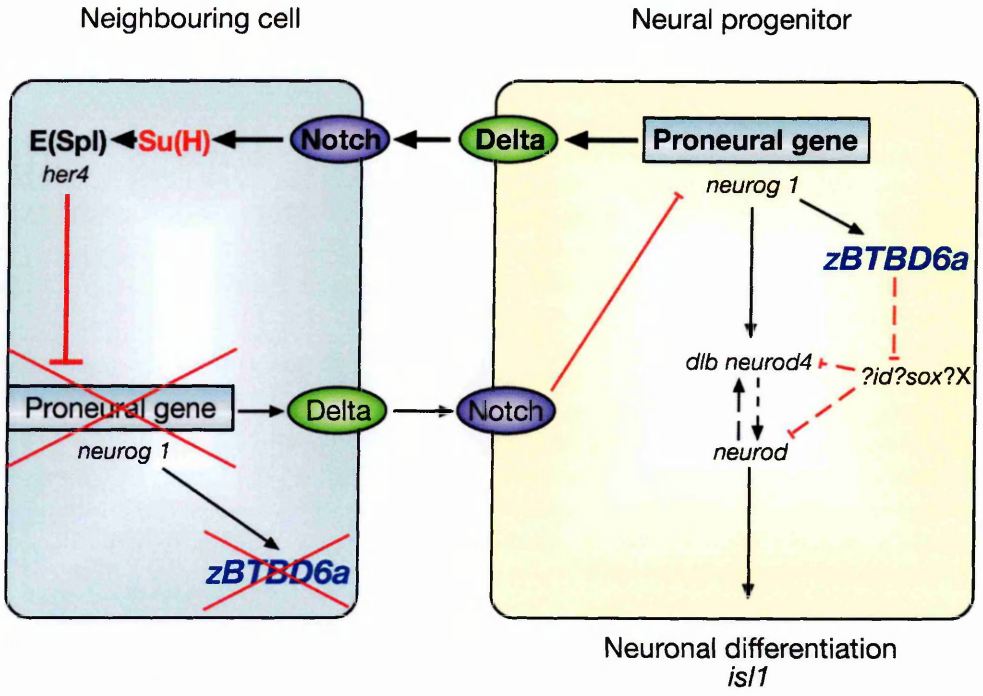


Figure 58. Working model for the role of zBTBD6a in zebrafish neurogenesis. Direct relationships are shown as solid lines, and unknown or indirect relationships as dashed lines. See the text for detailed descriptions.

misexpression and knockdown experiments demonstrated that *zBTBD6a* expression is regulated by Neurog1. Therefore, it is likely that the restriction of *zBTBD6a* expression to the neurogenic regions is spatially controlled by *her3* and *her9* via repression of *neurog1*.

Within the neurogenic regions, neurogenesis is initiated by high level expression of *neurog1* in a subset of progenitor cells (Bertrand et al., 2002; Blader et al., 1997; Chapouton and Bally-Cuif, 2004). The selection of progenitors relies on the process of lateral inhibition controlled by the Notch signalling pathway (Appel and Chitnis, 2002). Cells with high *neurog1* expression express the Notch ligands, e.g. Delta, which activate Notch signalling in the neighbouring cells (Appel and Eisen, 1998; Haddon et al., 1998). As a result of Notch receptor activation, these neighbouring cells express Her transcriptional repressors that, in turn, directly suppress *neurog1* expression (Takke et al., 1999). In this way *neurog1* restricts its own activity to single progenitor cells by inhibiting its expression in adjacent cells, thereby preventing these cells from differentiating. Thus, the initial uniform *neurog1* expression in proneuronal domains becomes restricted to single cells that enter a differentiation pathway. *zBTBD6a* expression in the posterior neural plate has a punctate appearance, similar to *neurog1*. Analysis of the relationship of *zBTBD6a* with Notch signalling suggests that *zBTBD6a* expression occurs in this pattern due to sensitivity to Notch-mediated lateral inhibition. The regulation of *zBTBD6a* expression by Notch signalling is executed through the activation of Notch target, E(spl) *her4* repressor which represses *neurog1* (Takke et al., 1999). As will be discussed below, *zBTBD6a* is downstream of *neurog1*, and this explains its regulation by Notch-mediated lateral inhibition. Since *zBTBD6a* knockdown does not affect *neurog1* or *dla* expression, it appears that *zBTBD6a* is not playing a direct role in the Notch regulatory feedback loop.

zBTBD6a1 expression during neuronal differentiation

Proneural genes promote neuronal differentiation by inducing a sequence of downstream regulatory genes implementing successive stages of neuronal-differentiation programme (Bertrand et al., 2002; Lee, 1997). They are the earliest markers of neurogenesis and are expressed transiently in the proliferating neural progenitors. In the vertebrate neural tube, proneural genes are downregulated before progenitor cells exit the proliferation zone and begin to differentiate (Gradwohl et al., 1996; Ma et al., 1996). *isl1* is one of the latest markers and is characteristic of a subset of terminally differentiated neurons (Inoue et al., 1994; Korzh et al., 1993). In the proneuronal domains of the neural plate, *zBTBD6a* expression is detected concomitantly with the proneural gene *neurog1* in neural cells that are at early phases of neurogenesis, as well as in differentiated neurons marked by *isl1*. *zBTBD6a* expression appears to be downregulated at later phases of neuronal differentiation, as some *isl1*-positive and *elavl3/HuC*-expressing neurons do not contain *zBTBD6a* transcripts. Analysis of the caudal spinal cord suggests that initiation of *zBTBD6a* expression in the neuronal progenitors precedes the onset of *elavl3* expression, concomitant with the withdrawal from the cell cycle, confirming that *zBTBD6a* expression accompanies early stages of neuronal differentiation.

