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Roles of Reelin and Disabled1 in Neural Development in Zebrafish

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Abstract

Roles of Reelin and Disabled1 in Neural Development in Zebrafish

Reelin and Disabled1 (Dab1) are known to be involved in neuronal migration in vertebrates and in particular in radial migration. Reelin is a secreted protein that can bind several receptors and the signal is intracellular transduced by Dab1. The final targets of the Reelin pathway are molecules that regulate cytoskeletal remodeling.

In my project I studied the expression pattern of *reelin* and *dab1* in several areas of the zebrafish CNS. The differences found in the telencephalic expression of *reelin* and *dab1* between teleosts and tetrapods are likely to arise from the process of eversion, which is specific for the teleost telencephalon. On the contrary, all the other regions of the CNS present a conservative pattern of expression, in comparable structures among vertebrates, with the only exception of the olfactory bulb that does not express *reelin* in zebrafish.

As the *dab1* gene shows a high degree of complexity in mammals and mice, I studied the genomic organization of the *dab1* gene in zebrafish and found a similarly complex organization.

In order to study the functions of the Reelin pathway in neuronal migration in zebrafish, I performed loss of function experiments with morpholino antisense oligonucleotides. I found defects at level of several

neuronal groups including the facial branchiomotor nucleus, the Mauthner neurons and neural crests.

To investigate whether the role of *dab1* in the migration of these neuronal groups may be that of conveying a Reelin signal, I attempt to rescue these phenotypes by overexpressing full length *dab1* or truncated forms of the protein that lack the Reelin or the CDK5 responsive domains. It appears that the presence of the tyrosine domain, but not of the CDK5 phosphorylation domain, is necessary for partial rescuing of most of these phenotypes. I also found that some populations of neurons that express *dab1* show defects in neurites growth in morpholino injected embryos.

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Abbreviations

A	anterior thalamic nucleus
aa	amino acid
ac	anterior commissure
Acc	nucleus accumbens (*Xenopus*)
Am	amydgala (*Xenopus*)
AON	anterior octaval nucleus
ap	area postrema
ATN	anterior tuberal nucleus
ALLN	anterior lateral line nerves
BST	bed nucleus of the stria terminalis (*Xenopus*)
cb	ceratobranchial cartilage
CbSg	granular layer of cerebellum
CbSm	molecular layer of cerebellum
CC	crista cerebellaris
Cce	cerebellar body
CG	geniculate body (*Xenopus*)
ch	ceratohyal cartilage
CNC	cranial neural crests
CON	caudal postirior octaval nucleus
CP	central posterior thalamic nucleus
CPN	central pretectal nucleus
cvr	ventral commissure of rhombencephalon
D	dorsal telencephalic area
DB	diagonal band area (*Xenopus*)
Dc	central zone of D
DCN	dorsal column nucleus (*Xenopus*)
Dd	dorsal zone of D
DH	dorsal horn
Di	diencephalon
DIL	diffuse nucleus of inferior lobe
Dl	lateral zone of D
Dld	lateral zone of D, dorsal part
Dlv	lateral zone of D, ventral part
Dm	medial zone of D
DON	descending octaval nucleus
DoP	dorsal pallium (*Xenopus*)

DP dorsal posterior thalamic nucleus

Dp posterior zone of D

Dpa dorsal pallidum (*Xenopus*)

dpf days post fertilization

DT dorsal thalamus

Dvc diencephalic ventro-caudal cluster

Dvr diencephalic ventro-rostral cluster

e etmoidal plate

ECL external cellular layer of olfactory bulb including mitral cells

EG eminentia granularis

En entopeduncular nucleus

Ep epiphysis

Fd funiculus dorsalis

Fld funiculus lateralis pars dorsalis

Flv funiculus lateralis pars ventralis

Fv funiculus ventralis

gc central gray

GL glomerular layer of the olfactory bulb

H hindbrain

Had dorsal habenular nucleus

Hav ventral habenular nucleus

Hc caudal zone of periventricular hypothalamus

Hd dorsal zone of periventricular hypothalamus

hpf hours post fertilization

Hv ventral zone of periventricular hypothalamus

Hy hypothalamus

ICL internal cellular layer of the olfactory bulb

IL intermedio-lateral column

IMRF intermediate reticular formation

in intermediate columns of the spinal cord

IZ intermediate zone

IoL infraorbital lateral

IO inferior olive

IRF inferior reticular formation

La lateral thalamic nucleus (*Xenopus*)

LA lateral amygdala (*Xenopus*)

LGE and from the lateral ganglionic eminence

lc locus coeruleus

LCa caudal lobe of cerebellum

LH lateral hypothalamic nucleus

llf lateral longitudinal fascicle

LTP long-term potentation

lot lateral olfactory tract

LP lateral pallium (*Xenopus*)

LS lateral septum (*Xenopus*)

LVII lobus of the VII

LX vagal lobe

M mesencephalon

m Meckel's cartilage

MeA medial amygdala (*Xenopus*)

MGE medial ganglionc eminence

MFn medial funicular nucleus

mlf medial longitudinal fascicle

ML mitral cell layer (*Xenopus*)

mn motorneurons

mo morpholino

MON medial octavolateral nucleus

mot medial olfactory tract

MP medial pallium (*Xenopus*)

NC nucleus of Cajal

ni nucleus isthmi

nin interpeduncular nucleus

nmlf nucleus of medial longitudinal fascicle

nlv lateral nucleus of valvula

OB olfactory bulb

oc optic chiasma

otc otic capsule

ot optic tract

pc posterior commissure

PG preglomerular nuclei

PGl lateral preglomerular nucleus

PGm medial preglomerular nucleus

PL postirior lateral line

PoA preoptic area

PPa anterior part of parvocellular preoptic nucleus,

PPd dorsal part of periventricular pretectal nucleus,

Pi pituitary

PPp parvocellular preoptic nucleus

PPv ventral part of periventricular pretectal nucleus,
pq palatoquadral cartilage
Pr pretectal nuclei
PI/PTB protein interaction/phosphotyrosine binding domain
PSm magnocellular superficial pretectal nucleus
PSp parvocellular superficial pretectal nucleus
PTN posterior tuberal nucleus
r rhombomere
RT rostral tegmental nucle
RF reticular formation
sac stratum album centrale
sfgs stratum fibrosum et griseum superficiale
sgc stratm griseum centrale
sgp stratum griseum periventriculare
sgt secondary gustatory tract
so stratum opticum
soL supraorbital line
spmo splice morpholino
SVZ subventricular zone
SC suprachiasmatic nucleus
SD saccus dorsalis
SRF superior reticular formation
St striatum (*Xenopus*)
T telencephalon
TeO optic tectum
Tg trigeminal ganglion
Tg tegmental area
Th thalamic nuclei
TL torus longitudinalis
Tla lateral torus
Tp posterior tuberculum (*Xenopus*)
TPp periventricular nucleus of posterior tuberculum
TS torus semicircularis
TSc central nucleus of torus semicircularis
TSvl ventrolateral nucleus of torus semicircularis
UTR untraslated region
v ventricle
v3 3rd ventricle
v4 4th ventricle

V ventral telencephalic area
Va anterior trigeminal nucleus
Val lateral division of cerebellar valvula
Vam medial division of cerebellar valvula
Vb posterior trigeminal nucleus
Vc central nucleus of V
Vd dorsal nucleus of V
VH ventral horn
Vl lateral nucleus of V
VL ventrolateral thalamic nucleus
VM ventromedial thalamic nucleus
Vpa ventral pallidum (*Xenopus*)
Vs supracommissural nucleus of V
vs vagus sensory ganglion
VT ventral thalamus
Vv ventral nucleus of V
III/IV oculomotor and throclear nerve nuclei
VI abducens nerve nucleus
VII facial nerve motor nucleus
VIIs sensory root of the facial nerve
VIII octaval nerve
VZ ventricular zone
IX glossopharingeal nerve nuclei
X vagal nerve nuclei
Xm vagal nerve motor nucleus
Xs vagal nerve sensory nucleus (solitary tract nucleus)
XI ipoglossal nerve nucleus
Zii zona limitans intrathalamica

CHAPTER 1

1.1. General Introduction

Neuronal migration is a process that starts at early stages of development of the organism. The nervous system of vertebrates is an extreme example of complexity and variety in the range and extent of neuronal migrations.

The vertebrate neural tube possesses a germinal zone, the ventricular zone (VZ), where neurons are generated through symmetrical and asymmetrical mitotic divisions. From the VZ, postmitotic neurons migrate radially and tangentially to reach specific positions, whence they grow axons to innervate their targets. This is a critical step in the development of the synaptic circuits of the brain.

The focus of my thesis is the involvement of *Danio rerio* Reelin and Disabled1 (Dab1) in neuron migration. The Reelin/Dab1 pathway has been studied extensively in the mouse brain in relation with the development of the cortical plate. Although the zebrafish brain does not present a telencephalic cortex, it shows subdivisions of the forebrain that are highly conserved among vertebrates (see fig.8 A and B paragraph 4.2), as are the molecules involved in migration and determining the dorso-ventral and anterior-posterior pattern of the brain.

I started my project looking at the anatomy of the zebrafish brain and comparing the zebrafish *reelin* expression pattern to what is known in the other vertebrates. Then, taking advantage of the potentialities of the zebrafish model I performed genomic and functional studies of zebrafish *dab1* that is a main effector molecule downstream of Reelin.

The study of the mechanisms and the molecules involved in neuron migration is of fundamental importance to understand how this development process works to shape the vertebrate brain.

1.2. Factors and signals involved in neuron migration

Neuronal migration is a process that requires a fine orchestration of factors and signals to allow the correct positioning of neurons. In general, neuronal migration is regulated by three important factors:

1. Cytoskeletal changes that permits movement
2. Factors that stimulate the movement and direct migrating cells toward their target (chemotropic molecules and cell surface receptors)
3. Structural elements that guide or constitute the substrate for neuron migration (*i.e.* axons or dendrites of other neurons and glia).

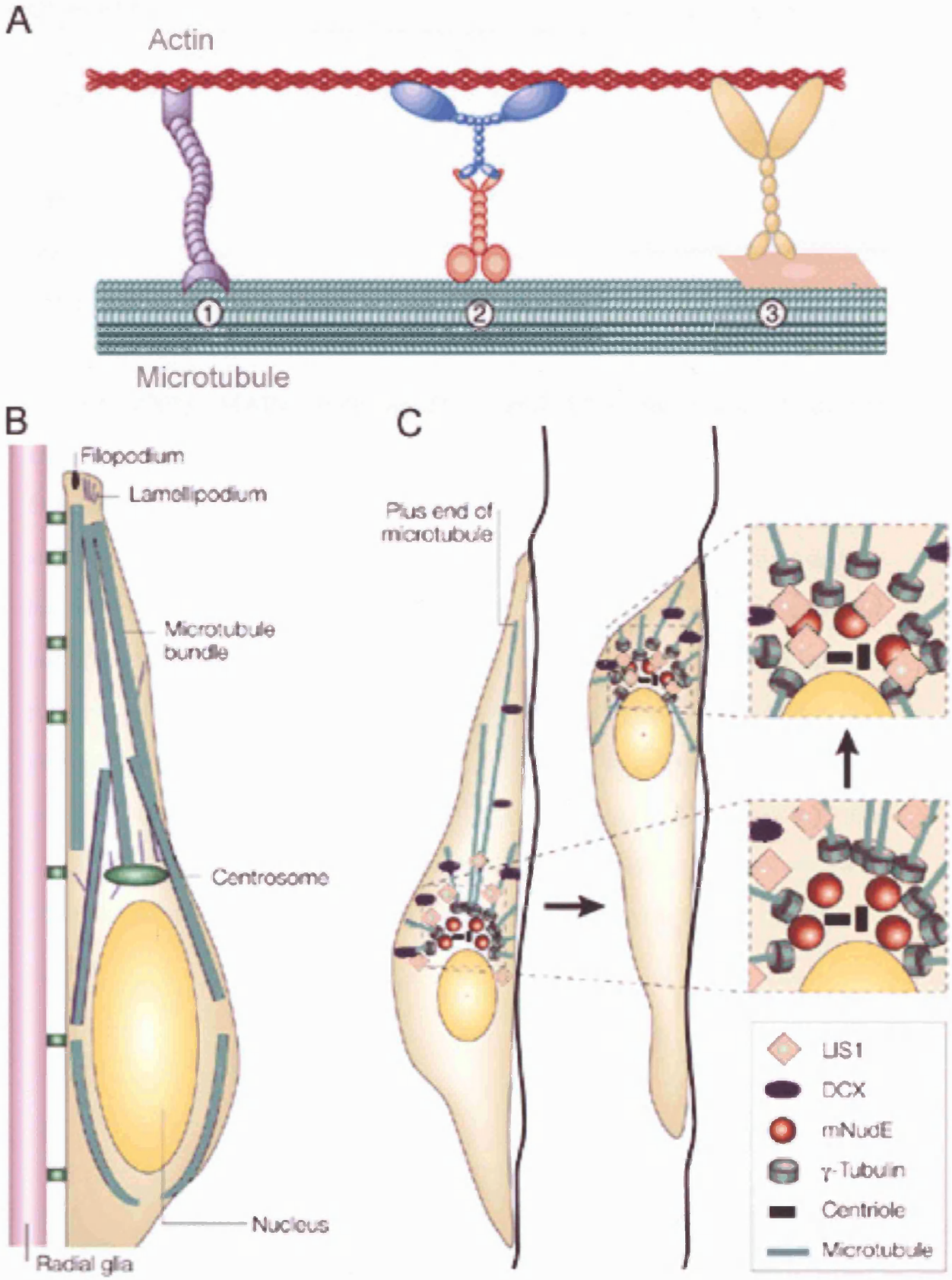
1.2.1. Molecules involved in cytoskeletal changes during migration

Molecules that control cytoskeletal changes are important players in neuronal migration. Migrating neurons are strongly polarized with a leading and a trailing process with a polarized organization of the underlying cytoskeleton (fig.1).

The polarity of the migrating neuron is given by the formation of cytoskeletal structures, such as microtubules and actin filaments that are constantly turning over. In the migrating cell, microtubules are arranged with the minus ends (the extremity that can depolymerize and became shorter) near the center of the cell, anchored at the centrosome, which in turn is usually located near the nucleus (Rodriguez, 2003). The microtubule plus end (the one that can polymerize and became longer) point towards the leading edge. At the level of the leading processes there are lamellipodia

Figure.1. Cytoskeletal changes and neuron migration.

A) Actin (red) and microtubules (green) can have three kinds of interactions. Interaction (1) shows a protein that displays both actin- and microtubule-binding sites and could provide a static crosslink between the two polymers, as hypothesized for MAP. Interaction (2) shows a complex between an actin-based motor (blue) and a microtubule-based motor (orange) like for example non muscular Myosin and Kinesin, whereas interaction (3) shows a complex between a motor (yellow) and a binding protein (pink), as in the case of Dynein and LIS1.The last two types of interactions could move actin and microtubules. B-C) Example of microtubule-based nuclear translocation during neural migration. In (B), scheme of a neuron; the migrating nucleus associates tightly with the centrosome. Filopodia and lamellapodia form at level of the leading hedge. (A) The migration starts in the leading process with the microtubule extension that might be regulated by DCX (blue) and other MAPs. Then, LIS1 (pink) interacting with mNudE at the centrosome reduced the polymerization of microtubules at the minus end, possibly by regulating the γ-tubulin-complex. As a result, microtubules shorten at the minus end and the nucleus is pulled towards the leading edge of the migrating neuron. A dark line depicts the membrane of the radial glial fibre, to which the migrating neuron is attached. Modified from Rodriguez et al., 2003 and Feng & Walsh 2001.


A
Actin
1
2
3
Microtubule
B
Filopodium
Lamellipodium
Microtubule bundle
Centrosome
Nucleus
Radial glia
C
Plus end of microtubule
LIS1
DCX
mNudE
γ-Tubulin
Centriole
Microtubule

and filopodia, formed principally by F-actin (fig.1B).

Actin filaments and microtubule dynamics are regulated by a wide variety of proteints including Rho family GTPases as well as the microtubule-associated proteins (MAPs). MAPs include proteins important for transient stability (Tau), motor proteins (Dyneins and Kinesins) and proteins (DCX and LIS1) that mediate the binding to actin filaments (Dent & Gertler, 2003). MAPs, such as DCX and LIS1 are involved in the translocation of the cell soma, which facilitates the migration (fig.1C). Overall the interactions between actin and microtubules or microtubules and MAPs determine the extension of the leading edge. The leading edge is formed by a meshwork of Actin, where microtubules converge. Inside the lamellipodia and filopodia, the contact between microtubules and actin, a process mediated by tip-associated proteins (tyrosine kinase-interacting proteins), is believed to signal to the cell to allow for dynamic modulation of actin structures. Specifically, Rho GTPases are important for transducing extracellular signals and for polarizing cells that migrate toward or away from the attractant or repellent source (fig. 2; Raftopoulou & Hall, 2004).

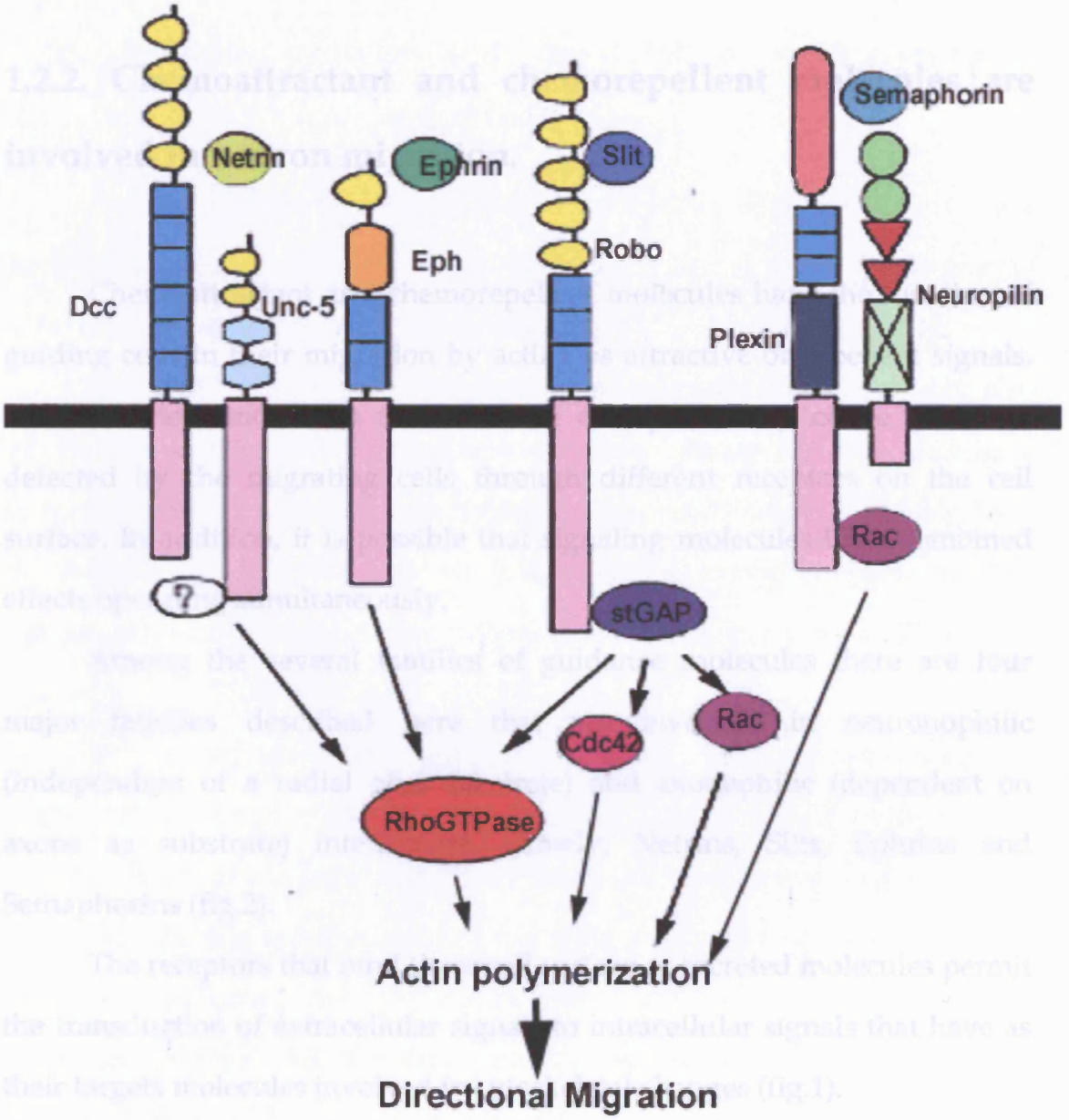


Figure 2. Scheme of the pathways involved in neuron migration.

Netrins Slits, Ephrins, Semaphorins activate or inactivate Rho, Rac and Cdc42 GTPases throught their receptors DCC, Unc-5, Eph, Robo and Neuropilin. The final target is the cytoskeletal Actin polymerization and finally the control of neuron migration. Modified from Park *et al.*, 2002.

1.2.2. Chemoattractant and chemorepellent molecules are involved in neuron migration.

Chemoattractant and chemorepellent molecules have the function of guiding cells in their migration by acting as attractive or repellent signals. Some of these molecules are diffusible and potentially create gradients detected by the migrating cells through different receptors on the cell surface. In addition, it is possible that signaling molecules have combined effects operating simultaneously.

Among the several families of guidance molecules there are four major families described here that are involved in neuronophilic (independent of a radial glial substrate) and axonophilic (dependent on axons as substrate) interactions, namely, Netrins, Slits, Ephrins and Semaphorins (fig.2).

The receptors that bind these cell surface or secreted molecules permit the transduction of extracellular signals to intracellular signals that have as their targets molecules involved in cytoskeletal changes (fig.1).

1.2.3. Neurite growth and its relation with neuronal migration

During migration, neurons use their leading edge to explore their surroundings and can use axons and dendrites of others neurons as substrates to reach their final destinations.

The leading edge processes are commonly short, but there are also migrating neurons, as for example the inferior olivary neurons, that have long processes identical in morphology to axons (Bourrat & Sotelo, 1988; Bloch-Gallego *et al.*, 1999). Finally it is also possible to find long leading edge processes, as in pontine neurons, where axonal markers are missing (Bourrat & Sotelo, 1988; Bloch-Gallego *et al.*, 1999).

In general, the growth cones of neuronal leading processes and of axons follow attractive and repulsive signals to arrive at their final target. In fact, chemoattractants and chemorepellents such as Netrins, Ephrins, Slits and Semaphorins are determinant for both axon pathfinding and neuron migration (Sugimoto *et al.*, 2001; Causeret *et al.*, 2002). Nevertheless, there is a substantial difference between a neuron that is migrating and a neuron that is only extending its axon and this is the process of nucleokinesis (Lambert de Rouvroit & Goffinet, 2001). Nucleokinesis describes the translocation of the nucleus towards the leading edge permiting the migration of the entire cell.

The reiterative elongation of the leading process and the subsequent translocation of the cell body are important events of neuron migration.

These coordinated movements are continuously repeated until the neuron reaches its final destination.

In summary, neuronal migration is the result of the fine orchestration of intracellular events that in response to extracellular signals allow the neuron to coordinate its cytoskeletal dynamics and find its correct position inside the brain. The type of neuron, the structural environment, through which it moves, and the signaling molecules in the extracellular matrix will determine the pattern of migration.

1.3. General modes of migrations

Four general migration modes have been described in the developing mammalian brain, namely, radial migration, tangential migration, chain migration and multipolar migration. All these modes of migration have been studied and described in the developing telencephalon.

1.3.1 Radial migration

Radial migration is defined as the movement of neurons in a direction perpendicular to the pial surface. This kind of migration can happen by somal translocation or can involve glia-guided locomotion (fig.3**a**, **b**).

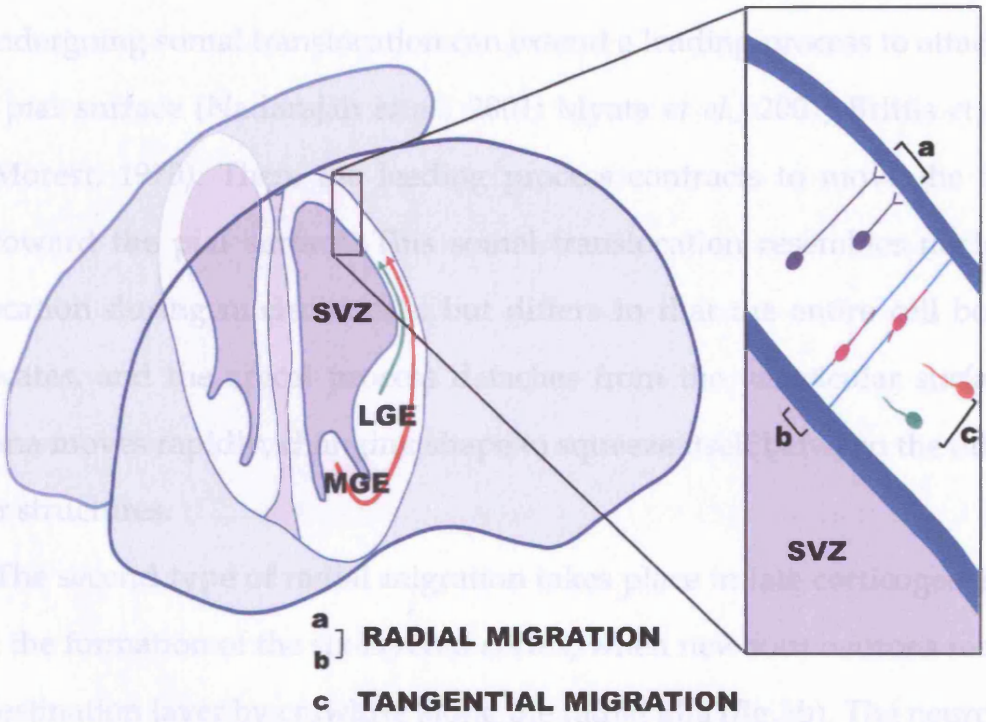


Figure.3. Radial and Tangential migration in mammalian neocortex.