Position of *zBTBD6a* in the neuronal differentiation gene hierarchy

As a step towards defining the role of *zBTBD6a* during neurogenesis, I have positioned *zBTBD6a* function in the gene cascade underlying neuronal differentiation. *zBTBD6a1* knockdown does not affect expression of *neurog1* or *dla*, early markers of neurogenesis which take part in the process of the determination and selection of neuronal progenitors. Moreover, *zBTBD6a1* expression is activated by Neurog1.

In contrast to the lack of effect on *neurog1* expression, *zBTBD6a1* knockdown leads to decreased expression of *neurod* and *neurod4*. *neurod* and *neurod4* are

bHLH genes that mark the transition between the proliferation and differentiation stages in the process of neurogenesis (Korzh et al., 1998; Park et al., 2003; Wang et al., 2003). Gain of function experiments in *Xenopus* embryos have revealed that *Ngnr1* (a Xenopus *neurog1* gene), *Xath3* (a *Xenopus neurod4* gene) and *NeuroD* (a *Xenopus neurod* gene) are expressed sequentially, and ectopic expression of *Ngnr1* induces the expression of both *Xath3* and *NeuroD*, whereas *Xath3* and *NeuroD* can cross-activate each other, but do not induce *Ngnr1* expression (Ma et al., 1996; Perron et al., 1999). Similarly, *NeuroM*, a *neurod4* homologue in chick, is transiently expressed in the spinal cord after *Neurogenin*, but before *NeuroD*, and labels differentiating neurons that have left the cell cycle but have not yet started migration to their final positions (Diez del Corral et al., 2002; Roztocil et al., 1997). The relative position of *neurod* and *neurod4* in the molecular hierarchy of neuronal differentiation in zebrafish has not been analysed. However, it is expected that there is the same epistatic relationship between these two genes, which places *zBTBD6a* upstream of *neurod4* followed by *neurod*.

The loss of zBTBD6a1 function also results in a reduction of the number of cells expressing *dlb*. This *delta* gene acts downstream of *neurog1* and marks committed progenitors after their withdrawal from the cell cycle (Haddon et al., 1998). However, the exact position of *dlb* in the neurogenesis cascade in relation to *neurod4* and *neurod* has not been established. The expression pattern of *dlb* in the early neurula embryo resembles the expression of *neurod4* rather than *neurod*, which appears to be expressed in a subpopulation of *dlb*- and *neurod4*-positive cells. Therefore, one would assume that *dlb* and *neurod4* mark similar stages of neuronal development and both act upstream of *neurod*. However, as discussed previously, the *zBTBD6a1* knockdown results need to be further confirmed with the rescue of the neurogenesis deficit in morphants by injecting *zBTBD6a1* RNA. This approach seems to be plausible, as zBTBD6a1 overexpression does not elicit any phenotype.

Although loss of zBTBD6a1 decreases, rather than completely inhibits neurogenesis, knockdown of both *zBTBD6a1* and *zBTBD6a2* splice variants almost completely prevents neuronal differentiation. Thus, the proteins encoded by both *zBTBD6a* isoforms appear to have overlapping functions and together are required for neural progenitors to undergo neuronal differentiation.

However, the relationships between the zBTBD6a1 and zBTBD6a2 proteins in their roles in neurogenesis are yet to be determined. Ablation of the *zBTBD6a2* splice variant alone results in a reduction or total absence of primary differentiated *isl1*-expressing neurons. It is not clear why the high level of *BTBD6a1* expression, as determined by RT-PCR, cannot compensate for the loss of BTBD6a2 in neurogenesis and this issue is difficult to resolve without a cell-resolution expression analysis. These expression data might also explain the basis for the morphological defects observed in some *BTBD6a2* morphants. If *zBTBD6a2* is required for some other earlier developmental processes, the observed neurogenic phenotype might be a secondary effect. Therefore, it is possible that *zBTBD6a* isoforms are expressed in different cells and/or alternatively, that there are quantitative and/or qualitative differences between these two proteins.

Overexpression of wild-type zBTBD6a1 does not alter the number of differentiated neurons labelled by *isl1*. Therefore, while zBTBD6a seems to be required for neuronal differentiation, it appears not to be sufficient to drive progenitors to a neuronal fate. This suggests that zBTBD6 plays a permissive role in neurogenesis. However, it should be noted that these overexpression experiments have yet to be carried out with both zBTBD6a1 and zBTBD6a2 proteins.

Both overexpression of truncated forms of zBTBD6a1 protein and combined loss of zBTB6a1 and zBTBD6a2, results in severe decrease in or total absence of *isl1*-expressing cells. Frequently, the loss of differentiated neurons is accompanied by an expansion of the neural plate. The same effect has been

reported after misexpression of a *Xenopus* Notch variant, and following misexpression of *her4* in zebrafish (Coffman et al., 1993; Takke et al., 1999). In these reports, similarly to my work, an enlargement of the neural plate is concomitant with a reduction in the numbers of cells expressing neuronal markers, including *isl1*. The explanation for this phenomenon is unclear. One possibility is that there is increased proliferation in the neural plate as the neuronal differentiation is blocked/decreased and/or delayed. Indeed studies of the *Xenopus* neural-specific transcription factor *XBF-1* (now termed *FoxG1*) have shown that misexpression of *XBF-1* at high doses results in suppression of neuronal differentiation and an expansion of undifferentiated neuroectoderm and this expansion is in part due to an increase in cell proliferation (Bourguignon et al., 1998; Hardcastle and Papalopulu, 2000). Another possibility is that there is a disruption of the early morphogenic movements that results in the neural plate being wider. If this was a case, there is a possibility that the observed loss of neurogenesis in embryos with impaired zBTBD6a function represents a secondary effect. However, currently such a mechanistic link between morphogenic movements and the progression of neurogenesis downstream of proneural genes seems a more complex model since similar enlargement of the neural primodium have been reported in experiments with molecules that have proven involvement in the neurogenesis. Further experiments would clarify this issue.

Proposed model of zBTBD6a function

Immunoprecipitation analysis showed that zBTBD6a1 associates with Cul-3. Cullins (Culs) are subunits of a class of RING ubiquitin ligases that are part of the ubiquitin system primarily directing substrates for proteosome-mediated degradation (Hershko and Ciechanover, 1998; Kipreos et al., 1996). Three enzymes are involved in the ubiquitin transfer reaction: E1, which mediates the ATP-dependent activation of ubiquitin, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase, which transfers ubiquitin to the target protein. Cullin-based E3 ubiquitin ligases recruit protein substrates to a

ubiquitin machine through substrate-specific proteins. Recent studies have identified members of the BTB-domain protein family as substrate-specific adaptors of Cul3-based E3 ubiquitin complexes (Furukawa et al., 2003; Geyer et al., 2003; Kobayashi et al., 2004; Krek, 2003; Pintard et al., 2004; Xu et al., 2003a). Since zBTBD6a1 immunoprecipitates with Cul-3, a component of E3 ubiquitin ligase, it may act as one of these adaptor proteins involved in the ubiquitination process.

The reported BTB adaptor proteins contain, in addition to the N-terminal BTB domain, an interaction motif located in the C-terminus: kelch, MATH or ankyrin. However, the interaction of BTB proteins without additional recognisable motifs with human Cul-3 has also been reported (Xu et al., 2003a). Two initial studies in human and yeast showed that the BTB domain interacts directly with Cul-3 (Furukawa et al., 2003; Xu et al., 2003a), while a study in *C. elegans* demonstrated that the interaction with substrate is mediated through a MATH domain (Pintard et al., 2003). However, a recent report on the ubiquitination of the transcription factor Nrf2 mediated by the BTB protein Keap1 identified yet another domain responsible for the association with Cul-3 (Kobayashi et al., 2004; Stogios and Prive, 2004). This domain, called the intervening-region or BACK domain is located in Keap1 protein between the N-terminal BTB and C-terminal kelch motifs. Therefore, the role of each of the domains in formation of the ubiquitin complex may differ in each specific BTB adaptor protein and may also depend on the substrate protein and/or other components contributing to the ubiquitin complex. zBTBD6a protein contains BTB, BACK and PHR domains. I found that removal of the PHR domain prevents zBTBD6a1 from interacting with Cul-3, while zBTBD6a lacking the BTB domain has a weaker interaction. Therefore, it appears that while zBTBD6a1 association with Cul3 is dependent on the presence of the PHR domain, the BTB domain appears to not be essential for this interaction. It is interesting that full-length zBTBD6a1 localises to the cytoplasm, while PHR- and BTB-deleted zBTBD6a1 proteins are confined either to the nucleus or both the nucleus and cytoplasm, respectively. Thus, perhaps interaction with Cul-3 ubiquitin ligase and/or

substrate is what keeps zBTBD6a in the cytoplasm. Hence, deletion of PHR domain prevents this interaction and as a consequence zBTBD6a protein localises to the nucleus; removal of BTB domain results in a weak interaction, which causes partial zBTBD6a localisation in the nucleus. Both zBTBD6a1 mutant proteins used in this study contain a BACK motif. At the time when the immunoprecipitation analysis was carried out, this motif had not yet been identified as a protein functional domain and therefore was not included in the analysis. Further investigation is required to elucidate the role of the BACK domain in zBTBD6a function.

An involvement of the protein domains of zBTBD6a in mediating an adaptor function is supported by the results of misexpression experiments. While ectopic expression of full-length zBTBD6a1 did not affect neuronal differentiation, overexpression of either the BTB- or PHR-deleted zBTBD6a1 proteins, resulted in decreased neurogenesis. Therefore, the removal of these zBTBD6a1 domains may act in a dominant negative manner as it elicited a similar result as MO-mediated *zBTBD6a1* knockdown. An adaptor function requires that the domains interacting with target protein and the degradation machinery are linked to each other. Thus, truncation of one or other of the domains is predicted to act in a dominant negative manner, as the target protein will not be brought to the degradation machinery in the cytoplasm. When the PHR domain is removed, the truncated zBTBD6a1 protein traverses to the nucleus, but low levels of the ectopic protein are still present in the cytoplasm. As described above, the degree of nuclear localisation of wildtype and truncated zBTBD6a1 correlates with the ability to bind Cul-3. The shift in localisation can be most easily explained as being consequence of the dominant negative action of truncated zBTBD6a1, in which it binds to a target protein (e.g. transcription factor) but fails to sequester it to the cytoplasm for degradation. Such a dominant negative effect is concordant with the phenotype being seen as caused by knockdown of zBTBD6a1.

Taking these data together, I propose that the role of zBTBD6a in neurogenesis is executed through Cul-3 mediated degradation of one or more proteins that affect formation of neurons. zBTBD6a positively regulates neurogenesis as loss of zBTBD6a results in an impaired neuronal differentiation. Therefore, it is likely that the molecule subjected to zBTBD6a-mediated ubiquitination affects neuronal differentiation in a negative manner. The zBTBD6a target may be a repressor of one of the bHLH transcription activators, involved in the neurogenesis cascade.