Diagram of the mouse cortex. Neural progenitors, born in the telencephalic ventricular zone, move radially towards the mantle area initially using their own cellular process to grip on the pial surface and translocate their soma **(a)** and at later stages using radial glial process as scaffold **(b)**. Tangential migration occurs parallel to the ventricular zone, neurons that migrate tangentially may use different substrates to guide their migration **(c)**. GABAergic neurons arise from the medial ganglionc eminence (MGE) and from the lateral ganglionic eminence (LGE). Subventricular zone (SVZ). Modified from Rao *et al.*, 2002.

Somal translocation occurs during early stages of corticogenesis (Morest 1970; Nadaraiah & Parnavelas 2002) for neurons that migrate from the ventricular zone (VZ), where they are produced, to the marginal zone

(Fig. 3**a**). At this time, the distance from the VZ to the pial surface is short, so cells undergoing somal translocation can extend a leading process to attach to the pial surface (Nadarajah *et al.*, 2001; Myata *et al.*, 2001; Brittis *et al.*, 1995; Morest, 1970). Then, the leading process contracts to move the cell body toward the pial surface. This somal translocation resembles nuclear translocation during nucleokinesis, but differs in that the entire cell body translocates, and the apical process detaches from the ventricular surface. The soma moves rapidly, changing shape to squeeze itself between the other cellular structures.

The second type of radial migration takes place in late corticogenesis, during the formation of the six-layered cortex, when newborn neurons reach their destination layer by crawling along the radial glia (fig.3**b**). The neurons that adopt glia-guided locomotion have short leading processes that are not attached to the pial surface and show a "saltatory" pattern of movement, *i.e.* short and rapid forward movements followed by stationary phases attached to the glial processes (Nadarajah *et al.*, 2001).

1.3.2. Multipolar migration

Multipolar migration is a kind of radial migration discovered through time-lapsed observations performed in the developing cortex (Tabata & Nakajima, 2003). Neurons migrating in multipolar mode do not acquire any fixed cell polarity, but extend multiple processes very dynamically while

progressing very slowly towards their destination. The freedom of movement allows the multipolar neurons to avoid obstacles, such as afferent and efferent fibers and other previously migrated cells. It has also been observed that occasionally multipolar neurons can jump laterally (tangentially) and so change their final destination, instead of remaining in the same column of neurons as their original neighbours. Contrasting with what happens in the others modes of radial migration, multipolar migrating neurons have only been observed in the subventricular zone (SVZ) and intermediate zone (IZ). Cell tracing suggests that multipolar cells are likely to change their mode of migration from multipolar to radial locomotion when they enter the cortical plate (Godement *et al.*, 1990; Halloran & Kalil, 1994; Tabata & Nakajima 2003).

In summary, the radial migration of neuroblasts from the ventricular zone to the developing cortex occurs in three ways, somal translocation, glial guidance and multipolar migration, depending on the time of development and on the specific guidance cues.

1.3.2. Tangential migration

Tangential migration refers to neuroblast migration that is not oriented vertically towards the developing cortical surface. The neuroblast extends a leading process in the direction of migration and then moves the

nucleus (nuclear translocation) through its long process (fig.3c). The elongation of the leading edge and the nuclear translocation movement is repeated untill the migrating neuron reaches its destination (Noctor, 2001; Moya & Valdeolmillos, 2004).

The guidance cues used by tangentially migrating neuroblasts are not clear. Tagentially migrating neurons move perpendicularly to the radial glia, gliding from one glial process to another, but this process is not radial glial dependent. Some tangentially migrating neurons appear to be associated with corticofugal axons in the intermediate zone (Denaxa *et al.,* 2001), but not all tangentially migrating neurons follow axons. From the sub-pallial, medial ganglionc eminence (MGE; fig.3) early and from the lateral ganglionic eminence (LGE; fig.3) at later stages, GABA-ergic interneurons migrate around the developing striatum and enter in the developing cortex via the intermediate zone. MGE interneurons and LGE interneurons move through the subventricular zone (Anderson *et al.,* 2001) and only later enter the deeper layers of the cortex (Lavdas *et al.,* 1999; DeDiego *et al.,* 1994, Denaxa *et al.,* 2001, Lavdas *et al.,* 1999). The neurons migrating from the subpallium to the pallium avoid the axon-rich zone, so they may use a neuronophilic migration by growth cone extension (Anderson *et al.,* 1997).

An additional tangential migration described outside the telencephalon is that of the cerebellar granule cells, pontine nuclei and inferior olive (IO) neurons along the rhombic lip that uses growth cone of other neurons as substrate to migrate (Gilthorpe *et al.,* 2002; Causeret *et al.,* 2002).

1.3.3. Chain migration

A specialized kind of tangential migration is chain migration. Chain migration does not involve either glial cells or an axonal scaffold, but does involve neuronophilic interactions. This means that the substrates are other neurons migrated previously. An example of this is the stream of neurons of the subventricular zone (SVZ) that at the pallial/subpallial border form the rostral migratory stream (RMS), and migrate attached to each other, forming a tight chain to populate the olfactory bulb (Altman, 1969; Lois & Alvarez-Buylla, 1994; Luskin *et al.*, 1988). It was shown that the polysialated form of neural cell adhesion molecule (N-CAM) provides a positive guidance cue on the cell surface of the rostral migratory stream neurons (Marin & Rubenstein, 2001; Sawamoto *et al*, 2006).

In general, cells moving tangentially do not seem to respect regional boundaries but move across different subdivisions of the forebrain (Heffron & Golden 2000; Letinic & Rakic 2001) or traverse long axonal pathways (Wray, 2001; Spassky *et al.*, 2002) to position themselves in the correct final destinations.

1.3. Radial and tangential migrations generate complexity of connections and circuitry in the developing brain

Radial and tangential migrations are important processes of neural development, which have added complexity to the structures of the brain during the evolution of vertebrates. Radial migration maintains the positional information acquired at early developmental stages and contributes to vertical laminar organization of the forebrain, particularly in mammals. In contrast, tangential migration enriches brain areas with distinct neuronal populations coming from different places and expressing different combinations of genes.

Comparison of the structure of the brain among vertebrates shows that glial scaffolding is important for brain architecture, where neuroblasts migrate vertically from the SVZ. However, the ratio of neurons migrating radially to those migrating tangentially differs from species to species. For example, in rodents most of the cortical interneurons migrate tangentially from the ganglionic eminence and represent 25% of the all-cortical neurons as opposed to 10% of the non-radially migrated neuron in the human cortex (Letinice *et al.*, 2002). In human and non-human primates vertical migration gives 90% glutamatergic pyramidal output neurons, while tangential account for the 10% of total cells that are GABA stellate interneurons. Therefore, the neuron populations that determine the size of the brain in mammals are mainly those migrated radially.

According to Rakic, the increased size of the mammalian brain (and in particular of primate neocortex) is due to an increase in progenitors in the ventricular zone happened during evolution (Rakic, 1995). In fact, the increment of progenitors determines an increment of neurons migrating along the radial glia, which horizontally form the 6 layers of the cortex (earliest born neurons in the deepest layers, while later born neurons in more superficial layers) and vertically form columns called "radial units" (Rakic, 1988; Rakic 1995; Noctor *et al.*, 2001). Thereby, the increment of the radial units gave rise to the enlargement of the surface and determined the formation of the convoluted structures of the cortex that are particularly developed in primates.

On the other hand, tangential migration adds complexity to the brain circuits, because it increases the variety of neurons that populate different regions of the brain. Examples are the GABAergic neurons that migrate from the ganglionic eminance to the layers of the cortex. Or outside the forebrain, the motoneuron precursors of the facial nucleus originate in rhombomere 4 and migrate tangentially to rhombomere 5-7 in both mammals and teleosts (but not in chicks; Studer, 2001).

Together radial and tangential migrations generate a level of complexity in certain brain areas, like the mammalian cortex, that facilitates the establishment of new connections and circuitries that could not otherwise be formed in a more homogeneous structure.

1.4. The Reelin signalling pathway and neuronal migration.

The Reelin pathway is one of the most studied pathways involved in neuronal migration and in particular radial migration. *Reeler* was one of the first neurological mutants isolated in the mouse (Falconer, 1951). It shows inversion of the 6 layers of the cortex, disorganization of the architecture of the hippocampus, cerebellulm and several others non–cortical structures such as the inferior olive, the facial nerve nucleus and other brain stem nuclei (Goffinet, 1992). Moreover, Reelin is involved in dendrite formation and synapse maintenance and consequently has a role in learning and memory (Chen *et al.*, 2005; Weeber *et al.*, 2002; Borrell *et al.*, 1999). Although several components of the Reelin pathway are known, the effect of Reelin signaling on migrating neurons is not well understood.

1.4.1. Reelin

Reelin is a secreted glycoprotein found only in vertebrates. The protein comprises about 3461 amino acids (aa) in size and is approximately 385-kDa. A cleavable peptide (for secretion) is located at its N-terminus. An F-spondin-like domain and a series of eight internal repeats comprising of aa 350-390 follow the signal peptide. These Reelin repeats contain two

subdomains, namely A and B, separated by a short sequence of 30 aa resembling an epidermal growth factor-like motif (fig.4A). This repeat structure is highly conserved through the evolution of vertebrates. Recent studies reveal that the repeats 3-6 are particularly important for receptor binding (Jossin *et al.*, 2003). In essence, Reelin can be cleaved at the level of repeats 2 and 3 as well as 6 and 7 (Lambert de Rouvroit *et al.*, 1999; Jossin *et al.*, 2003), to provide the functionally active segments, namely the central segment.

In the cortex of mammals, Cajal-Retzius cells, the superficial neurons of the marginal zone, express Reelin. In contrast, migrating neurons responding to the Reelin signal express high levels of Dab1 (Howell *et al.*, 1997). The newborn neurons leaving the ventricular zone use the radial glia as a guidance substrate to reach their final position in the cortex. This final position is thought to be determined by receiving the Reelin signal produced by Cajal-Retzius cells in the marginal zone. Thus, when a layer is formed, the neurons that have a later birthday pass through the previous layer to reach the zone where Reelin concentration is higher. They then leave the radial glia to form a new layer just below the marginal zone, above the previously generated layer (fig.4C). This model of cortex development suggests Reelin could be both an attractive signal and repulsive molecule that stops the migration of neurons, when they have arrived at the right place (D'Arcangelo & Curran, 1998). More recently, the *reeler* phenotype has been interpreted as being due to incorrect detachment of the migrating

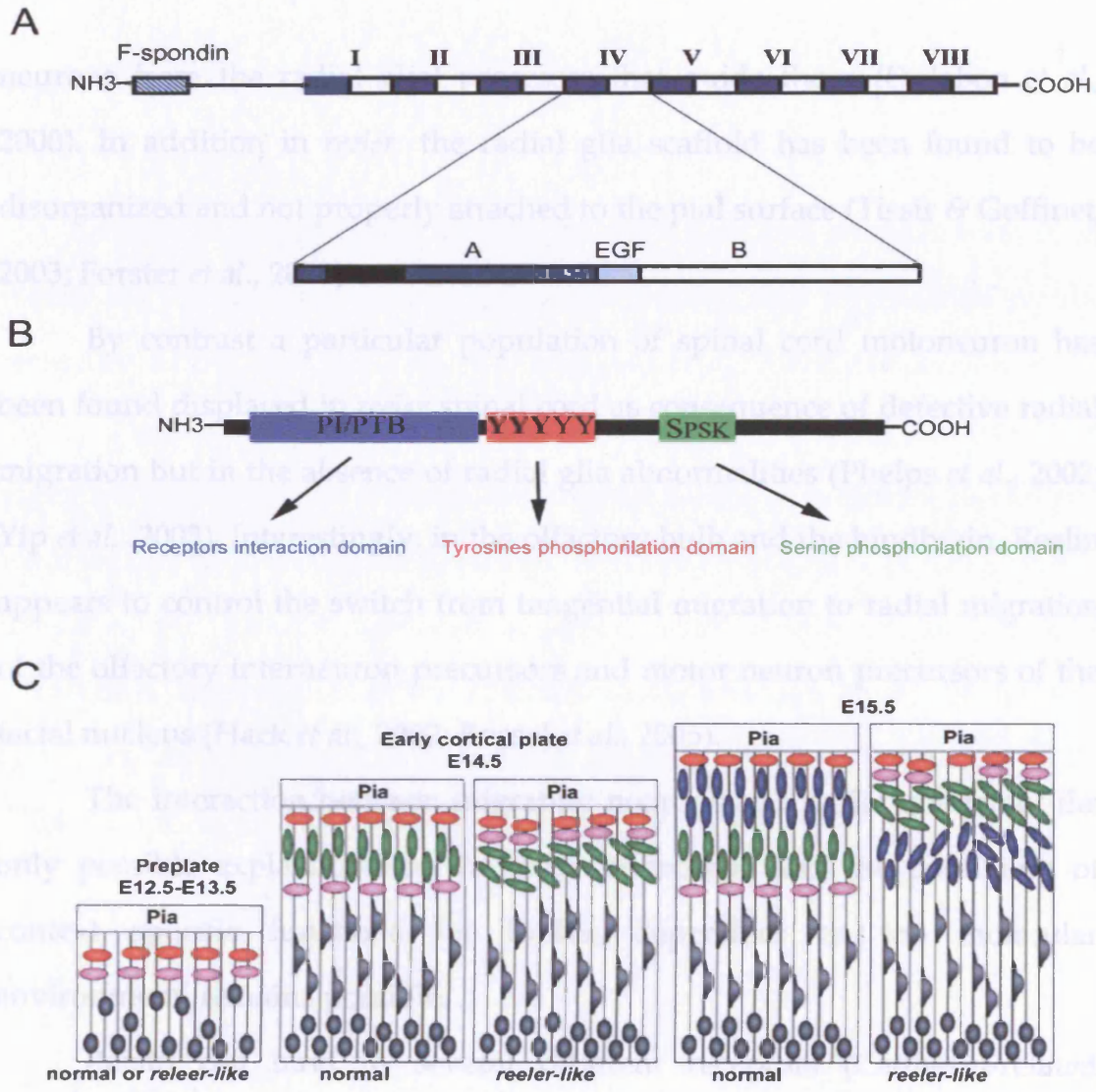


Figure.4. Mutations of Reelin or Dab1 give rise to a *reeler* and *reeler*-like phenotyphe in the neurocortex.

A) Scheme of Reelin, a glycoprotein of 385 kDa and composed by a cleavable F-spondin-like domain at N-terminus, eight internal repeat of 350-390 aa each. The internal repeats contain two subdomains, namely A and B, separated by a short sequence of 30 aa, resembling an epidermal growth factor-like motif. B) Dab1 is a cytoplasmatic protein of about 555 amino acids. It presents at its N-terminus a motif called PI/PTB (Protein Interaction/PhosphoTytosine Binding domain), here depicted in blue that can bind to an Asn-Pro-X-Tyr (NpxY) tetra-amino acid motif. Downstream of the PI/PTB domain a cluster of 5 tyrosines (in red) that can be phosphorylated by non-receptors tyrosines kinases in particular Fyn and Src (Kuo et al., 2005) and a serine phosphorylation domain (Spsk) in green. (C) Comparison between a normal 6-layerd cortex and *reeler*-like cortex. In *reeler* at E 12.5-E13.5 the preplate does not split in subplate and marginal zone and neurons that are rising from the ventricular zone accumulate in an inverted fashion compared to normal. Modified from Tissir & Goffinet, 2003.

neurons from the radial glial processes that guide those (Dulabon *et al.*, 2000). In addition in *reeler,* the radial glia scaffold has been found to be disorganized and not properly attached to the pial surface (Tissir & Goffinet, 2003; Forster *et al.*, 2002).

By contrast a particular population of spinal cord motoneuron has been found displaced in *reeler* spinal cord as consequence of defective radial migration but in the absence of radial glia abnormalities (Phelps *et al.*, 2002; Yip *et al.*, 2003). Interestingly, in the olfactory bulb and the hindbrain, Reelin appears to control the switch from tangential migration to radial migration of the olfactory interneuron precursors and motor neuron precursors of the facial nucleus (Hack *et al.*, 2002; Rossel *et al.*, 2005).

The interaction between migrating neurons and radial glia is not the only possible explanation of the *reeler* phenotype and the possibility of context specific functions for Reelin, dependent on the molecular environment, remains possible.

Reelin can bind to several different receptors (Cadherin-related Neuronal Receptors, VLDLr, ApoER2 and Integrins) and a link with the cytoskeletal machinery involved in neuronal migration was found through the discovery of a downstream adaptor molecule, Dab1. The relation between *reelin* and *dab1* was inferred from the similarities of *reeler* with two other natural mutants, *scrambler* and *yotari* (Sheldon *et al.*, 1997). Genetic studies that phenocopied the mutations with knockout experiments, further confirmed that *dab1* was the gene mutated in these two *reeler*-like mouse lines.

1.4.2. Reelin receptors

Reelin is a huge molecule that can aggregate and complex with itself within the extracellular matrix. It can be cleaved and internalised by the receptors VLDL and ApoERII (D'Arcangelo *et al.*, 1999; Heisberger *et al.*, 1999). These receptors transduce the Reelin signal, starting a cascade of phosphorylation events through the adaptor Dab1. In addition, Reelin can bind Integrins, and Cadherin-related Neuronal Receptors (CNRs). While Integrins are clearly involved together with Reelin in neuron-glial interaction, the function of the CNRs/Reelin interaction is not yet known. However, all the receptors that bind Reelin, including VLDL, ApoER2, CNRs and Integrins, are expressed by migrating neurons.

1.4.2.1 VLDLR and ApoER2

VLDLR (Very Low-densisty Lipoprotein Receptors) and ApoER2 (Apolipoprotein E Receptor 2) are members of the LDL (Low-density Lipoprotein) receptors family. Lipoproteins receptors are transmembrane glycoproteins involved in the metabolism of cholesterol and triglycerides. They consist of an extracellular domain, a single transmembrane domain and a cytoplasmic tail transducing signals important during embryonic development (Howell & Herz, 2001).

The extracellular domain of LDL receptors, formed by EGF precursor homology repeat motifs, is involved in ligand binding and in the dissociation of ligands from the receptor in endocytic vesicles. The transmembrane domain is important for anchoring to the membrane and the cytoplasmic domain, containing the NPXY (Asn-Pro-X-Tyr) motif, is required for directing the coated pits internalization.

The intracellular domains of LDL shows binding to a variety of proteins that takes part in cell adhesion, kinase signalling, cytoskeletal organization, vesicle transport and synaptic transmission *in vitro* (Gotthardt *et al.,* 2000; Hussain, 2001). Lipoprotein receptors can internalize their ligands through a mechanism involving the cytoplasmic NPxY motifs (Chen *et al.,* 1990; Herz *&* Bock, 2002). It was shown that Reelin, after binding to VLDLR, is internalized by vescicular endocytosis. This is thought to be part of the mechanism for Reelin's turnover (D'Arcangelo *et al.,* 1999).

Although Reelin can bind several kinds of receptors, only the double knockout of VLDLR and ApoER2 results in a phenotype almost identical to *reeler*. The only difference it is at level of the motor nuclei of the hindbrain, which are displaced in *reeler* but not in the VLDLR and ApoER2 double knock out mouse (Rossel *et al.,* 2005). The single knock out of VLDLR or ApoER2 does not cause a *reeler*-like phenotype, suggesting a redundancy of these Lipoprotein receptors (Hiesberger *et al.,* 1999).

1.4.2.2. Integrins receptors

Integrins are cell-surface glycoproteins involved in cell migration. These adhesion proteins mediate cell-cell interactions via the extracellular matrix (ECM) and the internal cytoskeleton. The name Integrin comes from the function of these molecules to integrate the extracellular and intracellular scaffold, allowing coordinated cellular movements.

The Integrins are formed by two distinct subunits α and β and the combinations of different α and β subunits enable the Integrins to bind differentially to extracellular molecules including Fibronectin and Laminin. While both α and β subunits bind extracellular molecules, only the β subunits connect to the cytoskeleton, via adaptor proteins such as Paxillin, Vinculin, or Talin, as well as cytosolic tyrosine kinases such as focal adhesion kinase (FAK). Integrins can promote (throughout Rho GTPases) Rac1 activation allowing Actin polymerization and protrusion of lamellipodia that push forward the leading edge to regulate cell migration (Barberis *et al.*, 2004).

Integrins are implicated in the association between migrating neurons and radial glia. Antibodies against integrins reduce the rate of migration and cause detachment from radial glial fibres *in vitro* (Anton *et al.*, 1999; Dulabon *et al.*, 2000). Reelin can bind only α3β1 integrin receptors expressed in radially migrating neurons (Dulabon *et al.*, 2000). It has been observed that neurons lacking the α3β1 Integrins are less sensitive to radial detachment induced by Reelin (Dulabon *et al.*, 2000), suggesting a role of

Reelin pathway in the separation of migrating neurons from the radial glia fibers in cortical layers formation. However, loss of α3 or β1 Integrins is not sufficient to give rise to *reeler* phenotype (Anton *et al.*, 1999; Graus-Porta *et al.*, 2001), suggesting that the full story is far from clear.

1.4.3. Dab1

Dab1 is a cytoplasmatic protein that shows a motif called PI/PTB (Protein Interaction/PhosphoTytosine Binding domain) at its N-terminus. The PI/PTB domain can binds to an Asn-Pro-X-Tyr (NpxY) tetra-amino acid motif present in several proteins including those of the LDL receptor family and amyloid precursor family (see fig.4B). Mouse *Dab1* encodes a protein of 555 amino acids. The sequence shows a cluster of 5 tyrosines downstream of the PI/PTB domain, with which Dab1 can bind the SH2 domains of non-receptor tyrosine kinases, in particular Fyn and Src (Arnaud, *et al.*, 2003; Kuo *et al.*, 2005). Dab1 is present in the developing CNS and, when tyrosine phosphorylated, can initiate a phosphorylation cascade with multiple targets (Howell *et al.*, 1997). These targets finally determine the phosphorylation of proteins associated with microtubules that can lead to cytoskeletal modifications.

In the mouse two mutants for *dab1*, *scrambler* and *yotari* (Sheldon *et al.*, 1997) show a phenotype very close to the mutant *reeler* (Sweet *et al.*, 1996; Yoneshima *et al.*, 1997), the only differences currently described being in the

distribution of callosal commissural neurons and pattern of layer V neurons (but not layer VI neurons) of the cortex (Aoki *et al.*, 2001; Yamamoto *et al.*, 2003).

During the development of the cortex, the expression of *reelin* and *dab1* are contemporaneous and complementary. The superficial layer of neurons expresses *reelin* and the migrating neurons express high levels of *dab1* (Howell *et al.*, 1997b). If *dab1* is mutated or not functional, the neurons (that usually produce it) cannot respond to Reelin signal, cannot migrate properly and cannot form the layers of the cortex in the normal inside-outside pattern.

The *dab1* gene is highly complex. It has 14 exons that give rise to at least five alternative tissue-specific splice variants in the mouse and several 5'-untranslated regions (UTRs) with different promoters (Bar *et al.*, 2003). Moreover, two isoforms ChDab1-L and ChDab1-E are involved in circuitry formation in chick retina (Katyal & Godbout, 2004).

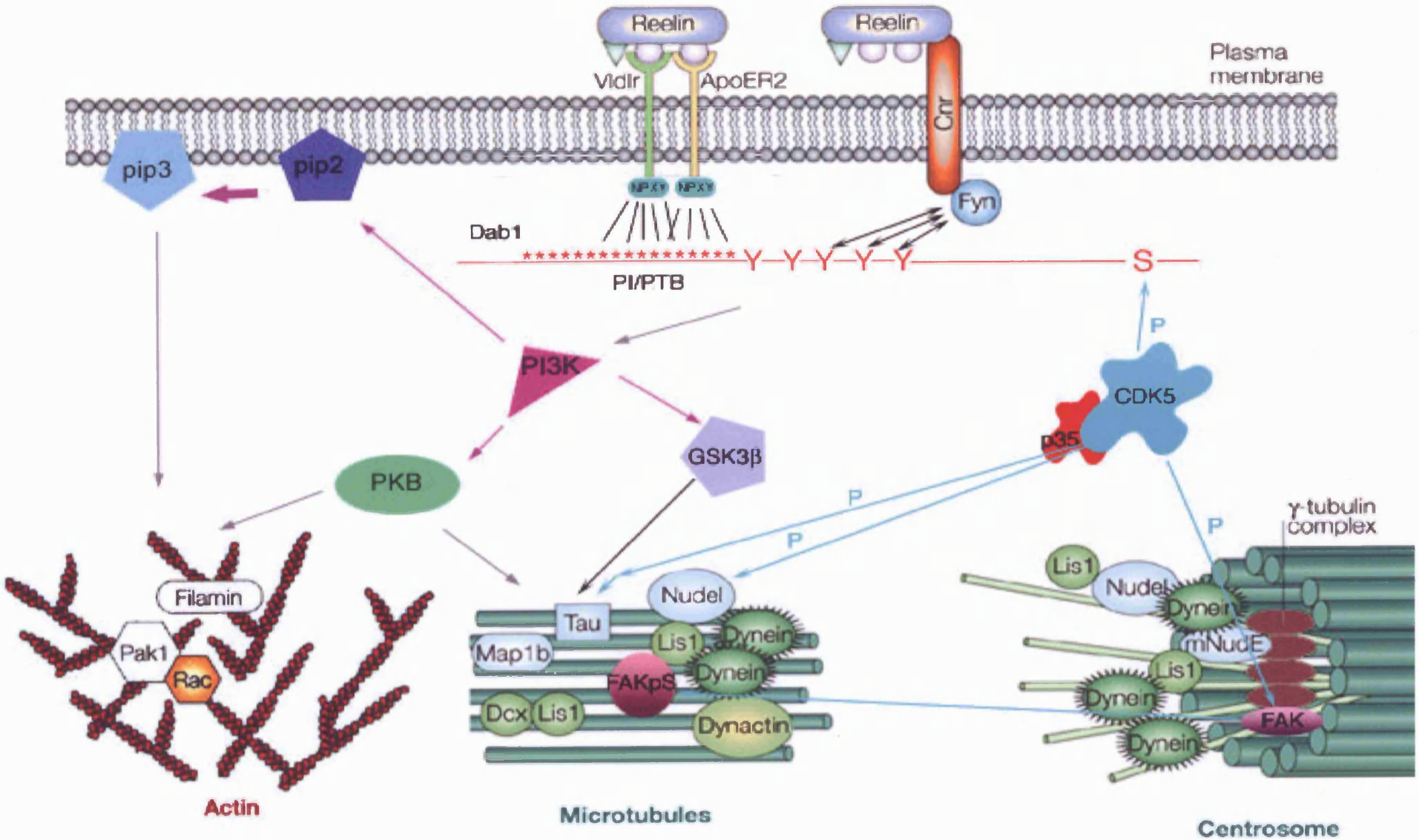
The complexity and different isoforms of the *dab1* gene may have impeded the identification of mutations in human *DAB1*, while in mouse the natural occurring mutations *scrambler* and *yotari* are not null mutations and might retain some vital functions (Sheldon *et al.*, 1997).