One example of a protein regulating transcriptional repression during neurogenesis that, similar to zBTBD6a, is required but not sufficient for neuronal differentiation, termed MTG, has recently been reported in chick (Koyano-Nakagawa and Kintner, 2005). The MTG gene family was originally identified at chromosomal translocation points and deletions associated with acute myelogenous leukaemia (Davis et al., 2003). Biochemical and molecular studies in mammalian cells have demonstrated that MTG proteins act as transcriptional corepressors by linking interactions between multiple proteins, including proteins with histone deacetylase activity (Amann et al., 2001; Lutterbach et al., 1998; Zhang et al., 2001). MTG proteins regulate transcription when recruited to the specific sites by DNA binding proteins as shown with the promyelocytic leukaemia zinc-finger (PLZF), growth factor independence-1 (Gfi-1), and B-lymphoma 6 (BCL-6) proteins (Chevallier et al., 2004; McGhee et al., 2003; Melnick et al., 2000). In the chick spinal cord, MTGR1, MTG8, and MTG16 are sequentially expressed during neuronal differentiation. A similar sequence of MTG gene expression induced by *Xngn1* during primary neurogenesis, has been reported in *Xenopus* (Cao et al., 2002). Misexpression of wild-type forms of MTG proteins in the developing chick spinal cord does not alter neuronal differentiation, whereas when a dominant-negative mutant of MTG proteins is expressed, the number of differentiated neurons is markedly reduced (Koyano-Nakagawa and Kintner, 2005). Although direct targets of the MTG proteins during neurogenesis have not been identified, it has been suggested that in the chick spinal cord MTG proteins, acting as corepressors, downregulate genes

whose expression needs to be extinguished for neuronal differentiation to occur. Therefore, zBTBD6a, similar to MTG proteins, may be involved in regulation of a repressor of neuronal differentiation, although through a different mode of action. Some potential candidates for zBTBD6a targets are discussed below.

Potential targets of the *zBTBD6a* promoted ubiquitination

Id proteins.

One family of candidates for the zBTBD6a-mediated degradation are Id proteins. These proteins have a HLH domain but lack an adjacent motif for DNA binding (Benezra et al., 1990; Ellis et al., 1990). As they have a preferential affinity for ubiquitously expressed E proteins, they compete with bHLH proteins by forming heterodimers that cannot bind DNA (Campuzano, 2001; Massari and Murre, 2000; Yokota, 2001). Therefore, Id proteins act as negative regulators of bHLH factors, including proneural genes and the downstream activators of neurogenesis, and consequently inhibit differentiation and stimulate proliferation (Campuzano, 2001; Massari and Murre, 2000; Yokota, 2001). Studies with Id knockout mice revealed that Id proteins are required to maintain the immaturity of neuroblasts and permit their proliferation until an appropriate time point during development (Lyden et al., 1999; Ruzinova and Benezra, 2003; Yokota, 2001). In Xenopus, Id proteins can differentially inhibit the activities of neurogenin and neuroD, as shown in the animal cap explant experiments (Liu and Harland, 2003). The distinct expression of some Id proteins (Id2 and Id4 in mouse) in both proliferating and differentiating neural cells implies that these proteins are involved in the timing of various stages of neuronal differentiation (Neuman et al., 1993; Riechmann et al., 1994; Tzeng and de Vellis, 1998). It is likely that each Id protein regulates neurogenesis by antagonising co-expressed cell-specific bHLH transcriptional activators that control successive stages of neuronal differentiation.

Regulation of the stability of the Id proteins within the cell is important for controlling the balance between E-box binding bHLH protein dimers and inactive dimers, allowing the cell to fine-tune the regulatory activities of the transcription factors. Id proteins are generally very short-lived, with half-lives ranging from 20 to 60 min depending on the cell types, and are stabilised by formation of dimers with bHLH factors (Bounpheng et al., 1999; Deed et al., 1996). The rapid degradation suggests the importance of precise regulation of Id protein function and this is achieved by proteolysis through the ubiquitin-proteosome pathway (Bounpheng et al., 1999). It is possible that zBTBD6a takes part in this regulation and mediates degradation of one or more of the Id proteins inhibiting neuronal differentiation by recruiting it to the ubiquitination machinery. However, currently, it is unknown whether the ubiquitination of Id proteins is promoted by Cul3 ubiquitin ligase.

Based on experiments with Id3, it has been proposed that the E proteins chaperone the Id proteins (which lack a nuclear localisation sequence) into the nucleus and increase the half-life of an otherwise unstable protein (Deed et al., 1996). In the absence of its E protein partner, the Id3 protein is localised exclusively in the cytoplasm/perinuclear region. Interestingly, zBTBD6a subcellular localisation is altered depending on the presence of BTB and PHR domains: wild type zBTBD6a localises to the cytoplasm, while PHR- and BTB-depleted zBTBD6a1 proteins are confined either to the nucleus or both nucleus and cytoplasm, respectively. Therefore, the presence or absence of these two domains is required to restrict zBTBD6a protein to specific compartments within the cell. This may reflect how zBTBD6a acts at the molecular level and where the potential molecules for zBTBD6a interaction, are located in the cell. Therefore, the proposal of Id protein traversing between nucleus and cytoplasm as a zBTBD6 binding partner is an appealing possibility.

Sox proteins

The HMG-box transcription factors Sox1, Sox2 and Sox3, which constitute the B1-subgroup of the *Sox* gene family, are co-expressed in most proliferating progenitor cells of the vertebrate embryonic and adult CNS. It has been demonstrated that Sox2 and Sox3 have a role in maintaining the undifferentiated state of embryonic chick neural progenitors and preventing them from neuronal differentiation (Bylund et al., 2003; Graham et al., 2003; Pevny and Placzek, 2005). Interestingly, it has been shown that Sox3 protein inhibits neurogenesis in the neural primodia by repressing the differentiation cascade downstream of proneural activity, whereas the capacity of proneural proteins to drive progenitors toward differentiation, in turn, is based on their ability to suppress *Sox3* expression (Bylund et al., 2003). For example, forced expression of Sox3 does not repress expression of the proneural genes *Cash1*, *Ngn1* and *Ngn2*, whereas the expression of *NeuroM*, a marker of subsequent step of neurogenesis, is reduced. Cells that are about to differentiate express higher amounts of preneural protein that inhibit *Sox3* expression. Thus, when proneural gene activity reaches the threshold necessary to repress *Sox3* expression, the neuronal differentiation cascade is activated. Transient transfection studies have shown that SoxB1 proteins primarily function as transcriptional activators and therefore it is suggested that the Sox2- and Sox3-mediated inhibition of neurogenesis is executed through induction of the expression of a factor/s that repress the expression of proteins required for neuronal differentiation (Bylund et al., 2003; Kamachi et al., 2000).

Therefore, there is a possibility that a member of SoxB1 protein subfamily or alternatively the downstream activated factor/s may represent a target for zBTBD6a function. The identification of six zebrafish *sox* genes, *sox1a*, *sox1b*, *sox2*, *sox3*, *sox19a* and *sox19b*, comprising apparently the full complement of zebrafish group B1 *sox* genes, have recently been reported (Okuda et al., 2006). The expression patterns of each of these genes suggest their involvement in the development of the nervous system. Further detailed

analysis of the expression and function of these genes would establish whether any of them might represent a candidate for interaction with zBTBD6a.

Groucho and Her proteins

Another family of repressors of neurogenesis are represented by Hairy- and E(spl)-related (Her) bHLH transcription factors (Davis and Turner, 2001; Kageyama and Nakanishi, 1997). These proteins have been shown to act as classical DNA-binding repressors of proneural-gene transcription, but they are also thought to inhibit the activity of proneural proteins by interfering with proneural-E-complex formation (Davis and Turner, 2001; Kageyama and Nakanishi, 1997; Ohsako et al., 1994). Their activity as sequence-specific transcriptional repressors depends upon their interaction with co-repressors of the Groucho/TLE family (Davis and Turner, 2001; Paroush et al., 1994). In zebrafish, *her2*, *her4*, *her12* and *hes5* (hairy and enhancer of split5), are expressed in proneural domains in the neural plate, similar to the expression of the *neurog1* and *zBTBD6a* (Bae et al., 2005; Raya et al., 2003; Takke et al., 1999). In addition, one of the zebrafish homologues of *groucho* co-repressor, *gro1*, is expressed in the neurogenic regions of the neural plate (Wulbeck and Campos-Ortega, 1997). Therefore, *gro1* and *her* genes of the first subgroup expressed at the sites of primary neurogenesis, could be classified as potential targets of zBTBD6a activity. However, these genes products act upstream of *neurog1* and are thus less likely candidates for interactions with zBTBD6a that acts downstream of *neurog1*.

GENERAL DISCUSSION AND PERSPECTIVES

This study identified a novel gene, *zBTBD6a*, encoding a putative substrate adaptor for the Cul-3 E3 ubiquitin ligase, which is involved in zebrafish neurogenesis. In this section, the general aspects of ubiquitination system and how my work relates to the published data on this subject are discussed. I also propose the future work required to gain further understanding of the role of *zBTBD6a* in neurogenesis and to get insight into the function of other members of the BTBD6/3 protein family identified in this study.

PRINCIPLES OF UBIQUITINATION

Proper control of growth and differentiation during development requires regulation of gene expression at many different levels. One level of regulation is achieved through the ubiquitin machinery.

Ubiquitination, a covalent attachment of one or more molecules of the protein ubiquitin to another protein, plays major roles in regulating a broad range of biological processes in eukaryotic cells, including cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, protein trafficking and quality control. Ubiquitin is a small protein of 76 amino acids that is highly conserved in all eukaryotes. Ubiqutination in general takes three steps: the first two involve activation and conjugation of ubiquitin and the third one promotes covalent ligation of ubiquitin to the target protein and is E3 ubiquitin ligase-dependent (Hershko and Ciechanover, 1998; Pickart, 2001). E3 proteins are important for the selection of specific targets for ubiquitination and consist of two main classes: HECT domain E3s and RING E3s.

Monoubiqutination, the linkage of a single ubiquitin molecule to a protein, has been shown to be important for regulation of endocytosis of cell surface receptors, DNA-repair mechanism, as well as for direct modulation of protein function (Bonifacino and Weissman, 1998). Polyubiquitination, that is

addition of at least four ubiquitin units to the substrate, is predominantly used to target proteins to the 26S proteasome for degradation. The proteasome-dependent ubiquitination pathway is known to degrade short-lived regulatory proteins and to remove abnormal or improperly assembled proteins, protecting cells against the potential toxic effects of protein aggregation. Protein degradation through the ubiquitin-proteasome system may be essential to ensure irreversibility of temporally controlled processes. In developmental processes, such as neuronal differentiation the rapid degradation of protein regulators is especially important when the regulator should act for a short period of time or when a process is initiated by the degradation of an inhibitor.