1.4.3.1. Molecules targets of Dab1

The binding of Reelin to its receptors initiates a phosphorylation cascade starting with the triggering of Dab1 by the Fyn and Src kinases (Arnaud *et al.*, 2003; Kuo *et al.*, 2005). The molecular targets of phosphorylated Dab1 are not well known, but new findings are showing new relationships between the Reelin pathway and molecules involved in cytoskeletal dynamics and neuron migration (fig.5). Molecules recently found as downstream to Reelin phosphorylation signaling are CrKL, C3G, Rap1 small GTPase and PI3K.

Figure. 5. Reelin pathway.

The pathway is composed of the signal, Reelin, a large glycoprotein secreted by specific groups of cells. Reelin can bind at least three different receptors, namely the Integrins, the Lipoprotein receptors VLDL and APOER2, which functions as heterodimers in this pathway, and the CNRs receptors and an intracytoplasmic adaptor, Disabled1 that appears to mediate all Reelin migratory functions. Unphosphorylated Disabled1 binds to the NPXY motif in the cytoplasmic tail of the Lipoprotein receptors and upon Reelin binding becomes phosphorylated, primarily by Fyn. Reelin regulate the phosphatidylinositol 3-kinase (PI3K). The phosphorilated Dab1 binds the PI3K that phosphorilates the phosphatidylinositol 4,5-bisphosphospate to phosphatidylinositol 3,4,5-trisphosphospate. The phosphatidylinositol 4,5-bisphosphate is involved in the regulation of cytoskeletal reorganization. Moreover, the activation of PI3K leads to the activation of the serine/threonine kinase (Akt or protein kinase B) and the inhibition of the glycogen synthase kinase 3β (GSK3β). The GSK3β activated by Reelin signaling is involved in the phosphorylation of proteins, such as Tau, implicated in axonal transport. The Cdk5 pathway is parallel to the Reelin pathway. Cdk5 can phosphorylate Dab1 on a serine at the C-terminus. Cdk5 is involved in cytoskeletal changes and in neuron migration. Modified from Gupta *et al.*, 2002.



Modified from: Gupta, Tsai and Wyshaw-Boris (2002) **Nature Reviews | Genetics**

1.4.3.2. Dab1/CrkL/C3G/Rap1 pathway coordinates neuron migration

Reelin via Dab1 stimulates tyrosine phosphorylation of C3G (guanine nucleotide exchange factor) activating the small GTPase Rap1. Rap1 (**R**epression and **a**ctivation **p**rotein) regulates cell-cell adhesion and cell migration (Caron, 2003). When Dab1 is phosphorylated on tyrosine, Y^{220} and Y^{232} interact also with the adaptor Crk-Like (Crk-L) proteins and these two particular sites of phosphorylation are involved in the migration of cortical plate neurons. As Crk-Like also binds C3G, the Dab1/CrkL/C3G/Rap1 pathway is believed to be involved, in response to Reelin signaling, in the coordination of the migrating neurons during development (Ballif *et al.*, 2004).

1.4.3.3. Reelin regulates PI3K

Phosphatidylinositol 3-kinase (PI3K), a membrane-associate molecule involved in several intracellular processes, is regulated by Reelin during the formation of the cortical plate in *in vitro* assays (Bock *et al.*, 2003). When Reelin binds the complex of its receptors the phosphorilated Dab1 binds the PI3K regulatory subunit p85α. The activation of the PI3K results in the phosphorilation of the precursor phosphatidylinositol 4,5-bisphosphate for PI 3,4,5-trisphosphosphate (Bock *et al.*, 2003). Phosphatidylinositol 4,5-bisphosphate is involved in the regulation of cytoskeletal reorganization (Yin & Janmey, 2003).

Moreover, the activation of PI3K leads to the activation of the serine/threonine kinase (Akt or kinase B) and the inhibition of glycogen synthase kinases 3β (GSK3β), a molecule downstream of Wnt signalling involved in the metabolism of the β–catenins, hence in neural induction and patterning (Niehrs, 1999; Heisenberg *et al.*, 2001). In addition, GSK3β activated by Reelin signaling is involved in the phosphorylation of Tau proteins involved in axonal transport and axon growth (Gordon-Weeks, 2004; Ballif *et al.*, 2004).

The discovery of new pathways downstream of Reelin are suggestive of new functions that the Reelin pathway might regulate, for example a possible role of there may be a role for the Reelin pathway in axon growth, in addition to the well-known functions of Reelin in neuron migration, in dendrite formation and maintenance (Niu *et al.*, 2004).

1.4.4. CDK5, Lis1 and DCX are in pathways parallel to the Reelin pathway

1.4.4.1. CDK5 pathway

Another pathway known to be involved in migration of cortical neurons is the Cyclin-dependent kinase-5 (Cdk5) pathway (fig.5). Cdk5 is a member of the family of cell cycle-related kinases that can activate two subunits: p35 and p39 that are expressed only in the brain (Lew *et al.*, 1994;

Tsai *et al.*, 1994; Tang *et al.*, 1995). The mouse mutated in Cdk5, p35 or both p35 and p39 show defects of cortical lamination similar to that in *reeler*.

When Cdk5 is mutated, the splitting of the preplate into a marginal zone and subplate is normal as in wild type situation (fig.4C), but the cortical layers show an interval pattern similar to that in *reeler*, where the splitting of the preplate never happens (Chae *et al.*, 1997, Gilmore *et al.*, 1998, Ko *et al.*, 2001, Kwon & Tsai 1998, Oshima *et al.*, 1996). Also the cerebellum of mouse *Cdk5-/-* shows laminar disorganization as in *reeler*, but the Purkinjie cells are displaced due to a cell-autonomous effect and *reelin* and *dab1* mRNA were found normal (Ohshima *et al.*, 1999). Inside the cell, Cdk5 phosphorylates Dab1 and it works as linker with Lis1, allowing interaction between Dab1 and Dynein (associated with Lis1). In this way Cdk5 may regulate nucleokinesis during the formation of cortical layers, which is dependent on Lis1 functions (Niethammer *et al.*, 2000).

Another link between the Reelin pathway and cytoskeletal re-organization is the phosphorylation of Tau proteins and the control of axonal transport (see above paragraph 3.3.1.2). Cdk5 may activate Tau phosphorylating directly GSK3β and cooperating with it at the level of the cytoskeleton (Beffert *et al.*, 2004). Both Reelin and Cdk5 are involved in neuron migration and axonal transport, but in a parallel and cooperative manner, because inhibition or activation of one pathway does not affect the other at the cellular level (Niethammer *et al.*, 2000; Beffert *et al.*, 2004).

1.4.4.2. Doublecortin (DCX)

DCX is a microtubule-associated protein (MAP) that interacts with and controls the microtubule cytoskeleton. Female patients with one copy of *DCX* mutated have a neuropathology of incorrect neuronal migration, called subventricular band heterotopia or doublecortex syndrome. A normal-looking layered extra cortex is formed in the white matter. For this reason the gene has been called "doublecortin" (Gleeson *et al.,* 1998; des Portes *et al.,* 1998). Male patients show a severe lissencephaly, due to the X-chromosomal location of the *DCX* gene.

In vitro DCX stabilizes microtubules in transfected cells (Yoshiura *et al.* 2000). The protein contains two repeats named DCX domains that mediate the binding to microtubules. Recent evidence shows that the N-terminal of this domain binds to microtubule polymers, whereas the C-terminal domain binds Tubulin dimers (Kim *et al.,* 2003). In this way the C-domain may catalyze microtubule growth by stimulating formation of new dimers of Tubulin, and together with its N-terminal can stabilize the microtubule polymer (Schaar *et al.*, 2004).

DCX is thought to be involved in nuclear translocation and axon growth. DCX is not localized along the whole microtubule network in the cell, but is associated with microtubules of growth cones and leading process of migrating neurons. A balanced level of phosphatase and kinase activities maintains this subcellular localization of DCX. *In vitro* studies show that a serine residue of DCX is required for the binding to

microtubules under the control of MARK, PKA kinases (Schaar *et al.*, 2004) and Cdk5 regulates the binding affinity of DCX for microtubules (Fig.3C; Tanaka *et al.*, 2004).

DCX is coexpressed with Lis1. They can interact and function in the same protein complex in cells of the developing brain (Caspi *et al.*, 2000). This means that both LIS1 and DCX may coordinate similar process in neuronal migration, for example somal or nuclear translocation during cell movement. Interestingly, DCX appears to be necessary for the transition from multipolar to bipolar shape in radially migrating neurons of the embryonic cortex (Bai *et al.*, 2003).

1.4.4.3. Lis1

Lis1 is the noncatalytic subunit of the platelet–activating factor acetylhydrolase *Pafah1b1*. However, a two cell-hybrid screen in human brain resulted in the identification of NUDEL (a substrate of Cdk5; M. Niethammer *et al.*, 2002) as a Lis1 partner and of other cytoskeletal components indicating that Lis1 is involved in multiple protein-protein interactions (Feng & Walsh, 2001).

The importance of Lis1 in neuron migration emerged through the finding of a human neuropathological condition that shows a severe lissencephaly called Miller-Dieker syndrome (Hattori *et al.*, 1994; Reiner *et*

al., 1993). Lis1 haplo-insufficient mutations result in a reduction in number of neurons migrating correctly to their lamination position.

In humans, Reelin mutations are associated with a form of lissencephaly that is similiar to that due to Lis mutation (Hong *et al.*, 2000). The relation between Reelin and Lis1 is not well understood, but it was shown that Lis1 and the Reelin adaptor Dab1 bound in response to Reelin signaling (Assadi *et al.*, 2003).

In mice, the expression of a truncated form of Lis1 gives rise to a delayed migration of neurons (Cahana *et al.*, 2001) in a manner similar to that of human lissencephaly. Mice, that completely lack Lis1 function, die very early because of defects in cell division (Hirotsune *et al.*, 1998).

Lis1 can bind proteins such as cytoplasmic Dynein, which interact with the microtubule network and bind directly to tubulin. This is seen in all organisms studied (Sapir *et al.*, 1997). Dynein is a microtubule–based motor protein that is involved in transport of organelles and retrograde axonal transport. Lis1 interacts with Dynein and Dynactin and is involved in mitosis of the cortical progenitors and in general in axon growth and cell migration (fig.1A, C; Niethammer *et al.*, 2000; Tsai *et al.*, 2005).

Reelin, CDK5, DCX and Lis1 are components of a large and intricate series of signaling systems that regulate neuronal migration, nuclear distribution and cortical layering of the mammalian brain.

1.5. Zebrafish as an animal model system for studying neuronal migration

The zebrafish (*Danio rerio*) is a small tropical freshwater fish (fig.6). It has a short generation time of 2-3 months and produces large number of embryos (the average is 100 per mating). The rapid development and external fertilization make the study of developmental processes easier to study than in other vertebrate models. Moreover, the transparency of the embryo (prolonged for more than 24 hours by inhibiting pigment development with 1-**p**henyl-2-**th**io**u**rea (PTU) in the medium water) facilitates imaging of developmental processes in wild type and mutant embryos. Mutagenesis of the zebrafish genome obtained using ENU (**e**thyl**n**itro**u**rea-mediated) and TILLING (**t**argeting **i**nduced **l**ocal **l**esions **in** **g**enomes) techniques gives the opportunity to analyse the function of target genes. Mutagenesis screens are powerful methods to understand gene function during development. In addition, the cloning of genes affected at the mutant loci is now easier due to the near completion of the zebrafish genome sequence (Talbot & Hopkins, 2000).

Molecular approaches, including injections of mRNA or morpholino oligonucleotides (see Chapter 4 and 5) at blastomeres stages (Heasman, 2002), are also possible, while embryological manipulations, such as cell transplantions, are becoming more and more refined techniques (fig.7).

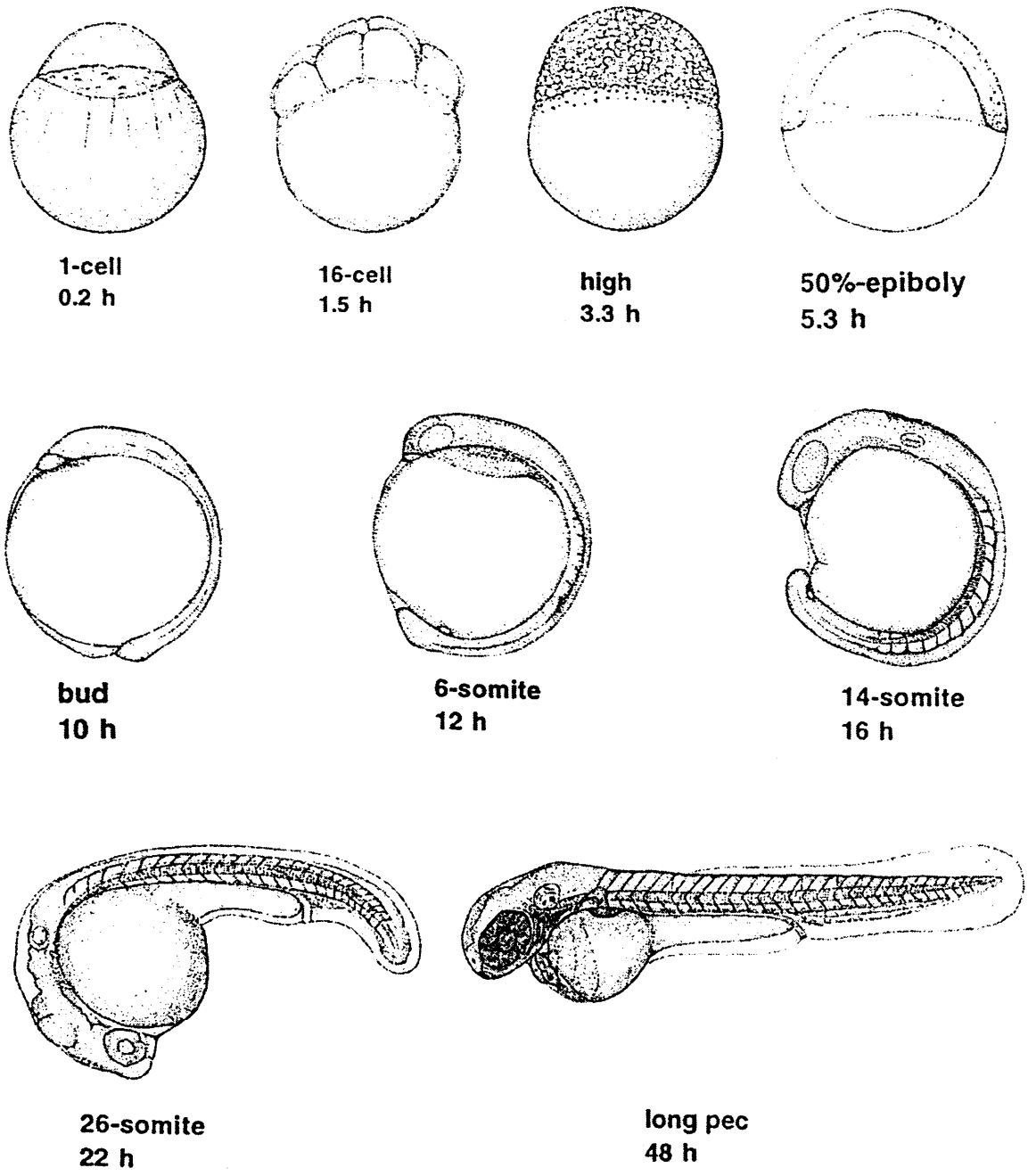


Figure.6. Zebrafish development from one cell stage to 48 hours post fertilization (hpf).

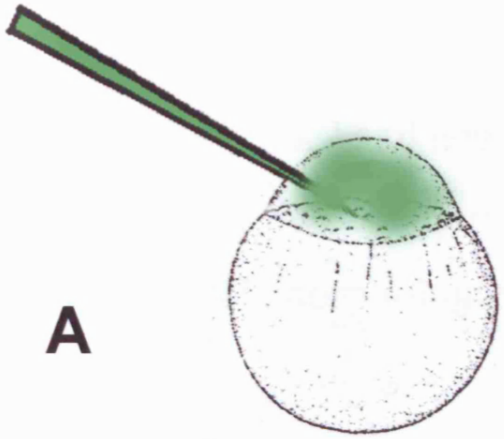
Zebrafish are also amenable to the creation of transgenic lines expressing the reporter green fluorescent protein (GFP) under the control of specific promoters (Jessen *et al.*, 1998). Zebrafish transgenic lines are now used especially for *in vivo* imaging. The fluorescence of the reporter protein is obtained by illuminating the live embryos with a UV lamp under the dissecting microscope or using confocal microscopy (with or without time-lapse photography).

1.5.1. The ancient duplication of the teleost genome is sometimes an advantage for functional analysis of the genes

Zebrafish, like several other species, possess duplicate copies of some genes. This is believed to be due to ancient whole-genome duplication about 350 million of years ago, probably just before the teleosts radiation (Postlethwait *et al.*, 1998). Zebrafish often presents two (or more) orthologs of human genes, *i.e.* gene descending from a single gene in the last common ancestor (Postlethwait *et al.*, 2004). This complexity in zebrafish is amazingly

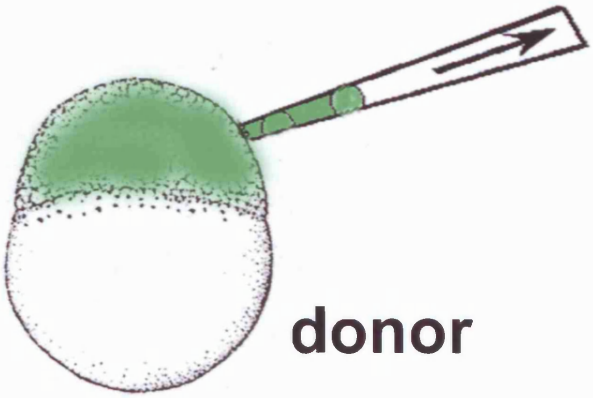
Figure.7. Transplant at early stages in the zebrafish.

A) The first step is the injection at one cell stage of the GFP mRNA as reporter, alone for lineage studies or together with a construct or morpholino of interest. B) At blastula stage the cells from the injected donor can be transplanted (C) in the host embryo at 50% epiboly or shield stage. In D, chimaeras of 30 hours post fertilization stage. This approach can be used for fate mapping, random labelling for tracking cell movements, for local or restricted expression of genes, whose product would be toxic or destructive if expressed in the whole embryo, and analysis of cell autonomy.



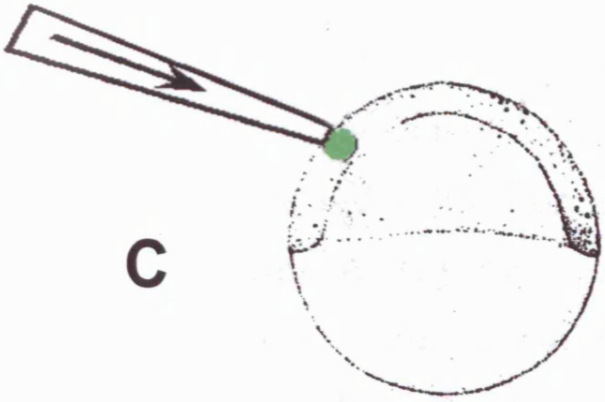
A donor

1-cell



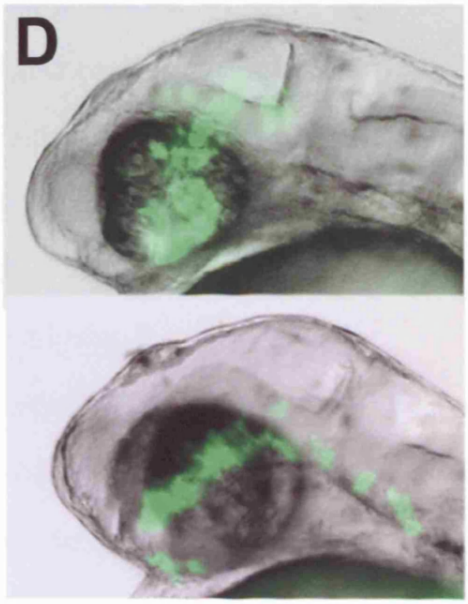
B donor

high



C host

50%-epiboly



D

becoming seen as a point of strength in the study of genes conserved among vertebrates, because of the "sub-function partitioning" common in zebrafish genes. The sub-function partitioning is the segregation of a function between two genes that originate from a common ancestral gene.

For example Nodal, a gene that in mouse is present as a single copy, in zebrafish is triplicated so that there are two Nodal genes functioning at early stages and a third at later stages: *cyclops*, *squint* and *southpaw*. Mutation of one or both the early expressed genes (*cyclops* and *squint)* showed that one is involved in mesoderm induction and the other in the later event of the neural plate formation (Feldman *et al.*, 1998; Sampath *et al.*, 1998). Unlike in zebrafish, the null mutation of Nodal in the mouse blocks the developing embryos at an early stage making it difficult to investigate Nodal function in later events (Varlet *et al.*, 1997). Abrogation of function of both Nodal genes in fish gives a more severe phenotype more similar to the single mouse knockout.

Another advantage is the functional analysis of genes that in mammals are haplo-insufficent. For instance, the mammalian heterozygote mutant for SOX9 dies because of defects in cartilages formation, as one allele of the SOX9 is not sufficient to support the normal development of the embryos (Bi *et al.*, 2001). In zebrafish there are two *sox9* and their mutations give rise to a recessive embryonic lethal phenotypes rather than dominant-lethal phenotypes as seen in mouse (Yan, *et al.*, 2002). The phenotypic analysis of the zebrafish mutants revealed that Sox9 is involved first in chondrocyte stacking and in cartilage formation.

Both cases of zebrafish sub-functional partitioning gave the opportunity to study the functions of genes that somewhat intractable in mice.

1.5.2. Neuronal migration in Zebrafish

The zebrafish CNS presents a subdivision of the main forebrain areas that is similar to the "more evolved" brains of other vertebrates (fig.8A, B). What is not well known is the pattern of neuronal migration during zebrafish brain development and whether this pattern is similar or different from that in other vertebrates.

The first difference to point out is the development of the zebrafish telencephalon that, as all the teleosts, is everted and not evaginated as in other vertebrates (fig.8 C; Hodos & Butler, 1996; Butler, 2000). The morphogenetic movements that give rise to an everted brain are completely different from that of an evaginated brain and do not result in a layered structure as in reptiles and mammals. However *dlx*, a vertebrate subpallial marker (Anderson *et al.*, 1997), is expressed in zebrafish telencephalon and olfactory bulb suggesting that a migration from the subpallial region to the pallial region may occur (as in the case of MGE tangential migration: see paragraph 1.1.3 and fig2).