NOTCH PATHWAY IS REGULATED BY UBIQUITINATION AT MANY LEVELS

A prominent example of ubiquitination in regulation of a molecular pathway has been demonstrated for the Notch receptor, whose activity is modulated by ubiquitin at different levels in the signalling pathway (Hirata et al., 2002; Lai, 2002).

Sel-10 is a negative regulator of Notch signalling first identified in *C. elegans*, and then in mammals (termed Fbw7) and *Drosophila* (named Archipelago [Ago]) (Maruyama et al., 2001; Moberg et al., 2001; Sundaram and Greenwald, 1993; Winston et al., 1999). Sel-10/Fbw7/Ago is related to the F-box/WD40 repeat proteins, which are components of Skp1 Cul1 F-box (SCF) E3 ubiquitin ligases (Hubbard et al., 1997). Sel-10/Fbw7/Ago inhibits Notch signalling by targeting the nuclear intracellular domain of Notch receptor for proteasome-dependent degradation (Gupta-Rossi et al., 2001; Oberg et al., 2001; Wu et al., 2001). Interestingly, in addition to regulating Notch, Sel-10/Fbw7/Ago facilitates the ubiquitin-mediated degradation of Cyclin E, c-Jun and Myc, indicating that a single F-box protein can facilitate ubiquitination of multiple target proteins that are components of multiple pathways that control growth and cell cycle progression (Koepp et al., 2001;

Moberg et al., 2001; Moberg et al., 2004; Nateri et al., 2004; Strohmaier et al., 2001; Tetzlaff et al., 2004; Welcker et al., 2004; Yada et al., 2004). Cyclin E is a key regulator of the G1-to-S-phase transition of the cell cycle, c-Jun is implicated in neuronal apoptosis, while Myc promotes cell growth and proliferation. All these proteins subjected to the Fbw7-mediated degradation have oncogenic potential and inactivating mutations in Fbw7 have been found in number of human malignancies, indicating that Fbw7 may act as a tumor suppressor.

Suppressor of Deltex [Su(dx)] and its homolog Nedd4 are HECT-type E3 ubiquitin ligases and act negatively on Notch signalling (Cornell et al., 1999; Fostier et al., 1998; Rotin et al., 2000). They are proposed to inactivate Notch molecules by directing them to an intracellular compartment for degradation (Sakata et al., 2004; Wilkin et al., 2004). Another molecule, Deltex (Dx) is a putative RING-type E3 ubiquitin ligase and has been shown to be a positive regulator of the Notch pathway (Hori et al., 2004; Matsuno et al., 1995; Takeyama et al., 2003). Suppression of the *dx* mutant phenotype by *Su(dx)* mutation indicates that these two proteins representing two different classes of ubiquitin ligase act antagonistically with regard to Notch signalling (Cornell et al., 1999).

In addition to proteasome-dependent ubiquitination, ubiquitination followed by endocytosis is also involved in Notch pathway regulation. *Mindbomb* (*mib*) and *neuralized* (*neur*) characterised in *Drosophila* and *Xenopus,* both encode RING-type E3 ubiquitin ligases (Deblandre et al., 2001; Itoh et al., 2003; Koo et al., 2005; Lai et al., 2001; Lai et al., 2005; Le Borgne et al., 2005). They monoubiqutinate Notch ligands, the Delta/Serrate/LAG-2 (DSL)-type proteins, leading to their endocytosis in signal-sending cells, which is necessary to activate Notch in adjacent cells (Lai et al., 2005; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). In addition, an endocytic adaptor protein, Epsin (that recognises the ubiquitin moiety), which in *Drososphila* is encoded by the *liquid facets (lqf)* gene, is required in the signaling cell for Notch activation and for DSL protein endocytosis (Overstreet et al., 2003).

REGULATED UBIQUITIN/PROTEASOME-DEPENDENT PROCESSING

The target protein subjected to ubiquitin-proteasome degradation is in most cases proteolysed into small fragments. However, in a few cases, the proteasome only degrades specific protein segments, leaving other parts of the substrate intact and this process is referred as regulated ubiquitin/proteasome-dependent processing. This is one of the mechanisms used by cells for activation of dormant transcription factors that are initially made as inactive, often membrane-bound precursors. It was first shown to be essential for the processing of p105, the cytosolic precursor of the p50 subunit of the NF-κB1 transcription factor, involved in immune, inflammatory, stress, and developmental processes (Baeuerle and Baltimore, 1996; Fan and Maniatis, 1991). Processing of ubiquitinated p105 leads to degradation of its C-terminal portion by the proteasome, whereas the N-terminal transcription factor portion, p50, is left intact. The processed transcription factor is retained in a latent form in the cytoplasm of nonstimulated cells via association with inhibitory protein IκBα. Upon receipt of appropriate signal, IκBα is phosphorylated, ubiquitinated and degraded by proteasomes, which permits the active NF-κB1 protein to translocate to the nucleus where it exerts its transcriptional acivity (Ghosh et al., 1998). Two distant homologs of NF-κB1 p105, SPT23 and MGA2, in the yeast *Saccharomyces cerevisiae,* are activated in a similar fashion. However, in contrast to p105, which is a cytosolic soluble protein, the precursors of SPT23 and MGA2 are anchored to the membrane of the endocytoplasmic reticulum. Upon ubiquitination, both proteins are partially cleaved to generate the active transcription factors, which can subsequently migrate into the nucleus to drive transcription (Hitchcock et al., 2001; Hoppe et al., 2000; Hoppe et al., 2001). A third protein that has been identified to be regulated in this way is *Drosophila* Cubitus interruptus (Ci), which is essential for Hedgehog-mediated cellular differentiation (Aza-Blanc et al., 1997; Jiang and Struhl, 1998; Noureddine et al., 2002). Ci is processed into Ci^{75}, in a reaction that requires the ubiquitin ligase SCF and the proteasome. Therefore, regulated ubiquitin/proteasome dependent processing can

control both the activation of transcription factors and their trafficking into different cellular compartments. zBTBD6a could promote neurogenesis by permitting, in a ubiquitin-dependent manner, activation of one of the factors required for neuronal differentiation. In addition, zBTBD6a subcellular localisation is altered upon removal of the functional domains, which might reflect the changes of the localization of its binding partner.

CULLIN-RING UBIQUITIN LIGASES

zBTBD6a interacts with Cullin-3 protein, a component of one type of the Cullin-RING E3 ubiquitin ligases (CRLs). The superfamily of CRLs comprises the largest known class of ubiquitin ligases found throughout eukaryotes. These multisubunit ubiquitin ligases are characterised by an enzymatic core that contains a cullin-family member and a zinc-binding RING-domain protein, which is known as either ROC1, RBX1 or HRT1 (Ohta et al., 1999; Seol et al., 1999; Tan et al., 1999). Human cells express seven different cullins (CUL1, 2, 3, 4A, 5, and 7), each used as scaffold in assembling various CRL complexes, which appear to share a similar modular architecture.

Different Cullin proteins present in a common enzymatic core can recruit numerous substrates by employing various adaptor proteins and substrate-receptors (or domains). Recent studies have demonstrated that in the case of CRLs nucleated by Cul3, substrate recognition is facilitated by BTB-domain-containing proteins (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2004; Pintard et al., 2003; Xu et al., 2003a). The association of zBTBD6a with Cul3 suggests that zBTBD6a may have this function. Several BTB Cul-3 adaptor proteins have been reported to date and most of them, similar to zBTBD6a, possess at least one more protein-protein interaction domain in addition to the BTB motif located at the N-terminus. However, no substrate recognition protein containing the same domains as zBTBD6a - BTB-BACK-PHR - have been identified yet. One example of a BTB-domain protein acting as an adaptor for Cul-3-based E3 ligase that has similar structure to zBTBD6a is Keap1. Keap1, like zBTBD6a has BTB and BACK domains but at the C-

terminus, contains a kelch repeat instead of the PHR motif. Keap1 has been found to be required for the degradation of the transcription factor Nrf2, which is a major regulator of genes encoding phase 2 detoxifying enzymes and antioxidant stress proteins (Furukawa and Xiong, 2005; Kang et al., 2004; Kobayashi et al., 2004; Zhang et al., 2004). In the absence of stress stimuli, Nrf2 is inactive as Keap1 sequesters it in the cytoplasm and targets it for Cul-3-mediated rapid degradation through the proteasome system. In this way when the toxic environmental stresses are not present, Nrf2 is repressed and the unnecessary gene activation is avoided allowing cellular homeostasis to be maintained. Keap1 and Nrf2 are so far the only reported mammalian substrate and adaptor, respectively, of the Cul-3-based ubiquitination system.

PHR DOMAIN-CONTAINING PROTEINS AND UBIQUITINATION

The PHR domain was first identified in human PAM and is also present in its highly conserved homologes: *Drosophila* Highwire (Hiw), *C. elegans* RPM-1, and murine Phr1 (Burgess et al., 2004; Guo et al., 1998; Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000). These large proteins are neuron-specific, contain RING domains, and act as E3 ligases. RPM-1, Hiw and Phr1 have been shown to be involved in regulating protein stability in presynaptic differentiation. Drosophila *hiw* is a negative regulator of synaptic growth and loss of Hiw results in synaptic overgrowth and impaired synaptic transition (Wan et al., 2000). Overexpression of the deubiquitinating enzyme Fat Facets (Faf) results in a nearly identical phenotype suggesting that a balance between the ubiquitination and deubiquitination of a key target molecule regulates synaptic growth. Similarly, mutations in *C. elegans rpm-1* disrupt synaptic morphology in the worm (Schaefer et al., 2000; Zhen et al., 2000), and *Phr-1* deficient mice show abnormalities in synapse organisation (Burgess et al., 2004). Biochemical studies in *C.elegans* have shown that RPM-1 associates into the CRL complex, which also includes Cul-1 and the substrate recognition F-box protein FSN-1. The RPM-1/CRL complex represents one example of the use of multiple domains and components

from other ubiquitin-ligase pathways, since RPM-1 possesses numerous RING domains and does not have a typical ROC1/RBX1/HRT1 E3 ligase structure (Liao et al., 2004). Interestingly, within the RPM-1/CRL complex, the PHR domain is located in the RPM-1 E3 ligase protein, whereas zBTBD6a is a putative substrate recognition module, representing a different CRL component. Thus, the domains involved in CRL assembly can be in different components of the complex.

UBIQUITINATION AND NEURODEGENERATIVE DISEASES

Ubiquitin-dependent processing and degradation of proteins has a fundamental role in cellular processes, and consequently aberrations in the ubiquitin system have been implicated in the pathogenesis of many diseases such as certain malignancies and neurodegenerative disorders. For example, a common feature of several chronic neurodegenerative diseases, for instance Alzheimer's disease, Parkinson's disease and motor neuron disease, is abnormal deposition of insoluble protein aggregates or inclusion bodies within CNS neurons (Ciechanover, 2006; Ciechanover and Brundin, 2003). These intracellular inclusions are believed to result from accumulation of ubiquitinated proteins that occurs due to the malfunctioning of the ubiquitin-proteasome pathway or changes in the protein substrates, which make them resistant to degradation. Impaired proteolysis might also contribute to synaptic dysfunction, which is also seen in neurodegenerative diseases, since the ubiquitin-proteasome pathway plays a role in synaptic function and plasticity.