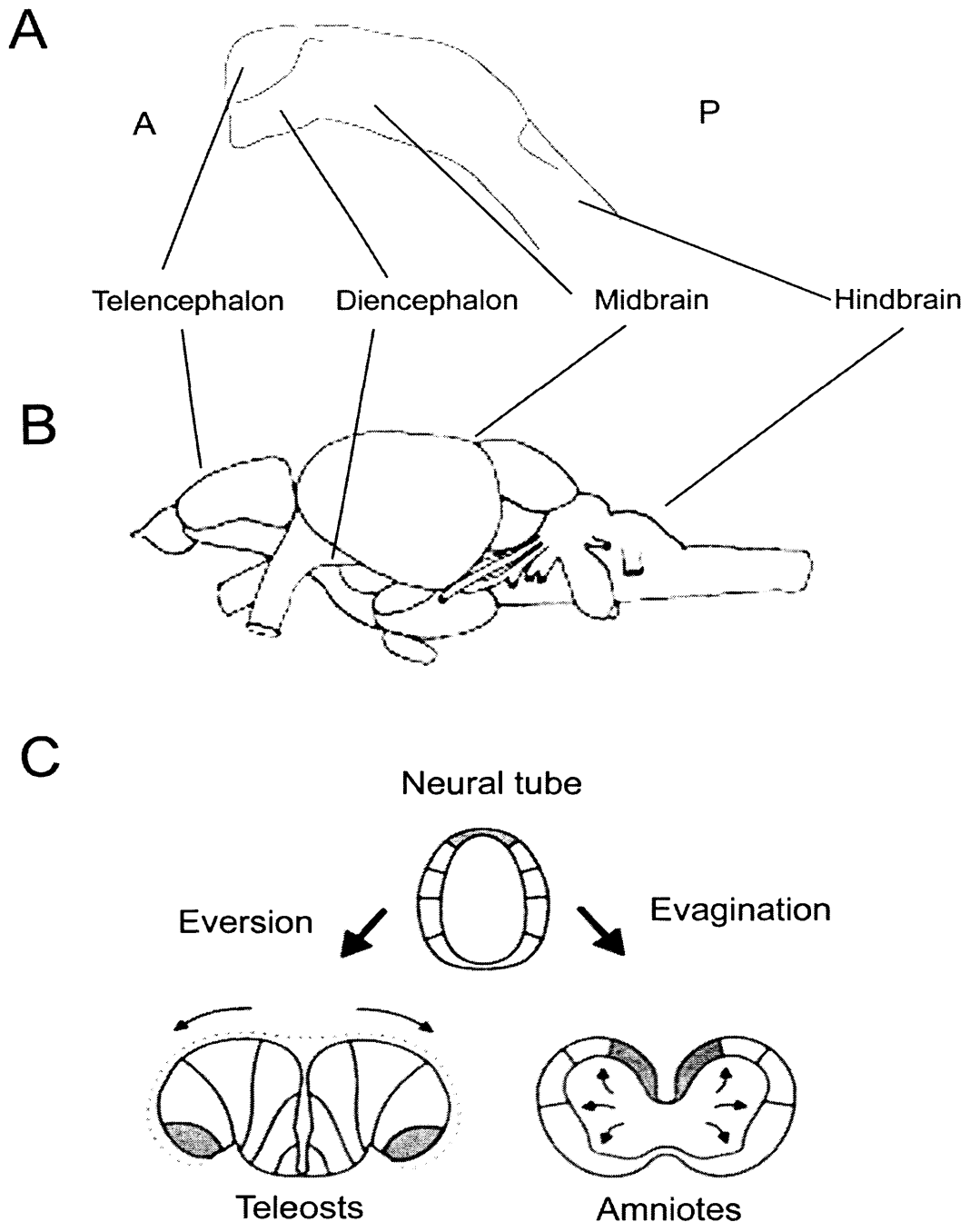


Figure.8. The zebrafish brain.

In A, schematic draw of 24hpf embryos. All the main subdivisions of the brain are already visible. In B, brain of adult zebrafish. C shows the comparison between the processes of evagination and eversion, adapted from Butler, 2000. The everted telencephalon is covered by a thin choroid tela, a structure that originates in the dorsal neural tube (in gray).

At the moment we do not have a clear understanding of the neuroanatomy of the zebrafish brain that is comparable to that of the mammals, but there are several tools for the studying *in vivo* neuron migration in zebrafish. For example the *dlx*4/6:GFP line produced by Mark Ekker (Park *et al.*, 2004) allows the observation *in vivo* of the migration of neurons in forebrain and midbrain/hindbrain. In fact, an enhancer of *dlx4* and *dlx6*, fused with coding sequence of GFP, marks cells of the ventral telencephalon, ventral thalamus to the hypothalamus and rhombic lip.

A clear example of tangential migration of zebrafish has been described for the first time *in vivo* (Higashjima *et al.*, 1998). They created a fluorescent transgenic line under the promoter of *islet1* gene (a marker of branchiomotor nuclei of the hindbrain) and were able to record the migration of the VII branchiomotor neurons from rhombomere 4 to their final location in rhombomeres 6-7.

In my work I have used both *dlx4/6*:GFP and *tg(islet-1-GFP)* lines to better characterize the involvement of Reelin pathway in neuron migration.

1.6. Aims of the work

In the first part of the introduction I have described the principal mechanisms and the principal molecules involved in neuron migration during development of the vertebrates CNS. In the last part, I introduced

zebrafish as a good model to study, *in vivo,* these important mechanisms and molecules.

Although the Reelin pathway has been extensively studied in mouse brain cortex formation, recent discoveries have suggested a more general but not less important roles for this pathway in vertebrate development (Hack *et al.,* 2002, Yip *et al.,* 2004).

In this present work I focused on three main points:

1. The description of the expression pattern of *reelin* and *dab1* in the zebrafish brain and other non–neuronal structures.
2. The genomic structure of *Danio rerio dab1* to obtain more insight into the role the Reelin/Dab1 pathway in vertebrate evolution.
3. The study of possible functions of dab1 isoforms using several *in vivo* techniques as morpholino injection, mRNA overexpression and visualization by confocal microscopy of neuronal migration using GFP constructs as specific reporter gene.

CHAPTER 2

2. Materials and Methods[1]

2.1. Isolation of the *Danio rerio reelin* and *disabled1* cDNA clones

2.1.1. *reelin* cDNA clone

A cDNA-arrayed library made from zebrafish adult brain constructed by J. Ngai (Berkeley) and distributed by the Resource Center Primary Database (RZPD http://www.rzpd.de) was screened with a 1.3 Kb probe corresponding to nt. 4630-5956 of mouse *Reelin*. The probe was obtained through PCR (using the following primers: 5'GAGATGTTCGACAGGTTTGAG3' and 5'GTGACTCCTCCACTGACAGAG3'). Two positive clones were identified, obtained from RZPD and sequenced by a commercial sequencing service (MWG, DE). The EST corresponding to *Xenopus reelin* (acc.no. AW158822) was obtained from Cold Spring Harbor laboratories, and sequenced entirely by MWG.

[1] For standard techniques see Appendix

2.1.2. *disabled1* cDNA clone

To identify zebrafish *disabled1* cDNA clones a microarrayed adult brain zebrafish library (distributed by RZPD; library no. 611) was screened a with a cDNA fragment corresponding to nucleotide (nt) 450-1311 of mouse *Dab1-555* (genebank acc. no. NM_010014). This fragment was obtained through PCR using an E13.5 mouse brain cDNA as template. Hybridization yielded a number of positive clones, which were analyzed for their sequence and expression pattern. Conceptual translation yielded an open reading frame of 538 amino acids, similar to that of mouse and human Dab1. Screening the available zebrafish EST databases for possible dab1 isoforms revealed only one entry with this feature (acc. No. gi: 38652136) corresponding to the first 184 nt in the 5'UTR region of our clone and then changing abruptly. No ORF was present in this clone. Other EST clones corresponded to parts of *dab1_tv1* (see below). However it was predicted and confirmed with RT-PCR the presence of different isoforms of zebrafish *dab1* on the basis of the analysis of the genomic sequence (see below).

2. 1. 3. RT-PCR

Total RNA was extracted at various stages from pools of approximately 50 zebrafish embryos and larvae at the following stages: 1-32 cells, 8 somites, 30 hpf, 48 hpf, 3 dpf and 5 dpf and from 3 adult brains,

using Trizol (Invitrogen). The synthesis of cDNA from 500 ng total RNA was primed by oligodT or random primers and performed with Superscript II RT (Invitrogen) for 1 h at 42°C following the manufacturer instructions.

The following primers were used:

Forward exon 3:	CGTTTTAAGGGAGATGGCGTTC
Forward exon 5:	CTACATCGCGAAGGATATCAC
Reverse exon 6:	ATCTCCTCCCTCGTTAATCTC
Reverse exon 8:	TGATATATGCTCTCCTCTGATGG
Reverse exon 9 (probe):	TCACTGGATGTCGCTTTGGGA
Forward exon 8 (probe):	CATTGTATTTGAGGCGGGACAC
Reverse exon 10:	GGACATGTCTCCAAAAAGCTC
Forward exon 10:	GACTTTTTGGAGACATGTCC
Reverse exon 11:	CAAGGGGTCCAGAGTGTTAGCC
Forward exon 12:	CAGAGACAGGCAAAGATGAGCAAG
Reverse exon 12:	CCAAACGCTAATGGAGCCTGAG
Reverse exon 15:	GATCTGGGCATAAAGAGGGTCCTTCC

2.1.4. Sequence and genome alignments

For the identification of the genomic clones containing the *dab1_tv1* sequences and the localization of the exons the Blast tool from NCBI (http://www.ncbi.nlm.nih.gov/blast) was used. The database used consisted of BAC clones fully sequenced by the Sanger Institute and deposited in

Genebank without annotations. The zebrafish sequences used for exon searching were selected on the basis of comparisons with the reported mouse exon/intron boundaries (Bar et al., 2003). For the identification of the sequences encoding for exon 8 and 9 was used mouse sequences to blast the clone BX248232. The same was done for the missing exons: 555*, 217*, and 271*.

For the comparative genomic analysis, it was used the Blat tool (Kent, 2002) at UCSC (http://genome.ucsc.edu/) and the Zv4 release at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/) for the zebrafish genome, the NCBI Build 35 release for the human genome, the NCBI Build 34 release for the mouse genome and the WUSTL Feb 2004 release for the chick genome.

2.2. Embryological Techniques

2.2.1. Experimental animals and tissue preparation

Danio Rerio embryos and adult fish from UCL fish facility were used in all experiments. Fish were raised in 20 liter tanks at 28°C with a 14 hours light/10 hours dark cycle. Eggs were collected daily and incubated in purified water containing 0.001% methylene blue at 28°C. The animals were handled in accordance with European Union regulations on laboratory animals.

Embryos to be processed for bromodeoxyuridine (BrdU) uptake were dechorionated and exposed to BrdU, 1 mg/ml in purified fish water for 2 hours before fixation.

Paraformaldehyde fixed embryos (stage 32 and 50) of *Xenopus laevis* were a gift of Les Dale, UCL. Animals were terminally anaesthetized with 0.3% tricaine methane sulphonate (MS222, Sigma) and those of 1 month or older were decapitated. All embryos, larvae and adult animals were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. Those to be processed for cryosectioning were rinsed in PBS and equilibrated in 10% and 20% sucrose in PBS at 4°C over 48 hrs. Tissue was embedded in OCT compound (Agar, UK), frozen on dry ice and cut serially at 20 um in coronal and saggital planes. Sections were collected on Superfrost slides (BDH, UK) and stored at –70°C in sealed boxes until ready to use.

Embryos to be processed for whole mount *in situ* hybridization were rinsed in PB after fixation, dehydrated in 50%, 70%, 90% and 100% methanol, and stored at -20°C in methanol.

2.2.2. Whole mount *in situ* hybridization

Embryos to be processed for whole mount *in situ* hybridization were rehydrated through methanol series and treated with 10 ug/ml proteinase K (Sigma) in 0.1 M phosphate buffer containing 0.1% Tween 20 for various

lengths of times (from 1 min to 1 hr) according to age. Embryos older then 30hpf were treated with proteinase K.

Embryos were pre-hybridised at 65-68^{0}C for a minimum of 2 hour in pre-hybridisation solution (50% formamide, 5xSSC (pH6), 50ug/ml heparin, 200ug/ml yeast RNA, 5mM EDTA, 1xDenharts and 0.1% Tween-20). This solution was replaced by hybridisation solution containing the anti-sense riboprobe in the best working concentration and the embryos incubated at 65-68^{0}C overnight.

Post-hybridisation washes were carried out at 65-68^{0}C. Embryos were washed first in hybridisation solution, and then 10 min washes with decreasing concentrations of hybridisation solution: 2x SSC (75%, 50%, 25%) followed by 2 x30 min washes in 2x SSC and by 2 x30 min washes in 0.2x SSC.

After re-hydratation, embryos were rinsed in antibody block at room temperature in MABT (0.1M maleic acicd, 0.15M NaCl, pH7.5 and 0.1% Triton) and then blocked in 2% Boehringer blocking reagent (Roche) in MABT plus 10% of lamb serum for at least 2 h. The embryos were incubated in the appropriate antibody, either anti-digoxigenin-alkaline phosphatase conjugated F_{ab} fragments (Roche) (1 in 5000) or anti-fluorescein-alkaline phosphatase conjugated F_{ab} fragments (Roche) (1 in 2000) overnight 4^{0}C.

The antibody was washed six times for 30 min in MABT at room temperature. The embryos were equilibrated with staining NTMT buffer (100 mM Tris-Hcl, pH 9.5, 100mM NaCl, 5mM $MgCl_2$, 0.1 % Tween-20) for

30 min. Then they were developed using NBT-BCIP (Gibco) in NTMT buffer.

For two color *in situ* hybridization following the development of the alkaline phosphatase activity with NBT-BCIP, embryos were dehydrated and rehydrated through ethanol series, treated with 0.1 M glycine, 0.1 % tween 20, pH 2.2 for 4 times, 10 minutes each, to inactivate the alkaline phosphatase activity, rinsed in phosphate buffer tween-20 and incubated with an anti-fluorescein Fab-fragment alkaline phosphatase conjugated antibody (Roche). The substrate for the second alkaline phosphatase reaction was INT-BCIP (Roche), which gives a red (instead of a blue) precipitate.

The reaction was stopped by rising with PBS and re-fixing with 4%PFA. Embryos were then gradually washed into glycerol 75% for storage and photographed.

2.2.3. Whole-mount antibody staining

The embryos fixed with 4% PFA were washed three times in PBS for 5 min each time. Embryos older then 24hpf were with trypsin treated in PBS as permeabilization treatment. After permeabilization the embryos were washed 3 times for 15 min in PBST (a solution of 0.1%Tween 20 in PBS) and blocked at least one hour in 10% goat serum, 1% DMSO (Dimethylsulfoxide), 0.5% Triton X-100, in PBS (IB). The embryos were incubated in the primary antibody in IB overnight at 4^0C.

The embryos were washed out of the antibody by rising several times with PBST and washed five times for 30 min in PBST on a shaker and block again at least one hour in IB. Then the embryos were incubated overnight at 4^{0}C with the secondary antibody Alexa 488 (green) or 555 (red) anti-rabbit diluted 1 in 200 (1:200) of solution of IB (IB stands for immunobloting: 10% goat serum, 1% DMSO, 0.8 Triton, in PBS) for fluorescent staining. The embryos were then rinsed several times in PBST to wash out the secondary antibody. Following the washes, the stained embryos were detected by fluorescence with a confocal microscope.

For revealing non-fluorescent staining a biotinylates secondary antibody and the ABC kit from Vector were used. To stop the reaction embryos were rinsed with PBS, refixed with 4%PFA and stored in 75% glycerol.

2.2.4. Plastic section (see Appendix).

2.2.5. Agarose mounting of live embryos

For all mounting, dechorionated embryos were anaesthetised according to the Zebrafish Book using tricaine (3-amino benzoic acid ethylester, Sigma) in embryo medium. Embryos were mounted in 1.5% low melting-point agarose (Sigma) dissolved in in embryo medium. Embryos for mounting were pipetted singly into a glass bijou bottle containing molten

agarose at approximately 40°C. They were then drawn up into a fire-polished glass pipette with excess agarose and expelled onto a slide or coverslip. Precise orientation of the embryos was performed within 30 seconds using a blunt tungsten needle.

For imaging procedures, live embryos were usually mounted in agarose on a coverslip and surrounded by a glass ring (Fisher). The well was then filled with embryo medium/tricaine into which water immersion lenses were directly dipped.

2.2.6. Injection of mRNA or morpholino into early stage embryos

Embryos at the 1 cell stage were aligned in a plastic through, still with their chorions, and microinjection targeted to the cytoplasm or to yolk syncytial layer or to the interface between them. Needles were pulled from glass capillary tubes by Clark Electromedical Instruments needle puller and injections were performed using a Picospritzer micro-injector.

Embryos from natural spawning were collected and injected from at 1 cell stage according to guidelines in the Zebrafish Book. Briefly, micropipettes were pulled from borosilicate glass capillaries (with filament) on a horizontal Flaming/Brown micropipette puller (model P-87, Sutter Instrument Co.), producing micropipettes with a long, fine, sealed tip. Micropipettes were backfilled with 1-2μl DNA or RNA or morpholino solution, made up in Danieau buffer, and the very tip of the micropipette

was broken off such that a short (< 0.5 second) puff from the Picospritzer (General Valve Corporation) would dispense a drop approximately 100μm in diameter.

Embryos were laid, in their chorions, along the edge of a glass slide glued to the inside of a petri dish lid. Excess liquid was removed so that the meniscus held the embryos along the slide. Embryos were then injected through the chorion, directly into a single blastomere with minimum disruption to the underlying yolk. The concentration of all mRNA injected was around 200pg and of morpholino was 2-6ng. Following injection, embryos were transferred to embryo medium and grown at 28.5°C.

2.2.6.1. Morpholino Preparation

Gene Tools provided Morpholino antisense oligonucleotides (http://www.gene-tools.com/) against dab1. The supplied 300nmol (approx. 2.5mg) of lyophilised powder was diluted as a stock of 4mM in Danieau buffer (5mM HEPES pH 7.5 and 200mM KCl).

2.2.7. Image acquisition and elaboration

A variety of microscope systems were used during the course of this project. DIC images of non-fluorescent *in situ* hybridised and immunolabelled specimens were taken on an upright Nikon microscope,

with a Micropublisher digital camera (Q imaging) run by Openlab 3.1.4 software (Improvision, UK). Epifluorescence and DIC imaging of live specimens was carried out on a Zeiss Axioplan 2 microscope, with water immersion lenses. Images were captured with a Hammamatsu Orca-ER digital camera run by Openlab 3.1.4 software (Improvision, UK). Confocal imaging of fluorescent specimens was carried out on Leica microscopes running Leica software.

2.2.7.1. Image processing

Fluorescence images from confocal microscopy were processed using the freely available NIH image v1.63 software (http://rsb.info.nih.gov/nih-image/Default.html) to assemble and project stacks of images. ImageJ v1.32 software (http://rsb.info.nih.gov/ij/) was used to create red/green overlays. Adobe Photoshop 7 was used to create fluorescence/DIC overlays, and for general image adjustment. Figures were prepared in Adobe Illustrator 10.

2.3. *In situ* hybridization and immounhystochemistry procedures on cryostat sections

The cryostat sections were air-dried at room temperature for 20 minutes to 3 hrs and postfixed with 4% paraformaldehyde in phosphate

buffer containing 0.1 M NaCl (PBS) for 20 minutes. Following 3 washes in PBS, sections were acetylated (Triethanolamina +hydroxide anhydride) and incubated in 50% formamide, 3X SSC until ready to be exposed to the riboprobe. Riboprobes were diluted to 100 ng/ml in warm (60°C) hybridization solution, containing 50% formamide, 5X SSC, 10 mM beta-mercaptoethanol, 10% dextran sulphate, 2X denhardt's solution, 250 ug/ml yeast tRNA, 500 ug/ml heat inactivated salmon sperm DNA. Hybridization was carried out in a humid chamber at 58°C for 16 hrs. Slides were rinsed in 50% formamide, 2X SSC at 58°C, treated with RNAse A and RNAse T1 at room temperature, rinsed twice with 50% formamide, 2X SSC at 58°C and incubated with anti digoxigenin antibody as above. Following development of the color reaction slides were dehydrated and mounted in DPX (BDH) or processed for immunohistochemistry using one of the following antisera: anti Islet-1 (clone 39.4D5, developed by T. Jessel, obtained from the Developmental Studies Hybridoma Bank, University of Iowa), Distalless (a gift of G. Panganigan, University of Wisconsin), Zebrin (a gift of Steve Davies, UCL) diluted 1:400 in PBS containing 5% normal goat serum and 0.1% triton X, or anti TH or Calretinin (Chemicon, UK), or GABA (Sigma, UK) diluted 1:2,000 (1:2000). The immunoreactivity was revealed using a biotinylated secondary antibody and the ABC kit from Vector. Diaminobenzydine was used as a substrate for the peroxidase. For BrdU immunohistochemistry sections were treaded with 2N HCl for 20' at room temperature before being incubated with BrdU antiserum (Sigma) diluted 1:500 and processed as above. Sections were then mounted in glycerol.

Images were captured with a Polaroid digital camera connected to a Nikon Optiphot-2 microscope, using x4, x10 and x20 Planapo lenses. Digital images were stored as 1,600 pixels x 1,200 pixels at a resolution of 300 dpi and manually arranged to form composite pictures using Adobe Photoshop 5.5. Labeling was added as separate layers.

2.4. Transgenic lines used

Danio rerio transgenic lines were from the UCL Zebrafish Facility. The *tg(islet-1-GFP)* line was donated by Higashijima Okamoto (Higashijima et al., 2000). The construct is under the promoter of *islet-1* gene that markes the neurons of branchiomotor nuclei of the hindbrain.

Mark Ekker donated the *dlx4/6*:GFP line. The generation of a GFP line is under the control of the *dlx4/6* intergenic region (equivalent to *Dlx5/6* in the mouse). For example the *dlx4/6*:GFP line allows the observation in vivo of the migration of neurons in forebrain and midbrain/hindbrain regions.

CHAPTER 3

3. Results 1. Conserved and Divergent Patterns of *reelin* Expression in the Zebrafish Central Nervous System

3.1. Introduction

During the development of the nervous system an elaborate program of neuronal migration and axonal growth ensures the correct positioning of neuronal cells and the establishment of synaptic connections. The large extracellular molecule Reelin plays a key role in these processes in the vertebrate brain.

Mutations in the gene encoding Reelin in mice (Caviness, 1976; D'Arcangelo *et al.*, 1995) and humans (Hong, 2000) have helped to elucidate the mechanisms underlying neuronal migration and axonal targeting in the CNS (D'Arcangelo & Curran, 1998; Bar & Goffinet, 1999; Gilmore & Herrup, 2000). Reelin receptors and intracellular effectors are highly conserved and are thought to play key roles in neuronal migration. Members of at least three families of receptors, Lipoprotein receptors (VLDL and ApoER2),

integrins (α3β1 Integrin) and Cadherin neural receptor (CNR) families can bind Reelin *in vitro* and *in vivo* (Hiesberger *et al.*, 1999; Senzaki *et al.*, 1999; Trommsdorff *et al.*, 1999). These receptors trigger the phosphorylation of the cytoplasmic adaptor protein Disabled-1 (Dab1; Howell *et al.*, 1999b). Interactions of the Reelin pathway with other pathways regulating neuronal migration, namely the CDK5/p35 pathway (Ohshima *et al.*, 1996; Chae *et al.*, 1997; Kwon & Tsai, 1998) and the Lis1/Dcx pathway (Hattori *et al.*, 1994; des Portes *et al.*, 1998; Gleeson *et al.*, 1998), have also been reported (Walsh & Goffinet, 2000).

Interestingly, Lipoprotein receptors, Integrins and Dab1 are found even in *Drosophila melanogaster* (Rice & Curran, 2001), while Lis1 has a homologue in *Aspergillus nidulans* (Xiang *et al.*, 1995). By contrast, no *reelin*-related sequences have been identified in the fully sequenced invertebrate genomes. This suggests Reelin may be a chordate-specific molecule involved in regulating the migratory behavior of neuronal groups and the synaptic organization of complex brains. In vertebrate species, *reelin* has been isolated and studied by *in situ* hybridization in the developing brains of chicks, turtles, lizards and mammals. In addition, Reelin immunoreactive cells have been detected in the brain of adult lampreys and teleosts (Perez-Costas *et al.*, 2000; Perez-Garcia *et al.*, 2001).

In tetrapods that have laminar/layered brain areas such as the dorsal, medial pallium or the cerebellum, differences in the pattern of expression of *reelin* have been correlated with different degrees of laminar organization. In the case of the telencephalon (Bar & Goffinet, 2000), the picture that is

emerging from these studies implicates the Reelin pathway in controlling the ordered radial migration of cortical neurons.

In this first chapter of Results is described the expression pattern of *reelin* in the developing and adult zebrafish CNS, with particular emphasis on the evolution of telencephalic development, in which the Reelin pathway is known to play an important role (D'Arcangelo & Curran, 1998). Reelin expression in the telencephalon of zebrafish is compared with that of the same gene in the developing amphibian *Xenopus laevis*, which has a non-laminar evaginated telencephalon where cells hardly migrate from the ventricular zone (Northcutt, 1981).

However, unlike teleosts and like amniotes, the anuran telencephalon does undergo evagination, allowing a comparison of *reelin* expression in the telencephalic vesicles of all major vertebrate classes.

3.2. Results

3.2.1. Isolation of zebrafish and *Xenopus reelin* cDNA clones

Two clones were isolated through screening of an adult zebrafish brain cDNA library. Sequence analysis showed that they corresponded to the same sequence. The longest clone (3272 bp) encodes for 1090 amino acids showing around 65% sequence identity with other Reelin proteins (fig. 1A) and corresponding to the mid 1/3 of mouse Reelin (acc. no. NP035391; aa 1550-2640).

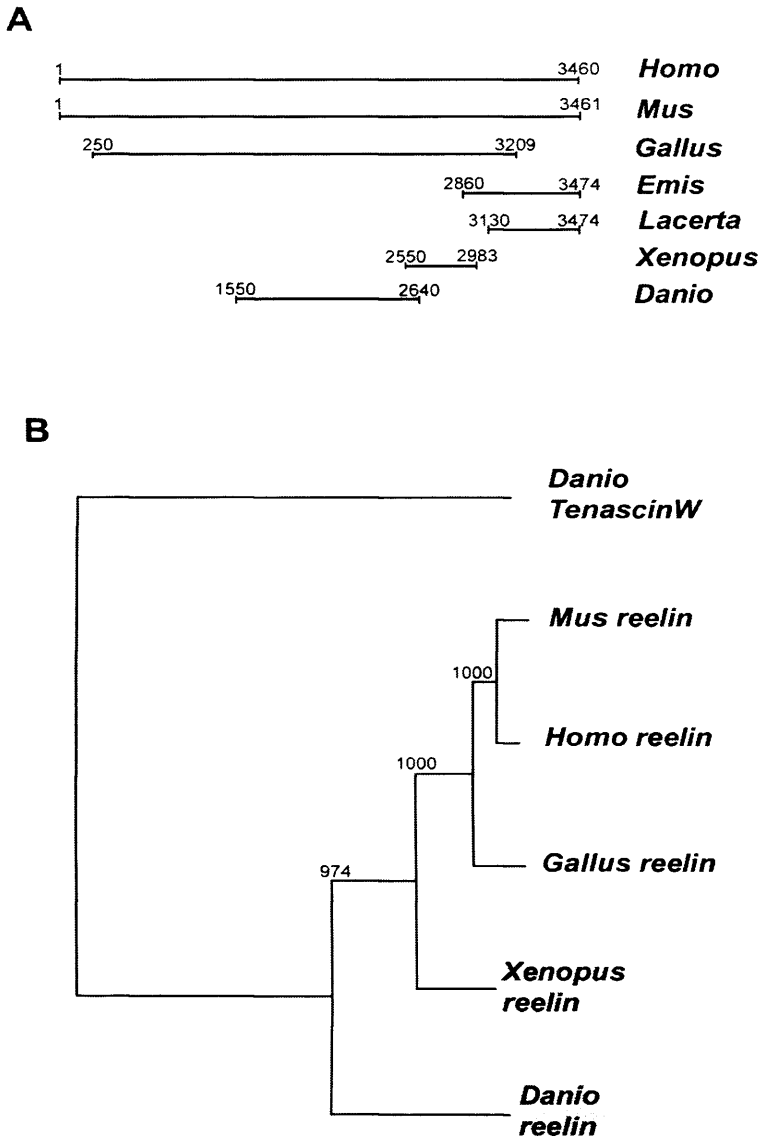


Figure. 1. Alignment of Reelin sequences.