FINAL PERSPECTIVES

Further investigation into the function of zBTBD6a

Identifying the zBTBD6a target protein(s) in the zebrafish nervous system will be one of the next crucial steps for understanding the mechanism of zBTBD6a function. One approach is to investigate interactions with the

candidates discussed in the previous chapter. However, since it may turn out that none of them are relevant, it is important to use a more general approach.

This could be achieved by protein affinity purification coupled with mass-spectrometric protein identification (proteomics) (Kaiser and Huang, 2005; Kirkpatrick et al., 2005). A potentially amenable approach would be to detect differences in protein concentrations between uninjected zebrafish embryos and embryos in which truncated zBTBD6a proteins, ΔBTB and ΔPHR, are overexpressed. zBTBD6a protein lacking specific domains presumably has an impaired ability to mediate degradation of its substrate. Therefore, a protein which is present in extracts of embryos expressing truncated zBTBD6a protein at elevated levels compared with the control could represent the potential zBTBD6a target. This analysis can be carried out using two-dimensional gel electrophoresis and mass spectrometry.

The two-hybrid assay has been successfully used to identify some substrates of ubiquitin ligases and is another method to identify zBTBD6a target proteins (Pintard et al., 2003; Uetz et al., 2000; Xu et al., 2003a).

There are also some other questions that need to be addressed to better understand the role of *zBTBD6a* in zebrafish neurogenesis. Further work is required to address the involvement of the splice variants of *zBTBD6a* in neuronal differentiation. For example, loss of both zBTBD6a isoforms leads to severe reduction or complete absence of terminally differentiated neurons labelled by *isl1*, but the effect of combined *zBTBD6a1* and *zBTBD6a2* knockdown on earlier markers of neuronal differentiation is still to be tested. Overexpression of zBTBD6a does not affect the number of differentiated primary neurons and it would be interesting to test whether or not the misexpression of both *zBTBD6a* splice variants elicits the same result. Elucidating the differential spatial expression of *zBTBD6a* isoforms is essential for this work.

Both the BTB and PHR domains are required for zBTBD6a function but the role of BACK domain remains to be assessed. This would involve ectopic expression of BACK-deleted zBTBD6a protein in zebrafish and analysis of the effect on primary neurogenesis. Testing whether zBTBD6a protein lacking BACK domain associates with Cul3 would determine the role of the BACK domain in forming the Cul-3-zBTBD6a complex.

Elucidating the potential role of cBTBD6 in chick neurogenesis

Similar to its homolog in zebrafish, *cBTBD6* is expressed at sites of neuronal differentiation. Establishing the identity of *cBTBD6*-positive cells and determining whether cBTBD6 is involved in neurogenesis in a similar way as zBTBD6a in zebrafish would be another interesting issue to address. This would involve electroporation of full length and truncated, domain-deleted, *cBTBD6* DNAs into the developing neural tube, and analysis of the outcome with the markers of neuronal differentiation. Another method to assess cBTBD6 function would be to use gene knockdown techniques, such as morpholino oligonucleotides or RNA interference (RNAi) (Brown et al., 2003; Heasman, 2002; McManus and Sharp, 2002).

BTBD6/D3 proteins - common mechanism of function, different targets?

Although BTBD6 and BTBD3 genes share some aspects of their expression during chick and zebrafish development, for example in the regions of neuronal differentiation, in general they have divergent expression profiles, indicating that they may play diverse roles in different tissues during embryogenesis. However, considering the highly conserved structure of these proteins, there is a strong possibility that their activities are executed through the same mechanism. Since all BTBD6/D3 proteins contain BTB, BACK and PHR domains, it is likely that they all serve as substrate recognition molecules for the ubiquitination process.

Another intriguing question is whether there is a common substrate for each of these putative adaptor proteins in the different tissues where they are expressed, or whether each BTBD3/D6 protein has different specific targets in various sites of their expression. Cases of adaptor proteins targeting multiple substrates for ubiquitination have been reported. One of them is the F-box protein Sel-10/Fbw7/Ago that, as described previously, has been found to mediate the ubiquitin-dependent degradation of four proteins: Notch, Cyclin E, c-Jun and Myc, components of various pathways (Koepp et al., 2001; Moberg et al., 2001; Moberg et al., 2004; Nateri et al., 2004; Strohmaier et al., 2001; Tetzlaff et al., 2004; Welcker et al., 2004; Yada et al., 2004). The phenomenon of one substrate-recognition protein having numerous targets may result from the combinatorial complexity of the E3 ubiquitin ligase complexes, in which the change of one binding partner may influence the affinity of the complex for a specific target protein. The proteins present in the ubiquitin ligase complexes may depend on the availability of a particular component in the specific cell type. Therefore, it is likely that BTBD6/D3 proteins have numerous tissue-specific targets rather than one common substrate protein. Identifying BTBD6/D3 target proteins will be a challenging and exciting task.

The identification of BTBD6/D3 genes and the experiments that have been presented in this thesis represent a significant first step in characterising this novel family of genes and elucidating their function during development. Clearly, further investigation is required to fully understand the roles of BTBD6/D3 genes in biological processes both during embryogenesis and in the adult and their relationship with or involvement in the ubiquitination process.

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