A) Optimal alignment of amino acid sequences of human (*Homo*), mouse (*Mus*), chick (*Gallus*), lizard (*Lacerta*), turtle (*Emys*), frog (*Xenopus*), and zebrafish (*Danio*) *reelin* clones was obtained with the Clustal-X program and checked manually. The corresponding amino acid sequences are numbered. The accession numbers of the sequences used here are as follows: human, NP005036; mouse, NP0035391; chick, AAC35559; turtle, AAC35993; lizard, AAC36362; zebrafish, AF427524; *Xenopus*, AF427525. B) A distance tree of the Reelin proteins isolated from different vertebrate species. The sequences isolated from zebrafish and *Xenopus* were compared with the corresponding amino acids 148-2983 of human, mouse, and chick Reelin and with zebrafish Tenascin-W protein. The distance tree was drawn with the Neighbor-joining program from the Phylip package and rooted on the zebrafish Tenascin-W. Numbers correspond to the bootstrap values (occurrence of presented branching after 1000 iterations). As the tree indicates, both zebrafish and *Xenopus* sequences belong to the Reelin family.

A *Xenopus reelin* clone was identified through a BLAST search. It corresponds to 1299 nucleotides (nt) sequence showing high similarity (79%) with nt 6884-8183 of a chick cDNA *reelin* clone (acc. no. AF090441).

Conceptual translation showed that it corresponded to amino acids 2550-2983 of the human/mouse Reelin (fig. 1A) with 84% identity to the corresponding fragments of other Reelin proteins. A phylogenetic analysis of the zebrafish and *Xenopus* Reelin protein sequences encoded by these clones showed that zebrafish Reelin is the most divergent of all Reelin clones isolated so far (fig. 1B). The accession numbers for the two clones described here are AF427524 for zebrafish and AF427525 for *Xenopus*.

3.2.2. *reelin* mRNA expression during embryonic and larval development of zebrafish brain

24hpf. At 24 hours post fertilization (hpf) *reelin* was expressed in distinct domains corresponding to neurons arising in different regions of the CNS (fig. 2A). Comparison with the expression of *eom* and *nk2.1a* (Marina Mione personal comunication) confirmed that both pallial and subpallial telencephalon (fig.2B, C; with the exception of the region that presumably later forms the olfactory bulbs) caudal hypothalamus, and ventral midbrain express *reelin*.

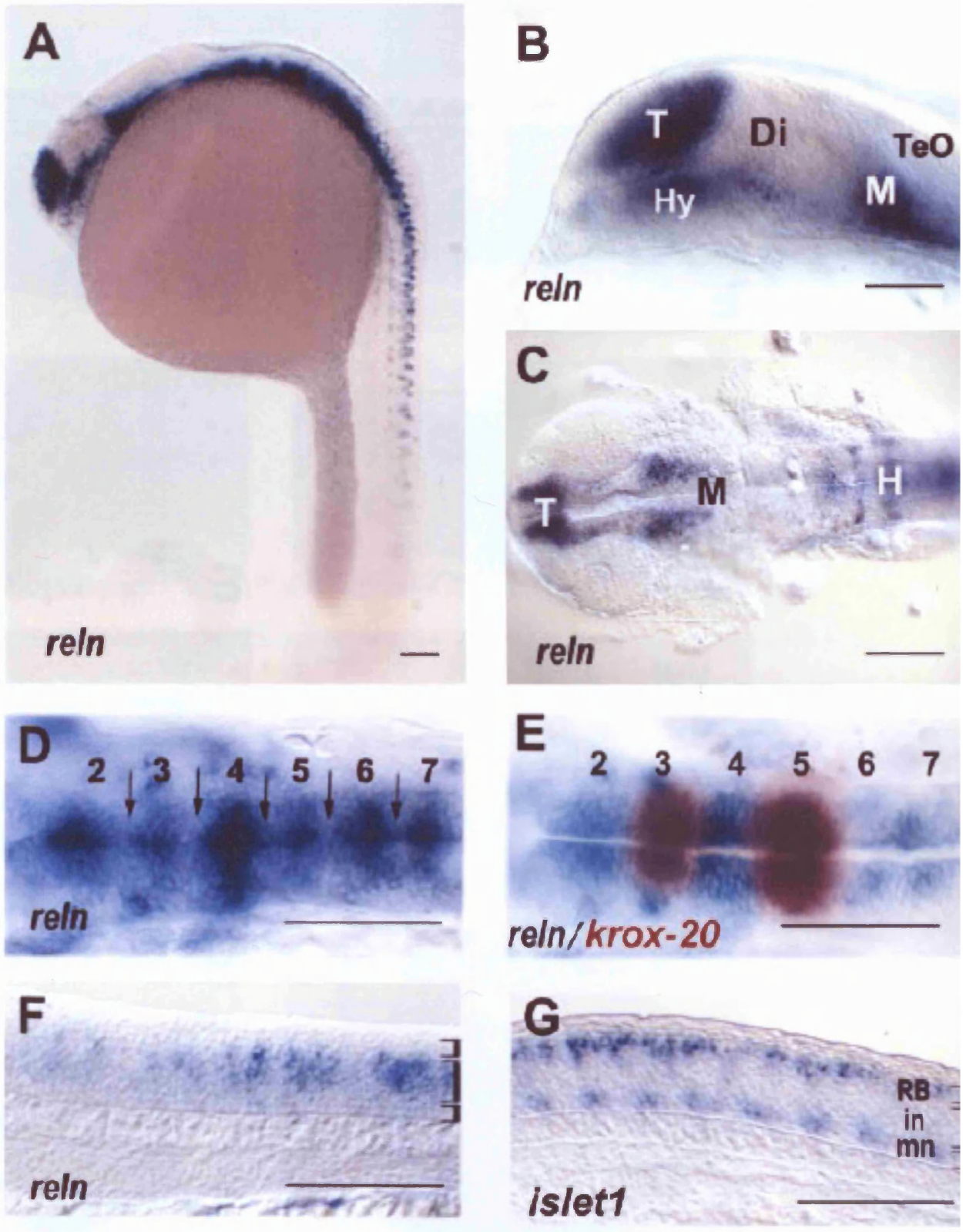
In the hindbrain, *reelin* was expressed in rhombomeres 2-7 and was excluded from the boundary zones (fig. 2D, E). In the spinal cord, *reelin*

transcripts primarily marked cells positioned between the ventral *islet1*-expressing motor neurons and the dorsal *islet1*-expressing Rohon-Beard cells (compare fig. 2F with G). This finding suggests that various spinal cord interneurons express *reelin*, a conclusion supported by analysis at later stages (see below).

40hpf. By 40 hpf the expression of *reelin* in the telencephalon became mainly restricted to the dorsal region (pallium, fig. 3A), and remains excluded from the prospective olfactory bulb. In the diencephalon, *reelin* was expressed mainly in the early differentiating neurons located along the tract of the postoptic commissure (fig.3A) and in the pituitary gland (not shown). Strong *reelin* expression was found in the presumptive pretectum and mesencephalic tegmentum (fig. 3A). Overall, this pattern resembles the distribution of the neurons that establish the early scaffold of axon tracts (fig. 3B). The postmitotic nature of the *reelin* expressing cells at this stage (as

Figure. 2. Reelin is expressed in several locations along the brain of 24 hours postfertilization zebrafish.

A–C: Lateral (A, B) and dorsal (C) views of *reelin* (*reln*) expression in whole embryos/brains showing labeling in the telencephalon (T), ventral areas in the diencephalons (Di), including the hypothalamus (Hy), mesencephalon (M), hindbrain (H), and in cell populations along the spinal cord. TeO, optic tectum. D, E) Dorsal views of hindbrains, comparing *reelin* and *krox-20* expression. *reelin* expression is present in the central domains of rhombomeres 2–7 (numbered) and excluded from rhombomeric boundaries(arrows in D). *Krox-20* expression marks rhombomeres 3 and 5 throughout their dorsoventral extension. F, G) Lateral views of spinal cords, comparing *reelin* and *islet1* expression. *Reelin* (F) is expressed in cells located in the intermediate columns of the spinal cord (in) and motorneuron (mn, marked by *islet1* expression) layers of the developing spinal cord and is not expressed by *islet1*-positive dorsal (presumably Rohon-Beard [RB] neurons) cells (G). Scale bars 200 μm in A–G.


A
reln
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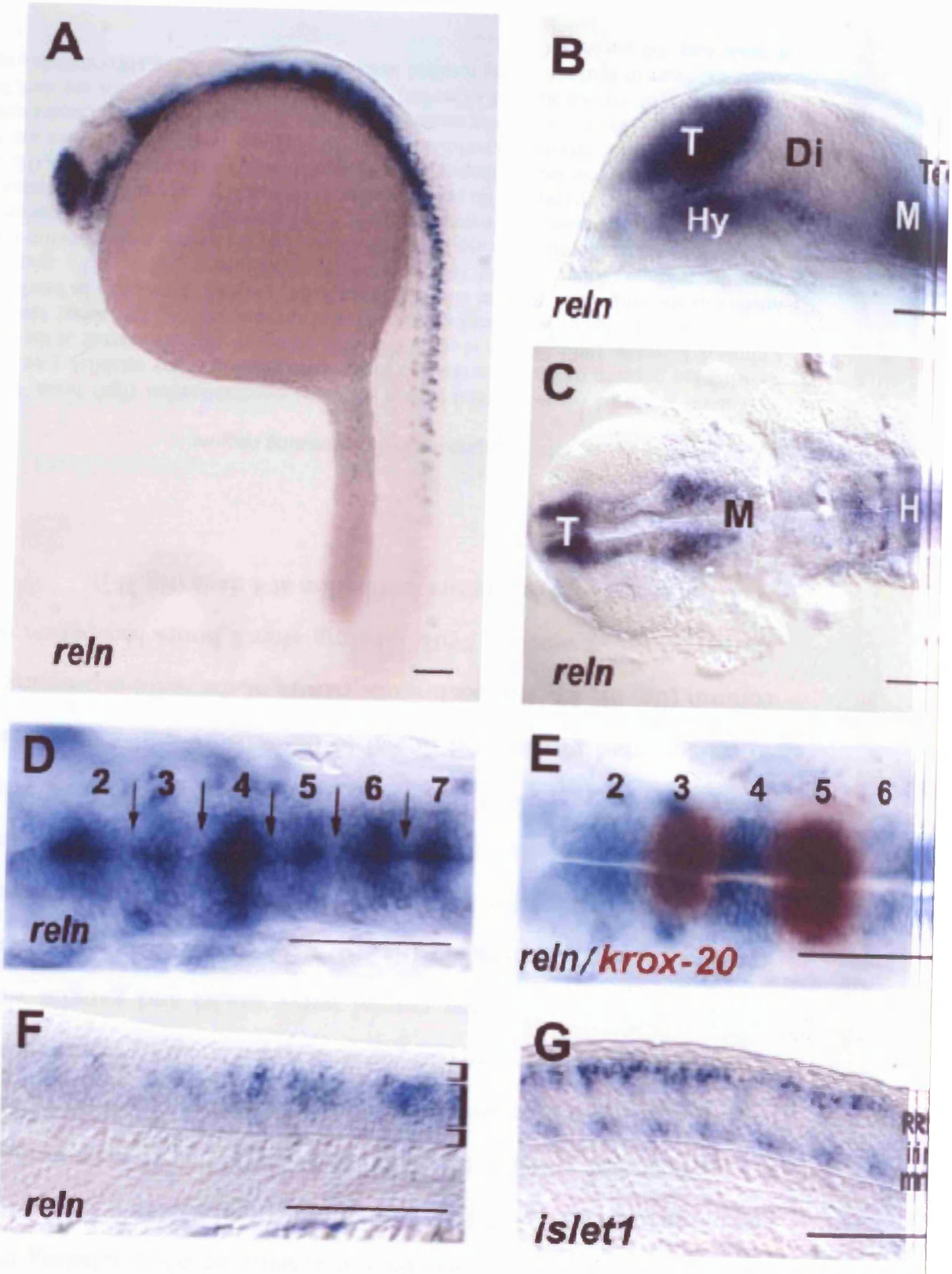
in
mn
islet1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reln
E
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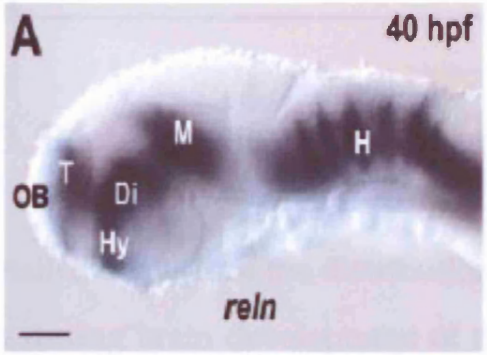
3
4
5
6
reln/krox-20
F
reln
G
islet1

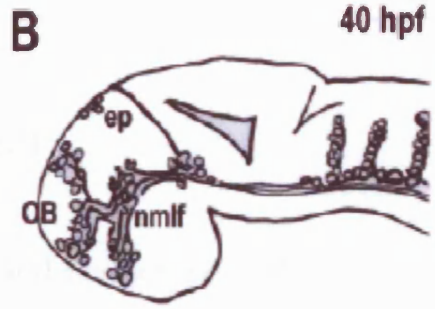
well as 5 dpf) was assessed by the absence of BrdU labeling in zebrafish embryos incubated with BrdU for 2 hours (figs. 3C, D).

To assess whether the segmental distribution of *reelin* in the hindbrain was indicative of an expression in the developing cranial nerve nuclei, *reelin* and *islet1* expression were compared in whole mount preparations at 40 hpf. At this stage, many of the cranial nerve nuclei and ganglia were clearly marked by *islet1* expression (fig. 3D). By contrast, *reelin* transcripts were found in six transverse stripes spanning the midline and appeared to be excluded from cranial nerve nuclei (fig. 3C). More detailed comparative analysis at 5 dpf showed that the segmental expression of *reelin* is confined to cells located medial and dorsal to the sensory and motor cranial nerve column (fig. 3E, F). The postmitotic nature of the *reelin*-expressing cells was shown by the absence of BrdU labeling after 2 hours incubation with BrdU at 40 hpf (fig.3G-H) or 6 hours incubation at 4 days (fig.3I-J).

Figure. 3. A–B: *reelin* (reln) is expressed in differentiating neurons.

A) Lateral views of *reelin* expression in a 40 hours postfertilization (hpf) brain. B) Schematic showing the position of early neurons and major axon pathways they establish (see, for instance, Chitnis & Kuwada, 1990; Wilson *et al.*, 1990). This pattern is very reminiscent of the expression of *reelin* in A. C–F) *reelin* is not expressed in cranial nerve nuclei. C, D) Dorsal views of 40 hpf hindbrains showing *reelin* (C) and *islet1* (D) expression. *reelin* expression is in bands of cells that span the midline, whereas *islet1* is expressed in cranial nerve nuclei. E,F) *islet1* and *reelin* expression in the hindbrain at 5 days postfertilization (dpf). F) Lateral view showing expression of *islet1* and level of section. G) Frozen section at 5 dpf showing that *reelin* expression (blue) and Islet1 immunoreactivity (brown) do not colocalize. G–J) *reelin* is expressed in postmitotic neurons. G, H) Frozen transverse section through the telencephalon (G) and mesencephalon (H) of a 40 hpf zebrafish stained for bromodeoxyuridine (BrdU; brown) and *reelin* (blue). There are no double-labeled cells. I, J) Frozen transverse section through the telencephalon (I) and mesencephalon (J) of a 4 dpf zebrafish stained for BrdU (brown) and *reelin* (blue). Dividing cells are dark brown. The longer exposure to BrdU (6 hours) resulted in a higher background. For abbreviations, see list page 9. Scale bars 200 μm in A, C–J.


A
40 hpf
M
H
T
OB
Di
Hy
reln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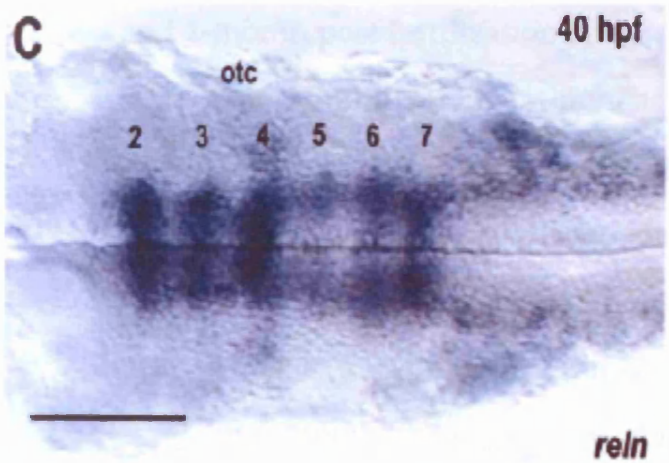
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2
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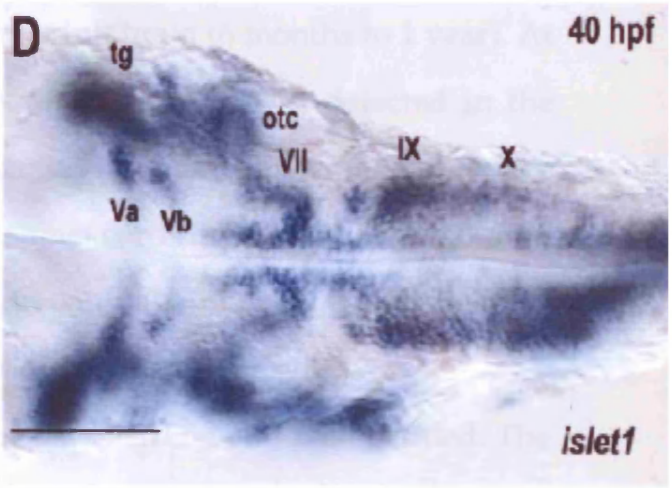
reln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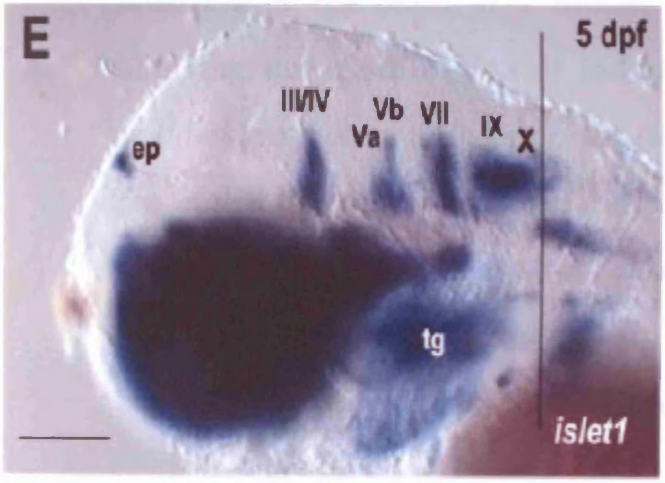
X
Va
Vb
islet1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III/IV
Vb
Va
VII
IX
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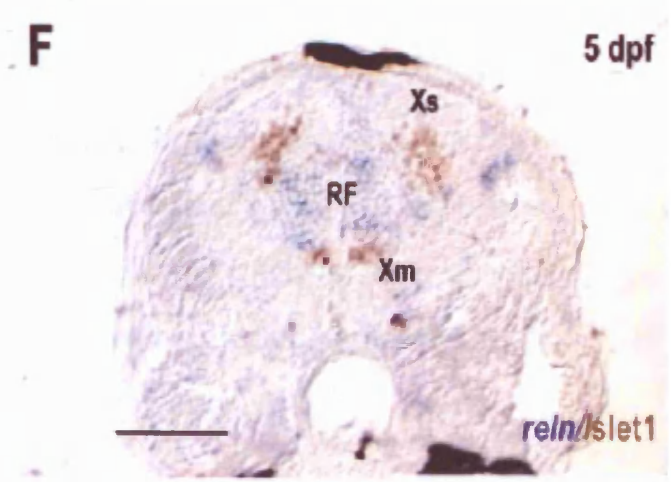
ep
tg
islet1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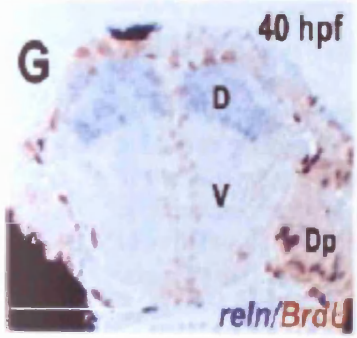
Xs
RF
Xm
reln/islet1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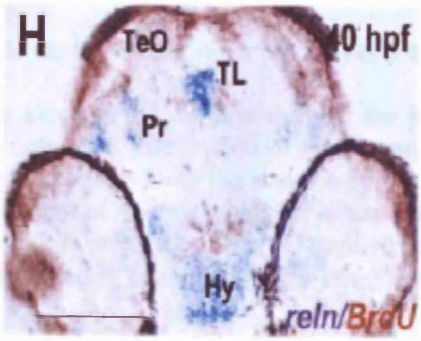
D
V
Dp
reln/BrdU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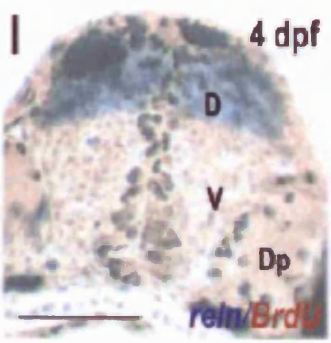
TeO
40 hpf
TL
Pr
Hy
reln/BrdU


I
4 dpf
D
V
Dp
reln/BrdU


J
4 dpf
Cce
reln/
BrdU

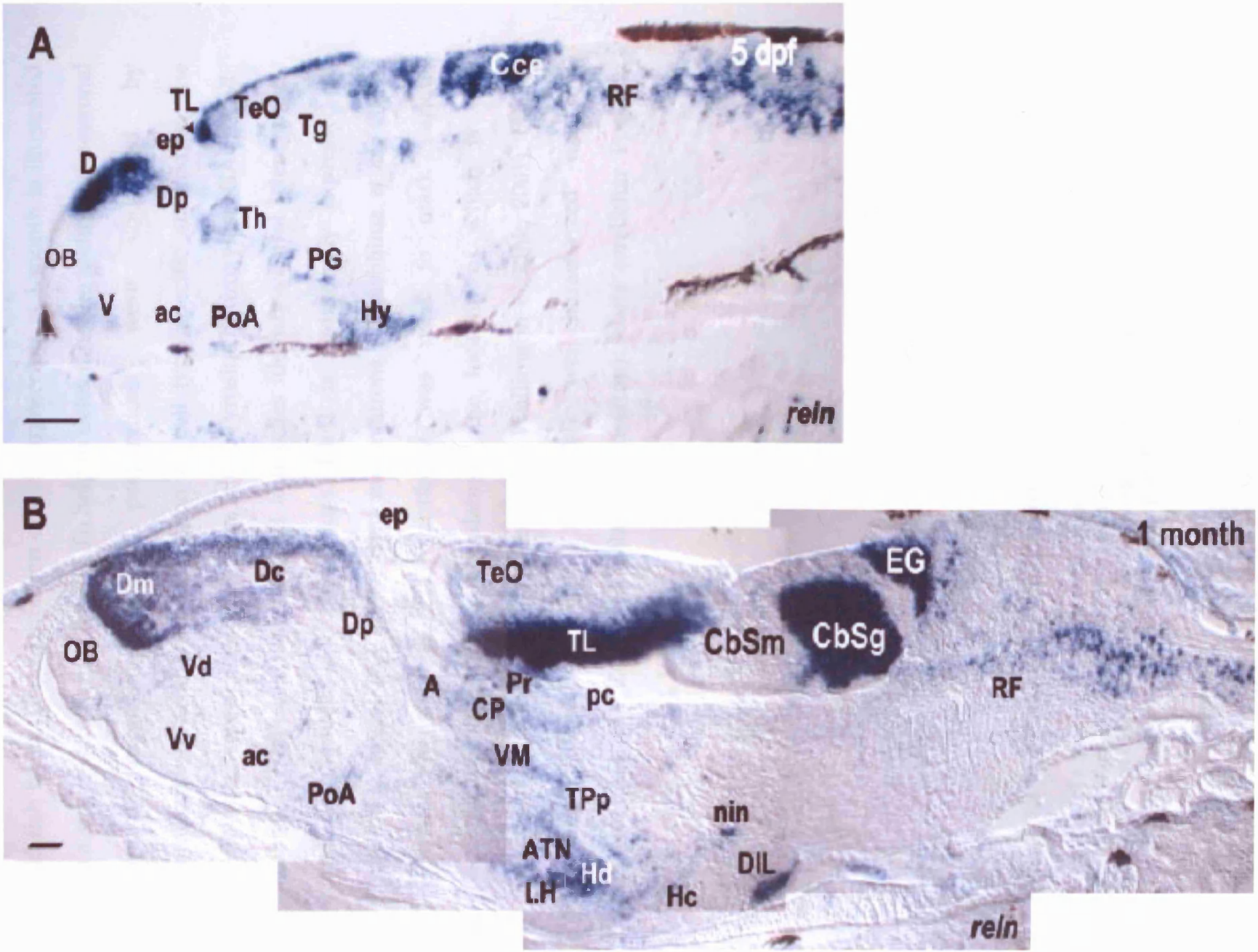
3.2.3. Post-hatching development

A detailed analysis of the distribution and phenotype of the cells expressing *reelin* during brain development of the zebrafish was carried out at 5 days and 1-month post fertilization and in the adult brain (6 months to 1 year). At 5 days post fertilization, prominent *reelin* expression was detected in the dorsal telencephalon, ventral hypothalamus, optic tectum, tegmental nuclei, developing cerebellum, hindbrain and spinal cord (fig. 4A). The distribution of *reelin* at this stage (as assessed both in coronal and sagittal sections) was very similar to that found in 1-3 months old zebrafish (fig. 4B).

At 1 month, the expression of *reelin* was strong and well defined. The anatomy of the brain at 1 month was very similar to the adult brain, allowing the identification of individual structures and nuclei. However, *reelin* expression in the adult brain (6 month-1 year old) was much lower, to the extent that it was undetectable in several structures that had expressed *reelin* during development. Therefore, in order to interpret the specific anatomical structures those were expressing *reelin* at earlier stages, the adult brain (table I, see below) was compared with expression in the 1-3 month

Figure. 4. *reelin* (reln) is expressed in similar patterns at 5 days (dpf) and 1 month postfertilization.

A, B) Sagittal sections close to the midline through the brain of 5 dpf (A) and 1-month-old (B) zebrafish processed to reveal the distribution of *reelin* transcripts. The pattern of *reelin* expression at the two stages is highly similar. Note the absence of *reelin* transcripts in the olfactory bulb (OB) and the rostrocaudal gradient of *reelin* expression in the dorsal telencephalon (D). At 5 dpf (A), the identities of thalamic (Th), preglomerular (PG) and hypothalamic (Hy) nuclei are ill-defined, whereas they can be identified at 1 month (B). For other abbreviation, see list. Scale bars 100 μm in A, B.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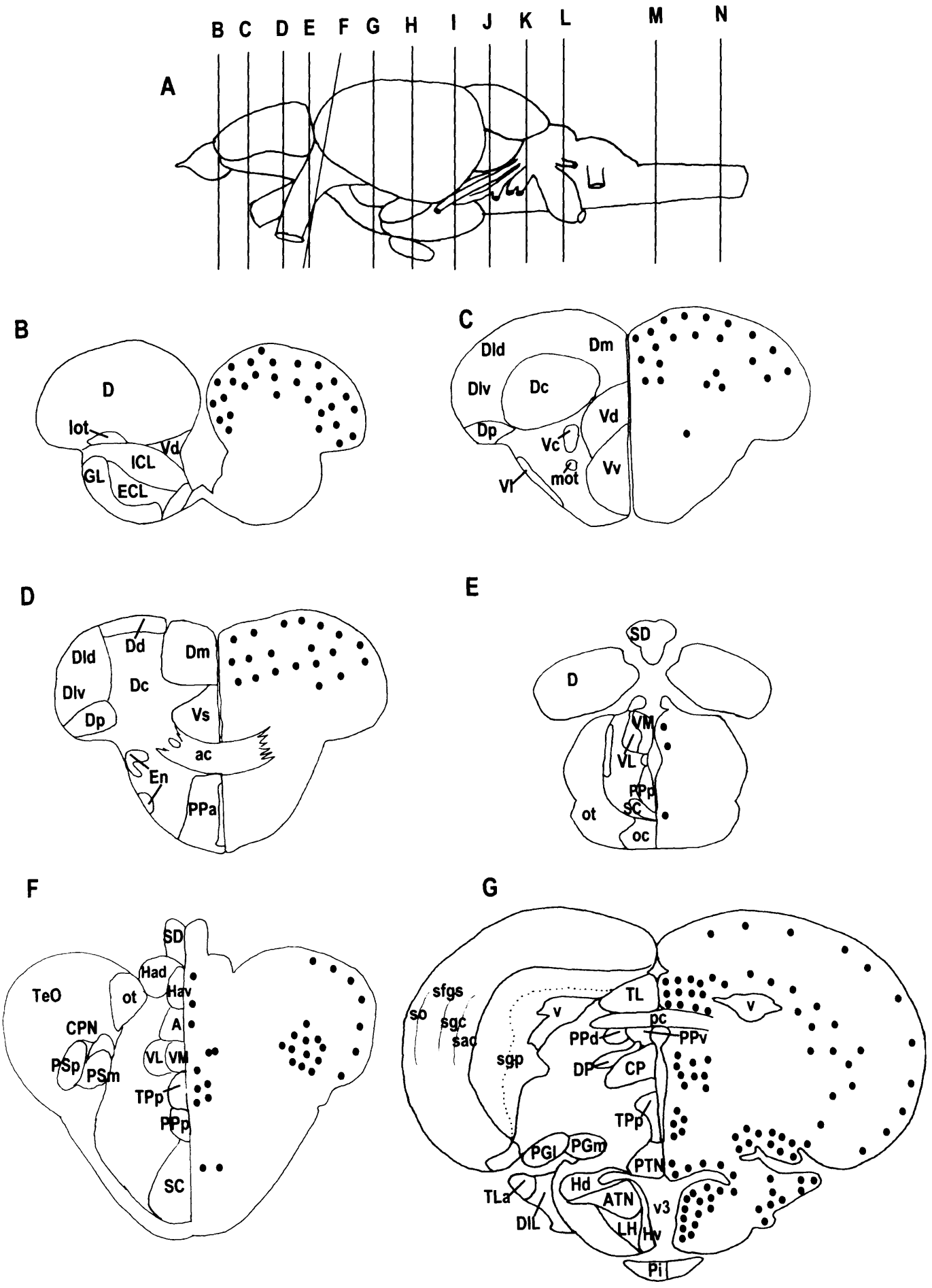
Dp
Th
OB
PG
V
ac
PoA
Hy
rein
B
ep
1 month
Dm
Dc
TeO
EG
Dp
TL
CbSm
CbSg
OB
Vd
A
Pr
pc
RF
CP
Vv
ac
VM
PoA
TPp
nin
ATN
Hd
DIL
LH
Hc
rein

old fish brain. The distribution of *reelin* transcripts at 1 month is illustrated in a series of drawings (fig. 5). To help to determine the identity of neuronal groups expressing *reelin*, preparations were co-labeled by immunohistochemistry with region or cell type specific antibodies. The antibodies were against: Distalless, Islet1, Tyrosine hydroxylase, GABA and Zebrin. Distalless (Dll) and Islet1 antibodies identify ventral telencephalic and diencephalic cell populations. Islet1 is additionally expressed in hindbrain nuclei and spinal cord motoneurons (Higashijima *et al.*, 2000). Tyrosine hydroxylase (TH) antibody was used to mark various catecholaminergic neural populations, the location of which is well documented (Rink & Wullimann, 1998; Wullimann & Rink, 2001). GABA antibody identifies several reasonably well-characterized neuronal populations and Zebrin (Aldolase Cantiserum) labels cerebellar Purkinje cells (Brochu *et al.*, 1990).

Figure. 5. Distribution of *reelin* mRNA transcripts.

Drawings of a series of transverse sections through the zebrafish brain, depicting the distribution of *reelin* transcripts. A) Schematic drawing of a lateral view of a zebrafish brain, to show the levels and orientation of the sections in B–N. *reelin* transcripts are represented by black dots on the right side of the sections. For abbreviations, see list page 9.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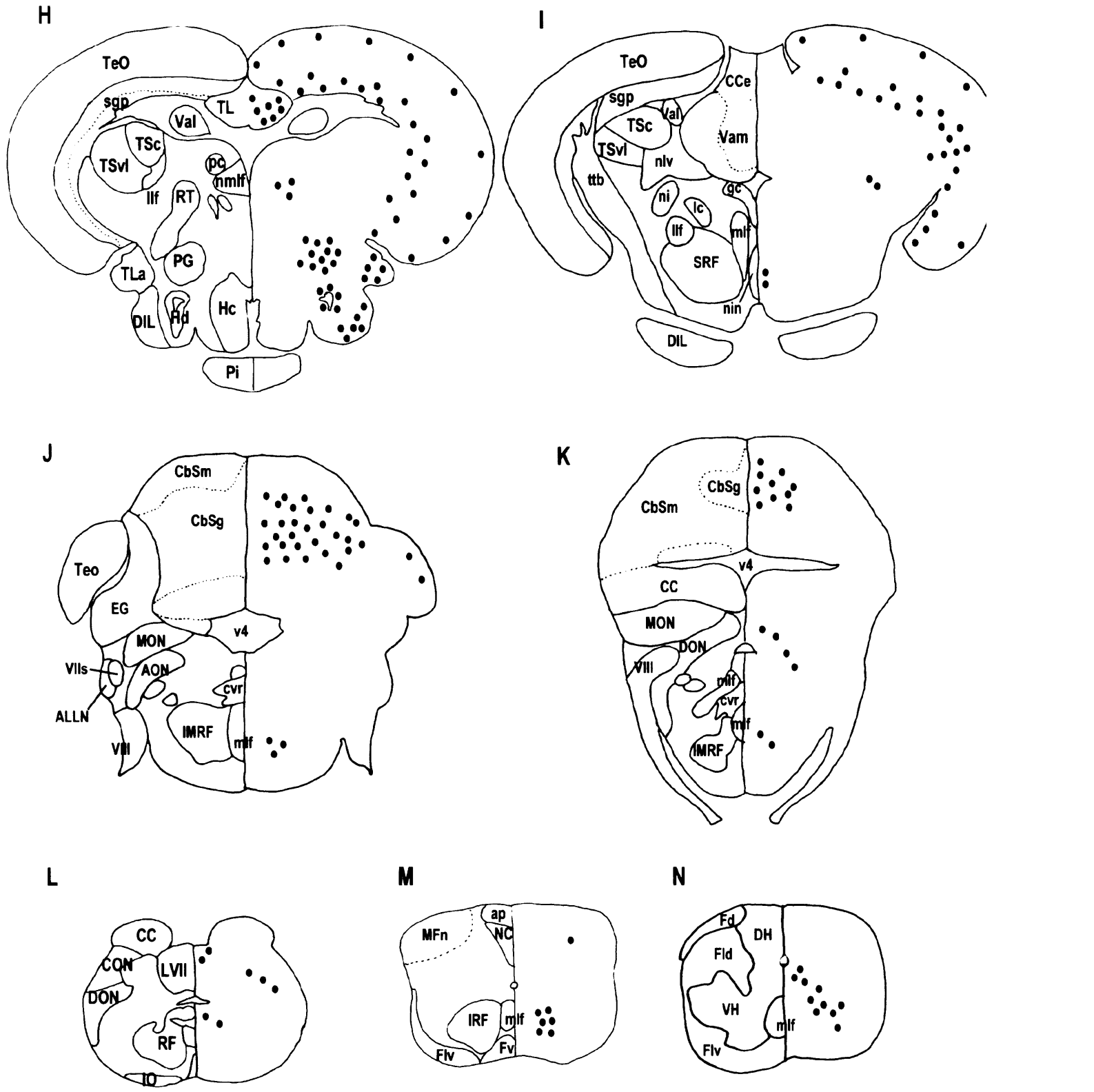
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Pi
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TeO
CCe
sgp
Val
TSc
Vam
TSvl
nlv
ttb
ni
gc
lc
llf
mlf
SRF
nin
DIL
J
CbSm
CbSg
Teo
EG
v4
MON
VIIs
AON
cvr
ALLN
IMRF
mlf
VIII
K
CbSm
CbSg
v4
CC
MON
DON
VIII
mlf
cvr
mlf
IMRF
L
CC
CON
LVII
DON
RF
IO
M
MFn
ap
NC
IRF
mlf
Flv
Fv
N
Fd
DH
Fld
VH
mlf
Flv

Table I. reelin expression in adult zebrafish brain

Area	1m	6m	Area	1m	6m	Area	1m	6m	Area	1m	6m
Telencephalon			VM	+	+	TPp	++	+/-	**Isthmus**		
Dd	++	-	**Preoptic area**			**Pretectum**			ni	+	+
Dld	+	+	PPa	-	-	PPd	-	-	***nin***	+	-
Dlv	-	-	PPp	-	-	PPv	-	-	**Cerebellum**		
Dm	++	++	SC	+	+/-	PSm	+	+	CbSg	++	++
Vc	+	+	**Hypothalamus**			PSp	+	+	CbSm	-	-
Epithalamus			ATN	++	+	**Optic Tectum**			Val	++	++
Had	+/-	+/-	DIL	++	+	TL	++	++	**Cranial n. nuclei**		
Hav	+/-	+/-	Hd	++	+	sfgs	++	++	AON	+	-
Dorsal thalamus			Hv	+	+/-	sgp	+	+	CON	+	-
A	+	+/-	LH	++	-	**Tegmentum**			DON	+	-
CP	++	-	**Posterior tuberculum**			TLa	++	+	LVII	+	-
DP	++	-	PGl	++	+	TSc	+	-	IRF	+	+/-
Ventral thalamus			PGm	++	+	TSvl	+	+	**Spinal cord**		
VL	+/-	+	PTN	+	+/-	***RT***	+	+	VH	+	+/-

Comparison of *reelin mRNA* expression in brains of 1 month and 6 month-old zebrafish. Expression levels range from – (no expression) to ++ (high expression).

3.2.4. *reelin* mRNA expression at 5dpf and 1 month old zebrafish brain

3.2.4.1. Telencephalon

Olfactory bulb. Neither putative interneurons (as marked by TH immunoreactivity, fig. 6E) nor putative mitral cells (as marked by *tbr1*

expression, fig. 6D) expressed detectable levels of *reelin* (Figs. 4A,B, 5B, 6C) at all stages studied. The lack of *reelin* expression in the olfactory bulb is in contrast to the consistently reported expression of *reelin* in the mitral cell layer of mouse and chick olfactory bulb.

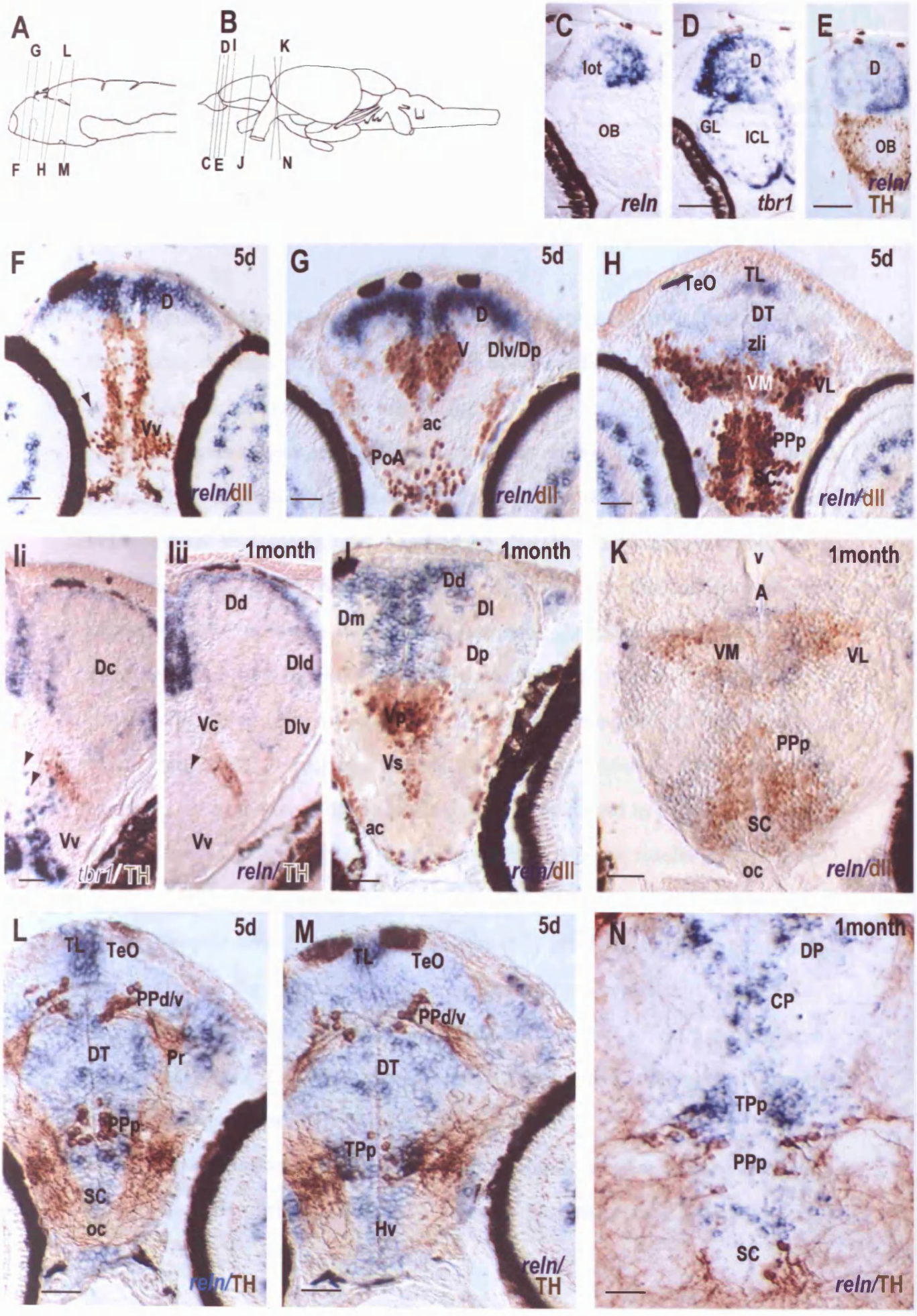
Telencephalon. Strong expression of *reelin* was detected in areas of the dorsal telencephalon (D in figures) mainly rostral to the anterior commissure (ac, figs. 4A, B; 5B-E; 6F, G, Iii, J). The subdivisions of dorsal telencephalon were not clearly defined at 5 dpf. Comparison of *reelin* expression at 5dpf and 1 month showed that *reelin* was expressed in all subdivisions throughout this developmental period, with the exception of the lateral-ventral and posterior area (Dlv and Dp respectively, figs. 4A, B; 5 C, D). At 1 month *reelin* mRNA was found in numerous cells of the medial and dorsal region of the dorsal telencephalic area (Dm and Dd, respectively). Large scattered cells contained *reelin* mRNA in the central area of the dorsal telencephalon (Dc, figs. 5C, D, 6I). Of the lateral areas of the dorsal telencephalon, only the dorsal one (Dld) had *reelin* expressing cells (figs. 5D, 6I).

reelin was also expressed at low levels in the ventral telencephalon (V), by a group of midline cells located at the level of the ventral nucleus (Vv; figs. 5C, 6F, 6Iii) at 5dpf and 1 month. In addition, weak expression of *reelin* at the level of the dorsal nucleus of the ventral telencephalon was present at 5 dpf but not at 1 month (fig.4B, 6Iii). The midline ventral telencephalic cells expressing *reelin* were not labeled with Dll or TH antisera at 5 dpf or 1 month post fertilization (fig. 6F and fig.6Iii). However, the *reelin*-positive

cells of the migrated nucleus are intermingled with TH-positive cells (arrowheads in fig. 6Iii). Both groups of *reelin*-expressin cells are included in a larger territory expressing *tbr1* (fig.6Ii). Based on these observations and the precommissural position of these *reelin*-expressing cells, the most convincing interpretation was that the midline groups are part of the central nucleus of V (Wulliman *et al.*, 1996). In all telencephalic regions the cells bordering the ventricle do not express *reelin* mRNA (see also BrdU labeled cells in Fig. 3G–I). In summary, *reelin* transcripts are enriched in the dorsal telencephalon but are excluded from the olfactory bulb region. Low expression of *reelin* is found in the ventral telencephalon mainly associated with midline cells of the ventral nucleus and with the central nucleus.

Figure. 6. Reelin expression in the forebrain.

A, B) Schematics illustrating the position of sections in C–N. A) At 5 days postfertilization (dpf), B) At 1 month postfertilization. C–N) Frozen transverse sections showing gene and/or protein expression (indicated bottom right) at 5 dpf or 1 month post fertilization. Dorsal is to the top. C–E) Olfactory bulb, 1 month. Neither mitral cells expressing *tbr1* (D) nor interneurons marked by Tyrosine Hydroxylase (TH) immunoreactivity (E) express detectable levels of *reelin* (C). F,G,I,J) Telencephalon. The highest expression of *reelin* in dorsal telencephalon (D) is observed in the medial (Dm) and dorsal (Dd) areas. In the ventral telencephalic areas, *reelin* transcripts are detected in a few cells at the level of the central nucleus (arrowheads in F, Iii) or near the midline of the ventral nucleus (Vv, arrows in Iii). These cells are located in a *tbr1*-positive area (6Ii). H,K) Diencephalon, rostral to the optic chiasm (oc). Both dll-positive (ventral) and dll-negative (dorsal) thalamic nuclei express low levels of *reelin*. Cells of the suprachiasmatic nucleus (SC) coexpress *reelin* and dll proteins. L) Diencephalon, section through the optic chiasm (oc) showing the distribution of *reelin* transcripts in relation to TH-immunoreactive cells and fibers. M) Diencephalon, section caudal to the optic chiasm, showing the ventral zone of the periventricular hypothalamus (Hv) and many of the TH-positive cells of the periventricular nucleus of posterior tuberculum (TPp) coexpressing *reelin* transcripts. N: At 1 month, the same nuclei as in L, M express *reelin*. For other abbreviations, see list page 9. Scale bars 100 μm in C–H, I (applies to Ii, Iii), J–N.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SC
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Ii
Dc
Vv
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Iii
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Dd
Dld
Vc
Dlv
Vv
reln/TH
J
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DI
Dp
Vp
Vs
ac
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TH
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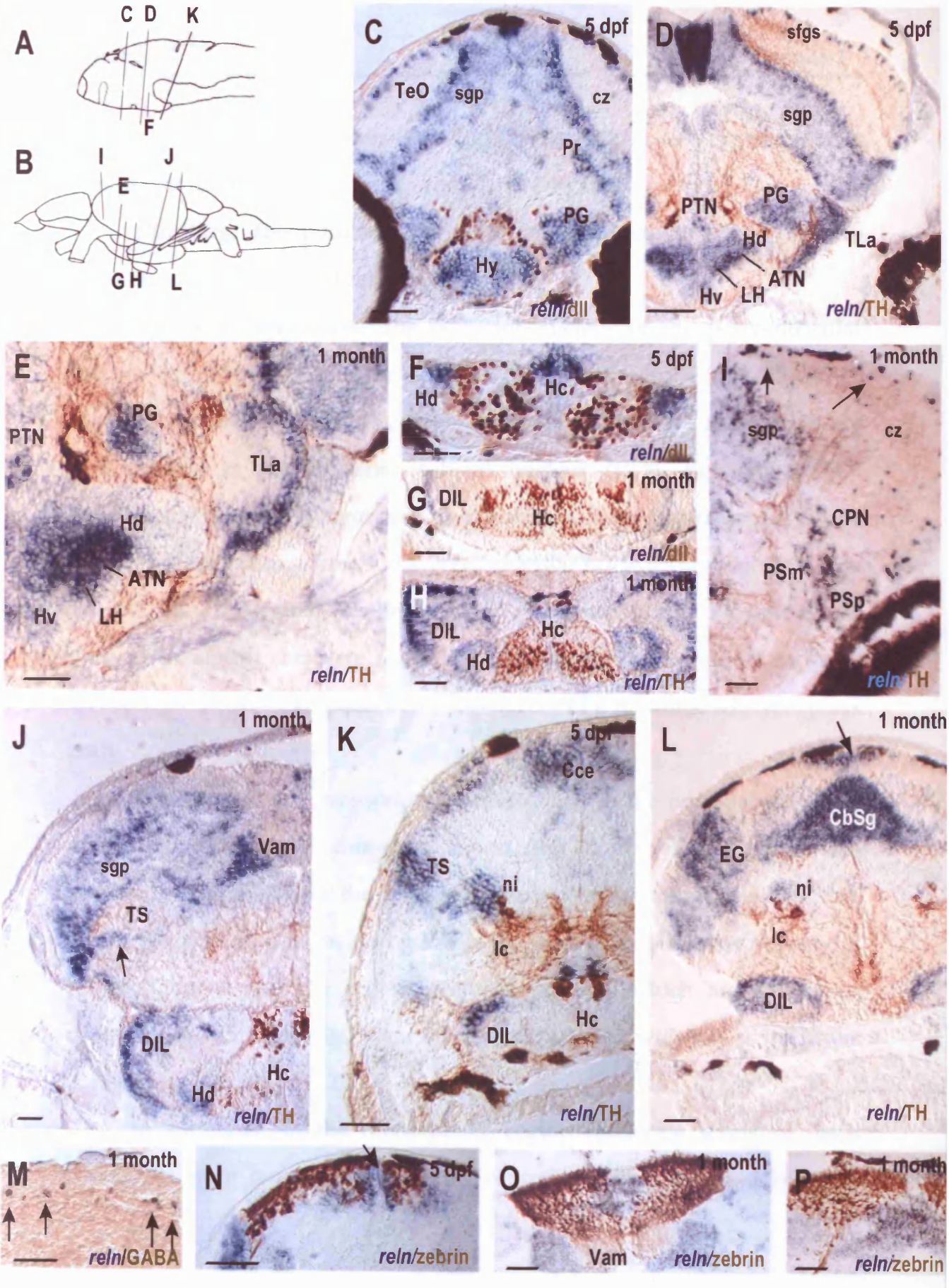
CP
TPp
PPp
SC
reln/TH

expressed *reelin* (figs. 5G; 7C,D). Strongly labeled cells were also observed in the medial (called PVO by Wullimann *et al* 1996) and lateral parts of the dorsal periventricular nucleus (Hd, figs. 7D, E). In addition, strongly labeled cells were present in the lateral hypothalamus (LH), the anterior tuberal nucleus (ATN) and the diffuse nucleus of the inferior hypothalamic lobe (DIL; 5G-I, 7D-H). At more caudal levels, *reelin* expressing cells were present mainly in the lateral DIL and Hd (figs. 5H, I, 7F-H). *reelin* was also strongly expressed by a small number of cells in the dorsal part of the caudal hypothalamic periventricular nucleus (Hc, fig. 7D). These cells did not co-express Dll or TH (figs.7G, H). Although *reelin* seemed to be expressed by most if not all hypothalamic cells, double labeling with TH and Dll antibodies clearly defines the ventral part of the caudal zone of the

Figure. 7. Reelin (reln) expression in the hypothalamus, mesencephalon, and cerebellum.

A,B) Schematics illustrating the position of sections in C–P. A) At 5 days postfertilization (dpf). B) At 1 month postfertilization. C–P) Frozen transverse sections showing gene and/or protein expression (indicated bottom right) at 5 dpf or 1 month postfertilization. Dorsal is to the top. C–E) Hypothalamus and posterior tuberculum. None of the hypothalamic *reelin*-positive nuclei express distalless or tyrosine hydroxylase (TH) at 5 dpf or later. The positive nerve fibers surround the preglomerular nuclei (PG), which express high levels of *reelin* transcripts throughout development. In the mesencephalon, cells of the lateral torus (Tla) and optic tectum (TeO) express high levels of *reelin*. (F–H) Posterior hypothalamus. Dll- (F, G) and TH- (H) positive cells in the ventral part of the caudal hypothalamic nucleus (Hc) do not express *reelin*. I) Pretectal nuclei and optic tectum. In the optic tectum, expression of *reelin* is also detected in the granule-like cells of the periventricular gray (sgp) and in large cells in the superficial layers (arrows). The latter cells are a subpopulation of the γ-aminobutyric acid (GABA)–immunoreactive interneurons, as shown by double labeling with a GABA antiserum in M (arrows point to double labeled cells). J–L) Isthmus. *reelin* expression in the torus semicircularis (Ts) and in the nucleus isthmi (ni), clearly visible at 1 month in J (for Ts) and L (for ni), can be traced back to 5 dpf (K). M) A subpopulation of GABA immunoreactive neurons in the optic tectum expresses *reelin* (arrows). N–P) Cerebellum. *reelin* expression is confined to the granule cell layer of the valvula (Vam in J), corpus cerebelli (CbSg in L), eminentia granularis (EG in L), and is excluded from Purkinje cells and molecular layers, labeled by zebrin immunoreactive Purkinje cell somata and dendrites (N–P). Zebrin is not expressed by Purkinje cells of the Valvula (Vam, O). Arrows in L and N point to *reelin* position external granular layer. For other abbreviations, see list. Scale bars 100 μm in C (applies to C–P).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sfgs
5 dpf
sgp
PTN
PG
Hd
TLa
ATN
Hv
LH
reln/TH
E
1 month
PTN
PG
TLa
Hd
ATN
Hv
LH
reln/TH
F
5 dpf
Hd
Hc
reln/dll
G
DIL
1 month
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1 month
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Hd
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PSp
reln/TH
J
1 month
Vam
sgp
TS
DIL
Hc
Hd
reln/TH
K
5 dpf
Cce
TS
ni
lc
Hc
DIL
reln/TH
L
1 month
CbSg
EG
ni
lc
DIL
reln/TH
M
1 month
reln/GABA
N
5 dpf
reln/zebrin
O
1 month
Vam
reln/zebrin
P
1 month
reln/zebrin

periventricular hypothalamus (Hc, fig.7F-H) as the only hypothalamic region that did not express *reelin*, from 5 dpf onward.

Pituitary. The pituitary did not express detectable levels of *reelin* transcripts in 1-month-old zebrafish.

Posterior tuberculum. The posterior nucleus of the periventricular tuberculum (TPp) was strongly labeled with the *reelin* probe (figs. 4B, 5F, G, 6L-N). Few *reelin* labeled cells, not immunoreactive for TH antibody, were also present in the posterior tuberal nucleus (PTN, figs. 5G, 7D,E). Moreover, strong *reelin* expression was found in the migrated nuclei of the posterior tuberculum, namely the preglomerular nuclear complex (PG, figs. 4A, 5G, 7C,D). Double labeling with a *reelin* probe and TH antiserum showed an almost complete reciprocal exclusion of the two markers at all levels, with the sole exception of a subgroup of TPp neurons, which express both.

Pretectum. *Reelin* transcripts were detected in the pretectal area at 5 dpf (Pr; fig. 7C) but at this stage it was not possible to distinguish the different nuclei that make the pretectal complex. At 1 month it was clear that all the pretectal nuclei, including the parvo- and magno-cellular superficial pretectal nuclei and the central pretectal nucleus, which are known to contain tectorecipient cells, were moderately labeled with the *reelin* probe (CPN, PSm, Psp; figs. 5F, 7I).

In summary, in the diencephalic region moderate levels of *reelin* transcripts were found in dorsal and ventral thalamic nuclei. Moreover,

reelin was enriched in basal plate derivatives, including the hypothalamic nuclei and the nuclei of the posterior tuberculum.

Eyes. *Reelin* was expressed in various neurons in the ganglion and inner nuclear layers of the retina (fig. 6F-J).

3.2.4.3.Mesencephalon

Optic tectum. *reelin* was expressed in a row of cells in the stratum fibrosum et griseum superficiale (sfgs) of the optic tectum (figs. 5G-J, 7C,D,I). These *reelin* expressing cells had round soma and large size and some of them were GABA positive (fig. 7M) and/or calretinin immunoreactive (not shown). Sparse large cells in the central zone (cz) at 5dpf also expressed *reelin* (cz; 7C, I). Small cells throughout the stratum griseum periventriculare (sgp) expressed low to moderate amounts of *reelin* (figs. 5G-I, 7C, D, I).

The highest amounts of *reelin* transcripts were found in the torus longitudinalis, which is a granular paired eminence specific to actinopterigian fishes (figs. 4A, B, 5G-H, 7C, D).

Tegmentum. In the torus semicircularis, two groups of cells contained *reelin* transcripts (TS; fig. 5H, I, 7J, K). One group was composed of medium size cells found in the anterior-medial torus semicircularis, close to the tectal ventricle. The other more caudal group consisted of large cells in layer 4 and

smaller cells in the ventral part (layer 1) of the lateral nucleus (layers as defined by Ito, 1974; arrow in fig. 7J).

In addition, the ventral part of the rostral tegmental nucleus (RT; fig 5H) express *reelin* and finally, the torus lateralis (TLa) was one of the most intensely labeled areas (figs. 5H, 7D, E).

In summary, many layered structures in the zebrafish mesencephalon, including the optic tectum and the torus semicircularis, expressed *reelin* in a laminar fashion.

Isthmus. In the mesencephalic subdivision of the reticular formation few labeled cells were observed. The interpeduncular nucleus, (Nin) which receives projections from the habenular nuclei, expressed *reelin* (fig. 5I, 7K). Dorsal to the interpeduncular nucleus, the raphe nuclei did not express *reelin*. Finally, the large catecholaminergic cells of the locus coeruleus did not express *reelin*, but a group of cells located just above them (corresponding to cells of the nucleus isthmi) had large amounts of *reelin* transcripts (figs. 5I, 7L). The group of *reelin* positive cells located just above the TH positive neurons of the locus coeruleus could be traced back in sections of 5 dpf zebrafish brains (fig. 4A, 7K).

3.2.4.4.Hindbrain

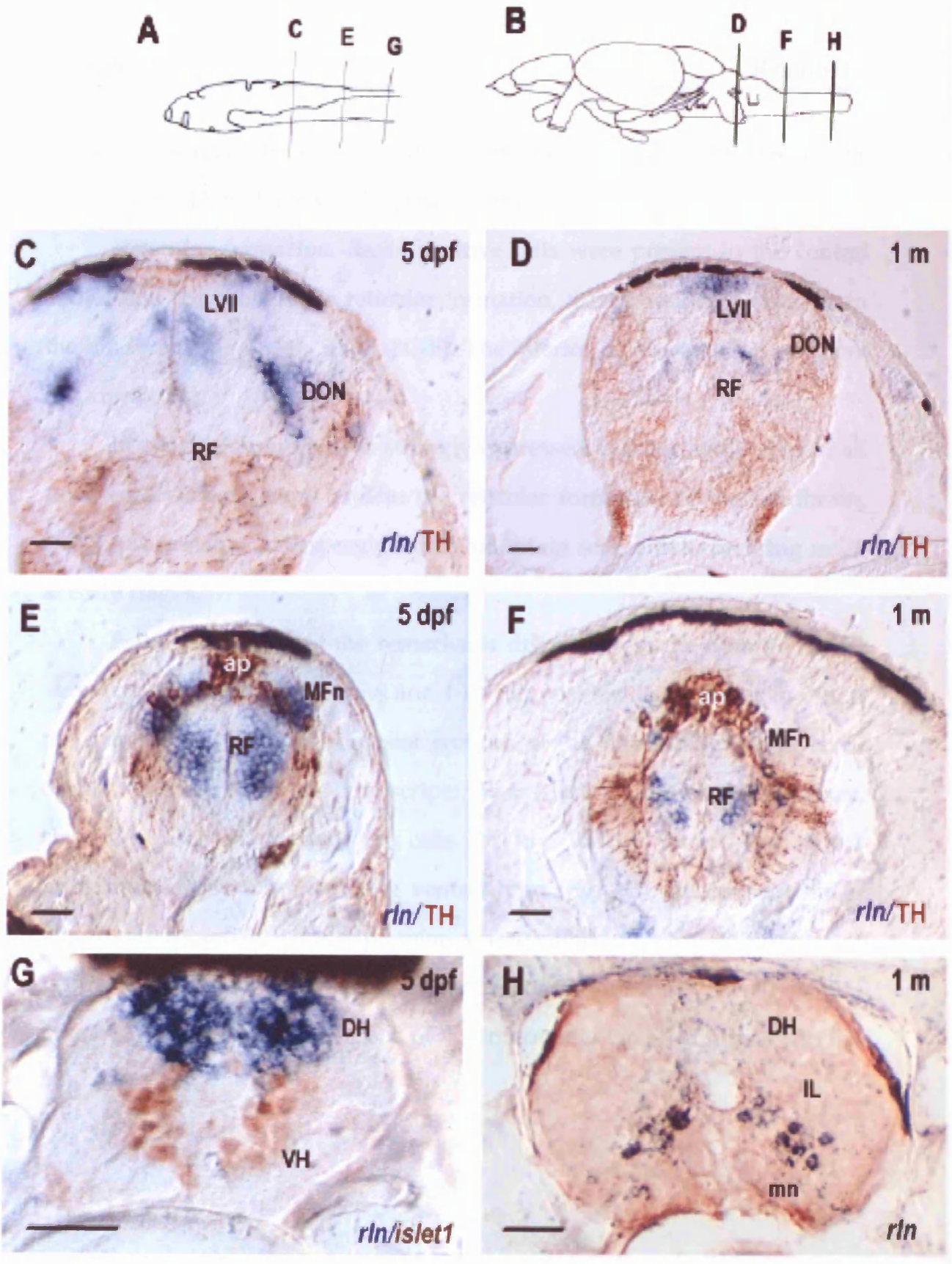
Cerebellum. All three parts of the fish cerebellum, namely, the valvula, the corpus cerebelli and the caudal lobe (vestibulo-lateralis) with

the lateral eminentia granularis, showed strong *reelin* expression confined to the granule cells (figs. 4A,B, 5I-K, 7J-L). In addition, *reelin* is expressed by cells of the external granular zone of the corpus cerebelli (arrow in fig 7L,N) both at 5 dpf and 1 month. *Reelin* expression was not found in Purkinje cells (labeled with a zebrin antiserum at 5 dpf, 7N, and 1 m, figs. O, P) and in the molecular layers of the corpus cerebelli. No gradients in *reelin* levels could be detected in the granule cells of the three-cerebellar parts. The crista cerebelli was devoid of *reelin* expression (CC; fig. 5K,L, 8C,D).

Cranial nerve nuclei. The motor nucleus of the trigeminal nerve, the facial motor nucleus and the abducens nucleus did not express *reelin* transcripts at detectable levels. In contrast *reelin* expressing cells were found in the octaval nuclei. Among them, the medial part of the dorsal octaval nucleus (DON) expressed moderate amounts of *reelin* mRNA throughout its rostrocaudal extension (figs. 5K, L; 8C,D). *reelin* expression in the dorsal octaval nucleus (DON) was stronger at 5 dpf (fig. 8C). In addition, few labeled cells were found in the anterior (AON) and posterior (CON) octaval

Figure. 8. Reelin expression in the hindbrain and spinal cord.

A,B)Schematics illustrating the position of sections in C–H. A) At 5 days postfertilization (dpf). B) At 1 month postfertilization. C–H: Frozen transverse sections showing gene and/or protein expression (indicated bottom right) at 5 dpf or 1 month postfertilization. Dorsal is to the top. C, D) Rostral hindbrain. Frozen transverse sections through the brainstem at the level of the lobus VII (LVII). *Reelin* (*reln*) expression is localized to cells of the reticular formation (RF), to the descending octaval nucleus (DON) and to midline cells in lobus VII. TH, Tyrosine Hydroxylase; cc, crista cerebellaris. (E, F) Caudal hindbrain. Frozen transverse sections through the area postrema (ap). *reelin* transcripts are excluded from the TH-positive cells of the ap but are detected in the region of the medial funicular nucleus (MFn) and in the RF. (G,H) Spinal cord. At 5 dpf (G), *reelin* transcripts are expressed by cells of the dorsal horn (DH), and absent in Islet 1-positive cells in the ventral horn (VH); at 1 month (H) a population of ventral interneurons dorsal to motorneurons (mn) and ventral to preganglionic sympathetic neurons of the intermediolateral column (IL) express *reelin*. Scale bars 100 μm.


A
C
E
G
B
D
F
H
C
5 dpf
LVII
DON
RF
rln/TH
D
1 m
LVII
DON
RF
rln/TH
E
5 dpf
ap
MFn
RF
rln/TH
F
1 m
ap
MFn
RF
rln/TH
G
5 dpf
DH
VH
rln/islet1
H
1 m
DH
IL
mn
rln

nuclei. The vagal lobe was devoid of *reelin* mRNA, whereas the facial lobe (LVII; fig.8C, D) had a few *reelin* positive cells.

Reticular formation. *Reelin* positive cells were present in the central and lateral columns of the reticular formation, throughout its extension in the hindbrain (fig.4A, B, 5K-M, 8C-F). The inferior olive was *reelin* negative (IO, fig. 4L 5L).

In conclusion, *reelin* was strongly expressed in the granule cells of all three cerebellar divisions and in the reticular formation of the hindbrain, which presumably corresponds to the hindbrain segments expressing *reelin* at early stages.

Spinal cord. One of the remarkable differences in the distribution of *reelin* transcripts between 5 days and 1-month post fertilization was found in the spinal cord. At 5-dpf, cryostat sections of the developing spinal cord, double stained for *reelin* transcripts and Islet1 immunohistochemistry, showed *reelin* expression in cells of the dorsal horn and Islet1 immunoreactivity in cells of the ventral horn (fig. 8G). By contrast, in 1-month-old zebrafish *reelin* expression was confined to a subpopulation of ventral interneurons located dorsally to motorneurons and ventrally to the preganglionic autonomic neurons of the intermedio-lateral columns (IL; fig. 8F).

3.2.5. Reelin expression in the adult zebrafish brain

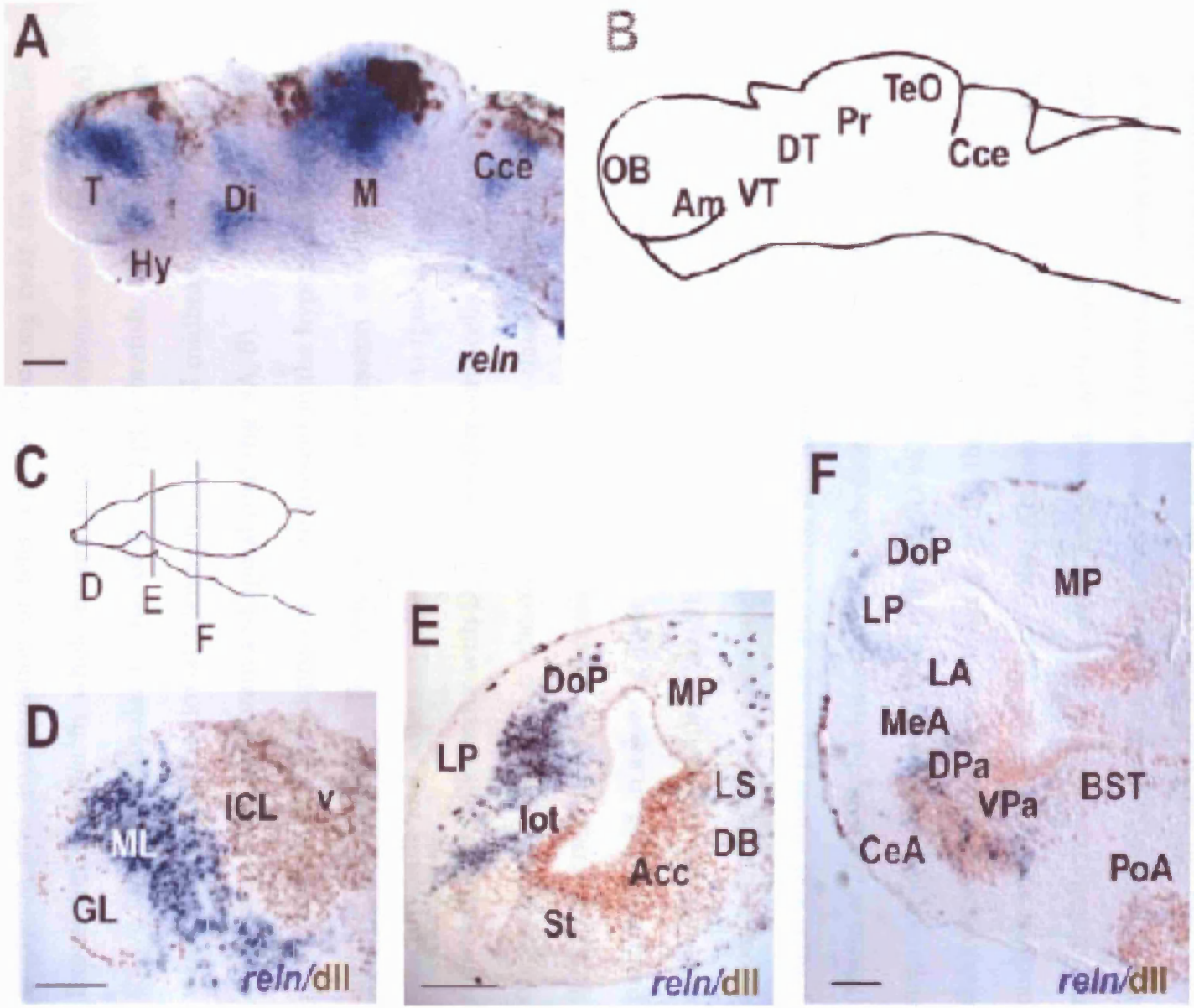
The expression of *reelin* was greatly reduced in adult zebrafish brains, but low levels of *reelin* transcripts continued to be detected in the same locations as described for 1-3 month old zebrafish. These locations included the telencephalic area dorsalis (D), the thalamic and hypothalamic nuclei and the nuclei of the posterior tuberculum, the optic tectum and the meso-rhombencephalic reticular formation. Strong expression persisted in the torus longitudinalis and in the granule cell layer of the corpus cerebelli.

3.2.6. Reelin expression in the brain of *Xenopus laevis*

The lack of *reelin* expression in the olfactory bulb and the diffuse expression in the dorsal telencephalon of fish were in contrast with the patterns reported for land vertebrates. These differences may be the consequence of the process of eversion or the absence of lamination or both. These are the major differences in morphogenesis of the dorsal telencephalon between teleosts and the other vertebrates. To investigate this issue further, the expression of *reelin* was studied in the developing brain of *Xenopus laevis*.

Figure. 9. Reelin expression in the brain of developing Xenopus laevis.

A, B) *reelin* (*reln*) expression (A) and schematic drawing (B) of stage 35 *Xenopus* embryo. C) Schematic drawing of a stage 50 *Xenopus* brain, illustrating the position of sections in D–F. D–F) Frozen transverse sections showing gene and/or protein expression (indicated bottom right). Dorsal is to the top. D) Accessory olfactory bulb. E) Rostral telencephalon. (F) Caudal telencephalon. For abbreviations, see list. Scale bars 100 μm in A, D–F.


A
T
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Iot
DB
Acc
St
reln/dll
F
DoP
MP
LP
LA
MeA
DPa
VPa
BST
CeA
PoA
reln/dll

Amphibia have an evaginated telencephalon (similar to amniotes) largely devoid of lamination (similar to teleosts) due to the absence of radial migration and accumulation of telencephalic neurons near the ventricle. Expression of *reelin* in whole mount stage 35 *Xenopus* embryos (fig. 9A) showed a pattern similar to that of 48-72 hpf zebrafish. Strong expression was visualized in the dorsal telencephalon, dorsal midbrain (optic tectum), cerebellar anlage, hindbrain and spinal cord (fig. 9A, B).

Patches of *reelin* expression were present in the hypothalamus, dorsal and ventral thalamus. By stage 50, *reelin* expression was strong in the olfactory bulb and accessory olfactory bulb of the tadpoles (fig. 9D), where *reelin* positive cells border with Distalless expressing cells. *Reelin* expressing cells were also abundant in the olfactory recipient (lateral) pallium (LP, fig. 9D 9E). Prominent *reelin* expression was present in large cells of the medial pallium aligned in a row or layer and in patches of *reelin* positive cells in the dorso-lateral region (fig. 9D 9E). Low levels of *reelin* expression were associated with basal ganglia nuclei, including striatum, nucleus accumbens, ventral and dorsal pallidum and lateral septum (nomenclature according to (Marin *et al.*, 1998; Nieuwenhuys *&* Nicholson, 1998). Ventrally to the *reelin* negative lateral olfactory tract (lot, fig. 9D 9E), cells of the lateral amygdala (LA) expressed high levels of *reelin,* as did the medially located cells of the nucleus of the diagonal band (DB). At more caudal levels (fig. 9E 9F), prominent *reelin* expression was associated with cells of the caudal amygdala (CeA), which also expressed Distalless proteins. Co-expression of *reelin* and Dll was also observed in the bed nucleus of the stria terminalis

(BST), whereas the Dll-positive cells of the ventral and dorsal pallidum did not express *reelin* transcripts.

In summary, the expression of *reelin* in the telencephalon of stage 50 *Xenopus* embryos is highly reminiscent of the pattern described in sauropsids (Bernier *et al.*, 1999; Bernier *et al.*, 2000).

3.3. Discussion

This chapter describes the identification of *reelin* sequences from zebrafish and *Xenopus* cDNA libraries, thus adding two new vertebrate *reelin* genes to the list of *reelin*-related sequences already identified in other vertebrates. The study of *reelin* expression in the developing zebrafish and *Xenopus* brain has revealed major species-specific differences in telencephalic expression (Bar & Goffinet, 2000). First, no *reelin* transcripts were found in the olfactory bulb. This difference could be due to the presence of an additional paralog *reelin* gene in zebrafish not isolated in this screen and expressed in the olfactory bulb. Second, in developing zebrafish, *reelin* transcripts were distributed to the majority of pallial cells with no indication of laminar localization, unlike in mammals and sauropsidian embryos. The only telencephalic region not expressing *reelin* in the early larval zebrafish are the ventrolateral and posterior regions (Dlv, Dp), which have been related with olfaction (Meek *et al.*, 1998).

During the examination of *reelin* expression in zebrafish it was observed: a) the pattern of *reelin* expression was highly dynamic during the

first 48-72 hours, reflecting the onset of *reelin* expression in newly born postmitotic neurons; b) by 5 dpf the pattern of *reelin* expression became restricted to specific CNS regions and cell populations where expression persisted unvaried to 1-3 months of age; c) the expression patterns that were conserved among vertebrate species include those related to laminated CNS structures and those where complementary expression of *reelin* and Reelin receptors/effectors may be responsible for the correct positioning of nuclei and cell groups. This suggests that the function attributed to the Reelin pathway during development in positioning cell groups and nuclei is conserved in anamniotes; d) *reelin* expression in the brain of 1-3 month old zebrafish was prominent in regions of high synaptic remodeling. This suggests that Reelin may play different/additional roles when development/migration of CNS structures is completed.

3.3.1. The dynamic expression of *reelin* during the early phases of CNS development reflects the pattern of neurogenesis.

Domains of *reelin* expression appear in quick succession throughout the CNS, in areas where differentiation of neuronal groups is known to take place (Wullimann & Puelles, 1999; Wullimann & Knipp, 2000). These include the telencephalon, the early differentiating neurons of the ventral diencephalon, the area of the posterior commissure and the nucleus of the

medial longitudinal fascicle. The absence of *reelin* expression in dividing (BrdU labeled) cells is a further indication of the association between *reelin* and differentiating neurons. This finding suggests that *reelin* may be important for the migration of newly born neurons, for the formation of the nuclei, and/or for the growth of their axons, which form the early scaffold of the future descending/ascending axon tracts (Chitnis & Kuwanda, 1990; Wilson *et al.*, 1990; Ross *et al.*, 1992).

Mammalian Cajal-Retzius (C-R) cells of the pallial marginal zone are among the first generated neurons in the cortical plate and are characterized by strong *Reelin* expression (Ogawa *et al.*, 1995). Expression of *Reelin* in C-R cells and in the early generated zebrafish nuclei peaks after their migration is completed, suggesting a role for *reelin* in axonal growth or signalling to adjacent cells, rather than in their migration. Migrating cells, which use the Reelin pathway, usually express one or more Reelin receptor(s) and the Reelin effector Dab1 (Howell *et al.*, 1997b), but not Reelin itself. This complementary association of cells expressing *reelin* on one side and cells expressing Reelin receptors/Dab1 on the other has been observed in the mammalian cerebral cortex, retina, cerebellum, hindbrain and spinal cord (Goffinet *et al.*, 1999; Carroll *et al.*, 2001).

3.3.2. Restricted expression of *reelin* from 5 dpf to adulthood suggests specific roles for the reelin pathway in neuronal positioning

By 5 dpf, the pattern of *reelin* expression has acquired its permanent distribution. There are specific cell populations that, in zebrafish as in all the other species, express *reelin* throughout development. These may be grouped in two categories: those belonging to laminated structures and those that are responsible for the positioning of other cell groups or nuclei (based on observations carried out in *reeler* mice: Fujimoto *et al.*, 1998; Gallagher *et al*, 1998; Lambert de Rouvroit & Goffinet, 1998; Deller *et al*, 1999; Yip *et al.*, 2000; Rice & Currant, 2001). There are several laminated structures in the fish brain, including the optic tectum, the torus semicircularis, the cerebellum, and the retina. In all these structures, *reelin* is expressed in a layer-specific manner from 5 dpf to 3 months or later. In the optic tectum, two of the seven layers have *reelin*-expressing cells. Most of the large type I interneurons of *stratum fibrosum et griseum superficiale* (sfgs) express high levels of *reelin*, whereas all of the granule cells of the periventricular stratum express varying levels of *reelin*, depending on the developmental stage (higher at earlier stages). *reelin* is not expressed by any of the other 10 or more cell types of the optic tectum, including the projection neurons. At present, it is not known whether the *reelin*-expressing cells of the *stratum fibrosum et griseum superficiale* play any function in providing positional information to other migratory cell populations. In *reeler* mice, the layering

of the superior colliculus is disrupted, suggesting a role for the Reelin pathway in the structural organization of mesencephalic tectal derivatives across species (Frost *et al.*, 1986). It is interesting to note that another fish mesencephalic structure, the torus semicircularis, which is organized in layers (Ito, 1974), also expresses *reelin* in a layer-specific pattern and specifically in the periventricular layer 1 and in the most peripheral layer 4.

In the mouse cerebellum, the role of Reelin and its signaling pathway in positioning Purkinje cells and in the architectural organization of cerebellar layers has been extensively studied (Goffinet, 1983; Miyata *et al.*, 1997). In mammals, Purkinje cells express high levels of Dab1 and a complement of Reelin receptors (CNR, VLDL/ApoER2; Rice & Curran, 2001) and respond, possibly by halting their migration, to the high levels of Reelin expressed/secreted by granule cells precursors in the overlying external germinal layer (Herrup, 2000). One of the first observed defects in the *reeler* mouse was the disruption of the Purkinje cell layer and the disorganization of cerebellar structure (Mariani *et al.*, 1977). The distribution of *reelin* transcripts in the zebrafish cerebellum (*i.e.*, in the granule cell layers of all major cerebellar divisions, including the external granular cell layer, which persists throughout fish life, described by Pouwels (1978) suggests that *reelin* may play a similar role in fish. The role of Reelin signaling in positioning nuclei and cell groups is further documented by the cytoarchitectural defects found in the hindbrain and spinal cord of *reeler* mice (Goffinet, 1984; Yip *et al.*, 2000). Investigations carried out in mice have clarified the mutually exclusive domains of expression of *reelin* and CNRs/Dab1 in the developing

mouse hindbrain and spinal cord (Carroll *et al.*, 2001). *reelin* expression is excluded from cranial nerve nuclei and CNRs and/or Dab1 are expressed by the trigeminal motor, facial, and hypoglossal nerve nuclei. In the mouse spinal cord, CNRs and Dab1 are expressed in subpopulations of motor neurons, whereas *reelin* is expressed by ventral interneurons located just dorsal to the motorneurons (Yip *et al.*, 2000; Carroll *et al.*, 2001).

In the developing zebrafish hindbrain, *reelin* transcripts was not found in the cranial nerve nuclei, with the sole exception of the octaval nerve complex. However, *reelin* was expressed by at least two (intermediate and lateral) columns in the rhombencephalic reticular formation. This pattern of expression is suggestive that reelin secreted by the cells of the reticular formation has a role in creating an "avoidance" zone for most cells/axons of the branchiomotor, somatomotor, and sensory nerve nuclei. It is likely that the segmental expression of *reelin* in the hindbrain during development represents an early marker of these neurons, which are known to have a segmental distribution (Trevarrow *et al.*, 1990).

The pattern of *reelin* expression in the spinal cord of zebrafish is extremely dynamic. At 24 hpf, cells throughout the spinal cord expressed *reelin*. By 5 dpf, *reelin* was mainly expressed by neurons of the dorsal horn and is excluded from the Islet1-positive ventral horn cells. Conversely, in 1-month-old zebrafish, it is a population of interneurons, located in the intermediate gray matter that expressed *reelin*. This latter distribution corresponds to the localization of *reelin* transcripts in the developing mouse spinal cord, where *reelin* expressing interneurons guide the positioning of

the preganglionic autonomic neurons of the intermediolateral column (Yip *et al.*, 2000).

3.3.3. *Reelin* expression in the developing telencephalon of anamniotes

The expression of *reelin* in the telencephalon of developing zebrafish differed greatly from that found in other species. Firstly, it was absent from the olfactory bulb throughout its development. Secondly, *reelin* was not expressed in a layer or laminar pattern seen in the telencephalon of most vertebrates.

The pattern of *reelin* expression in zebrafish contrasts with that found in developing mammals, where *reelin* is primarily expressed in a layer of early born cells in the marginal zone of the cortical plate (Ogawa *et al.*, 1995), and with the various telencephalic *reelin* expression patterns described in the developing reptile and avian brain (Bar & Goffinet, 2000). In zebrafish, strong expression of *reelin* is found in the majority of cells of the dorsal telencephalon (pallium) from 24 hpf. These cells are densely packed at early stages (see D in Fig. 6F, for example) and subsequently migrate to reach their final position in the mature telencephalon, at the periphery of the centrally located white matter tracts (compare D of Fig. 6F with Dd and Dm of Fig. 6I; Wullimann *et al.*, 1996).

In mammals, Reelin has been associated with the radial migration of cortical cells (Aboitiz, 1999). On the contrary, little is known about the mode of migration in the everted telencephalon of teleosts and a possible role of Reelin in this process remains to be elucidated. Although the pattern of *reelin* expression within the telencephali differs, the presence of *reelin* transcripts in the dorsal domain (pallium) is highly conserved in all vertebrates. According to recent interpretations of gene expression patterns, the developing vertebrate pallium is composed of four divisions: the medial, dorsal, lateral, and ventral pallium (Puelles *et al.*, 2000). On the basis of this model, *reelin* shows remarkable conservation of expression pattern in tetrapods (Bernier *et al.*, 1999, 2000; Goffinet *et al.*, 1999; Bar *et al.*, 2000). The gradient of increasing larger areas of *reelin* expression from medial to ventral pallium suggests a similar gradient of the signal(s) that regulates and maintains *reelin* expression in the developing dorsal telencephalon.

A candidate regulator of *reelin* expression, *tbr1*, is expressed in the same territory, both in mice (Bulfone *et al.*, 1995) and zebrafish (Mione *et al.*, 2001). In a study in the mouse, Tbr1 was shown to activate the expression of a luciferase reporter driven by t-box binding sites from the *reelin* promoter (Hsueh *et al.*, 2000). In the zebrafish dorsal telencephalon, the expression domains of *tbr1* and *reelin* overlap almost completely from early stages to adulthood (Mione *et al.*, 2001). Furthermore, additional domains of *reelin* and *tbr1* expression are present in the ventral telencephalon (this chapter). Here *reelin* is expressed in a few cells close to the midline in the ventral nucleus of the ventral telencephalic area (Vv), a nucleus that may correspond for

location and gene expression (Mione and Wilson, unpublished observations) to the septal area of amniotes.

In mammals and birds, strong *tbr1* expression is associated with the superficial septal area and with the vertical and horizontal limbs of the diagonal band (Puelles *et al.*, 2000). In addition, *tbr1* and *reelin* are expressed in a migrated group of cells located at the level of the central nucleus of V (Vc). The functions, connections, and origin of Vc are largely unknown. Wullimann *et al.* (1996) describe it as a migrated nucleus of the area ventralis, perhaps suggesting an origin from the subpallial ventricular zone.

In the plainfin midshipman (*Porichtys notatus*), a ventral telencephalic small nucleus located in the same position as Vc is known to receive auditory afferents (Bass *et al.*, 2000). In bird, auditory inputs to the ventral telencephalon terminate in the paleostriatum and in an area of the dorsal ventricular ridge, called "field L," which is strongly positive for *reelin* (Bernier *et al.*, 2000). According to recent comparative analysis of gene expression in the forebrain (Fernandez *et al.*, 1998; Puelles *et al.*, 2000), the dorsal ventricular ridge of birds, including the auditory recipient "field L," is part of the ventral pallium.

The *reelin* expression in zebrafish Vc raises the question of the homology of this nucleus with other acoustic recipient, ventral pallial nuclei of vertebrates. Two scenarios are possible: Vc could be a migrated part of the dorsal nucleus (Vd), and, therefore, be subpallial in nature. Alternatively, it could have a pallial origin and share homologies with other ventral pallial derivatives, such as "field L" of birds or amygdaloid areas of mammals. The

expression of *tbr1* in Vc supports the hypothesis of a pallial origin of this area.

As in other vertebrates, the *tbr1*-positive territory in fish is larger than the *reelin*-positive domain. In chicks the entire dorsal ventricular ridge is marked by *tbr1* expression, whereas strong *reelin* expression is only detected in "field L" (Bernier *et al.*, 2000). In the mouse both centromedial and basolateral divisions of the amygdala express *reelin* (Alcantara *et al.*, 1998), whereas ventral telencephalic *tbr1* expression extends to other cell groups beyond the amygdala (Bulfone *et al.*, 1995). In *Xenopus* (see above), the lateral, medial, and caudal amygdalar populations express *reelin*, but no detailed data are available on T-box gene expression in the telencephalon. Altogether these observations suggest that the conservation of spatial domains of *reelin* expression in the forebrain may be due to conserved expression and activity of upstream T-box genes.

The differences in *reelin* expression in the telencephalon of the zebrafish compared to the other vertebrates are not easy to explain and could be related to the morphogenesis of this structure. To gain insights into these issues, it is important to clarify several points: (1) if the distribution of *reelin* transcripts in the dorsal telencephalon is related to the eversion process. (2) If the expression of *reelin* in the ventral telencephalon is due to the presence of ventrally positioned pallial cell groups within the subpallial territory. The expression of *reelin* was analyzed in another anamniote, *Xenopus laevis,* in which the telencephalon evaginates but no major migration or laminar organization of dorsal telencephalic cells have been

reported to occur. As expected, *reelin* expression is found in the dorsal telencephalon in *Xenopus*. Transcripts are present in the olfactory bulb and accessory olfactory bulb. Therefore, unless a paralogous *reelin* gene is found in zebrafish, the absence of *reelin* expression in the zebrafish olfactory bulb might be a peculiarity of teleosts.

In the *Xenopus* telencephalon, a single layer of large neurons in the medial pallium expresses *reelin,* as blocks of cells do in the dorsal and lateral pallium. This pattern is highly reminiscent the expression of *reelin* in the sauropsid brain (Bar *et al.*, 2000) and suggests that the distribution of *reelin* transcripts in the developing telencephalon of vertebrates is similar for all evaginated telencephali. The pattern of *reelin* expression, thus, may be related with the morphogenetic process (eversion vs. evagination) underlying telencephalic formation, rather than with the degree of radial migration of telencephalic cells.

Indeed, no radial migration of telencephalic neurons occurs in *Xenopus* (Northcutt, 1981). However, the pattern of *reelin* expression hardly differs from that found in the telencephalon of birds and reptiles, where radial migration does occur (Bar & Goffinet, 2000). Cells that express *reelin* in the ventral telencephalon of *Xenopus* are found in the nucleus of the diagonal band and in the lateral and caudal amygdala, whereas the subpallial striatum and pallidum are mainly devoid of *reelin* transcripts. This suggests that the domains of *reelin* expression identified in the ventral telencephalon of zebrafish and other vertebrates, which also express *tbr1*,

may be associated with the territory corresponding to the nuclei of the ventral pallium of Puelles *et al.* (2000) rather than with subpallial nuclei.

3.3.2. Why there is so much *reelin* in the young/adult brain of vertebrates?

The presence of large amounts of *reelin* mRNA in the adult brain is constantly reported in all vertebrates (Ikeda & Terashima, 1997; Alcantara *et al.*, 1998) including the adult zebrafish. In the postnatal telencephalon of mammals, Reelin is associated with GABAergic inhibitory interneurons of various classes (Pesold *et al.*, 1998) and with developing axon tracts, including hippocampal entorhinal afferents, which are severely disrupted upon removal of local Cajal-Retzius cells or application of Reelin blocking antiserum (Del Rio *et al.*, 1997; Nakajima *et al.*, 1997; Borrell *et al.*, 1999).

Reelin localization and function in the adult brain are related with modulatory or inhibitory synapse stabilization and dendritic re-modeling. Reelin molecules, possibly secreted by GABAergic interneurons, assemble multimeric complexes through their F-spondin domains at the level of the dendritic spines of cortical pyramidal neurons, where they are held together by membrane bound alpha3beta1 Integrins and CNR molecules (Rodriguez *et al.*, 2000; Utsunomiya-Tate *et al.*, 2000). These complexes, in addition to stabilizing the organization of the synaptic cleft, are thought to signal through the receptor molecules and promote the stabilization of dendritic spine microtubules through Dab1 phosphorylation and interactions with

various cytoskeletal components (Stockinger *et al.*, 2000; Feng & Walsh, 2001). It is interesting to note that in zebrafish, high levels of *reelin* are again associated with regions of dendritic spine re-modeling, such as the well developed spiny dendritic trees of type I neurons in the optic tectum. Most of the regions expressing *reelin* in 1- to 3-month old zebrafish are multisensory integration centers, suggesting an involvement of the Reelin pathway in synaptic organization or plasticity in these centers. A relation between *reelin* and multisensory integration centers is also suggested by the expression of this molecule in the zebrafish telencephalon. Clearly, in teleosts *reelin* expression cannot be associated with the development of lamination; a process that itself has been proposed to lead to more efficient integration of multisensorial information (Reiner, 2000). For example, the dorsal pallium exhibits laminar organization (cortex) in amniotes but is organized into a rather simple and compact group of small cells in zebrafish. However, in this structure too, it is likely that *reelin* expression plays a role in the integration of sensory afferent information received by dorsal telencephalic areas from tectal or diencephalic sources.

Indeed, *reelin* transcripts are also found in the dorsolateral (Dld) and medial (Dm) part of the dorsal telencephalic area, regions suggested sharing homologies (in functions, connectivity, cell types, or origin) with the medial pallium and lateral amygdala of other species (Bradford, 1995; Kapsimali *et al.*, 2000). Although it is not clear to what extent the organization and function of actinopterygian Dld and Dm resemble those of the medial and ventral pallium of other vertebrates, their pattern of connections suggest that

they are centers of integration of multisensory information. They receive and integrate acoustic, mechanosensory, somatosensory, and viscerosensory stimuli from preglomerular, thalamic, and hypothalamic sources (Nieuwenhuys *et al.*, 1998). As with other integrative areas of the zebrafish brain, they express *reelin*, reinforcing the hypothesis of a link between Reelin, sensory information processing and complex synaptic organization. As suggested by Perez-Garcia *et al.* (2001), the different distributions of *reelin*-expressing cells in the developing telencephalon of vertebrates may correlate with differences in the orientation and distribution of the dendritic trees of the principal neurons, which differ greatly between species. This interpretation also establishes a link between the pattern of *reelin* expression during development and in the adult, and suggests that manipulations of *reelin* expression may lead to abnormalities in the distribution of dendrites. This hypothesis is testable in zebrafish and required further investigation in *reeler* mice. In fact, it is clear that defects in synaptic organization may have not been evident in *reeler* and *scrambler/yotari* mutants, which die before the vast majority of synaptic circuitries are refined, additional focused studies may be required.

With the establishment of the zebrafish as a model for behavioural studies and with the development of specific tests, it will be possible to address the role of the Reelin pathway in sensory processing, exploiting the wealth of molecular and genetic techniques available in this species. Moreover, the study of molecules downstream the Reelin signaling will help

to elucidate the functions of this molecule that is highly expressed throughout the development of the vertebrates.

In the next chapters I will present results concerning zebrafish *disabled1* (*dab1*) that is the main intracellular effector of Reelin. I will focus on the genetic organization of the coding region and I will describe preliminary studies about the functions of this molecule during zebrafish development.

CHAPTER 4

4. Result 2. Danio rerio dab1 is alternatively spliced and gives rise to tissues-specific isoforms

4.1. Introduction

Disabled1 is the main intracellular effector of Reelin. The mutant mice *scrambler* and *yotari* display phenotypes similar to those found in the mouse mutant *reeler* at the level of neuron migration. Recent analysis of human and mouse *Dab1* genomic sequence has revealed a very complex gene structure. Mammalian *dab1* is composed of 14 exons, spanning over 1.1 Mb and giving rise to at least five alternative tissue-specific splicing events in mice plus several 5'-untraslated regions (UTRs) with different promoters (Bar *et al.*, 2003).

In this chapter I present a study of zebrafish *dab1* gene that shows a high degree of complexity in its genomic structure, while its different isoforms show temporal and tissue specific expression similar to Mammals (Bar *et al.*, 2003).

4.2. Results

4.2.1. Cloning of zebrafish *Dab1*

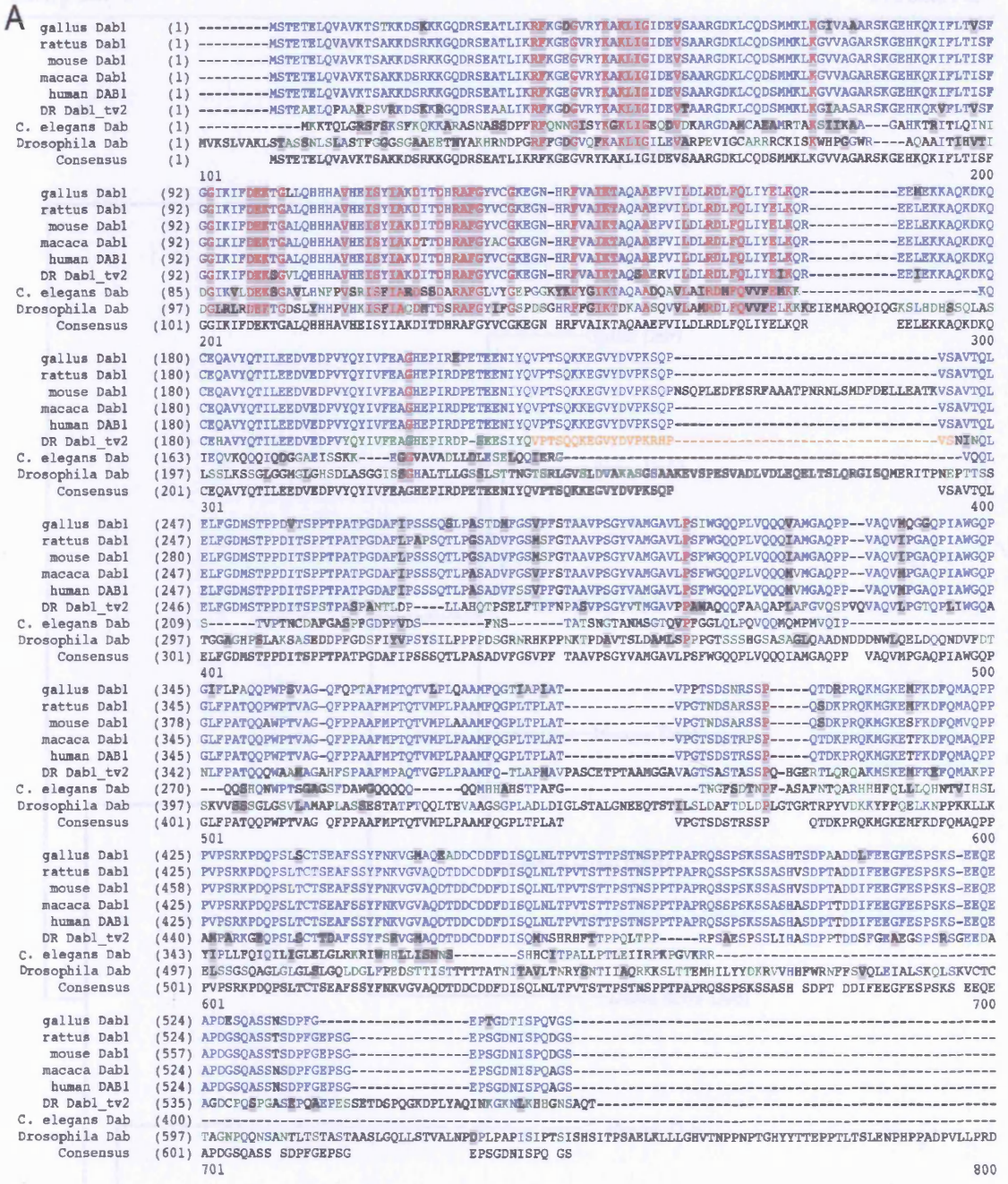
A zebrafish *disabled1* (*dab1*) cDNA clone was identified through library screening. This clone encoded for a protein of 538 amino acids showing high similarity to other Disabled1 proteins (fig.1, 2 and3A) and was named *danio rerio disabled1, transcriptional variant 1* (*dab1_tv1,* genebank, acc. No. DQ166810*)*. RT-PCR analysis (see below) revealed the existence of a second transcriptional variant (*dab1_tv2*, genebank acc. No. DQ166811). Comparison of the longest isoforms of Dab1 from several species yielded the phylogenetic tree shown in fig.2.

The genomic structure of the zebrafish *dab1* gene was analyzed using the genomic sequence provided by the Sanger Institute and the predicted exon/intron structure was validated with RT-PCR and *in situ* hybridization.

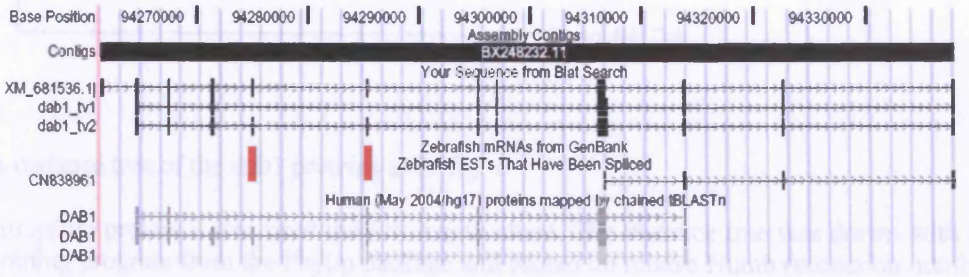
Figure.1. Sequence analysis of zebrafish dab1.

A) Alignment of amino acid sequences of human, primate, mouse, rat, chick, worm, fly (truncated at aa 693) and zebrafish clones was obtained with the Clustal-X program and checked manually. The accession numbers of the sequences used here are as follows: *Homo* NP_066566; *Mus* P97318; *Rattus* NP_705885; *Gallus* NP_989569; *Macaca* Q9BGX5; *C. elegans* NP_495732; *Drosophila* NP_066566 and zebrafish. aa identical in all species are shown in red in a grey background, aa identical in all vertebrate sequences are shown in blue. Conserved aa are shown in black on a grey background, Consensus sequence is at the bottom. Note that aa belonging to exon 8 in zebrafish Dab1 are in green, whereas aa belonging to exon 9 are in orange. B) UCSC Zebrafish Genome Browser Blat alignment, representative view (http://genome.ucsc.edu). Genome mapping and alignment of Dab1-tv1 and Dab1-tv2 on the contig BX248232.11. The red bars underline the two exons skipped in Dab1-tv1. The sequence XM_681536.1 is predicted by the automated computational analysis program GNOMON at NCBI (http://www.ncbi.nlm.nih.gov/) and supports the structure of Dab1-tv1 and Dab1-tv2 for the last 2 exons, together with the Zebrafish EST CN838961. The last three rows represent a 'chained alignment' of human DAB1 ESTs with *D.rerio* Dab1_tv1 and Dab1_tv2.

A


gallus Dabl
rattus Dabl
mouse Dabl
macaca Dabl
human DAB1
DR Dabl_tv2
C. elegans Dab
Drosophila Dab
Consensus

B


Base Position
94270000
94280000
94290000
94300000
94310000
94320000
94330000
Contigs
Assembly Contigs
BX248232.11
Your Sequence from Blat Search
XM_681536.1
dab1_tv1
dab1_tv2
Zebrafish mRNAs from GenBank
Zebrafish ESTs That Have Been Spliced
CN838961
Human (May 2004/hg17) proteins mapped by chained tBLASTn
DAB1
DAB1
DAB1

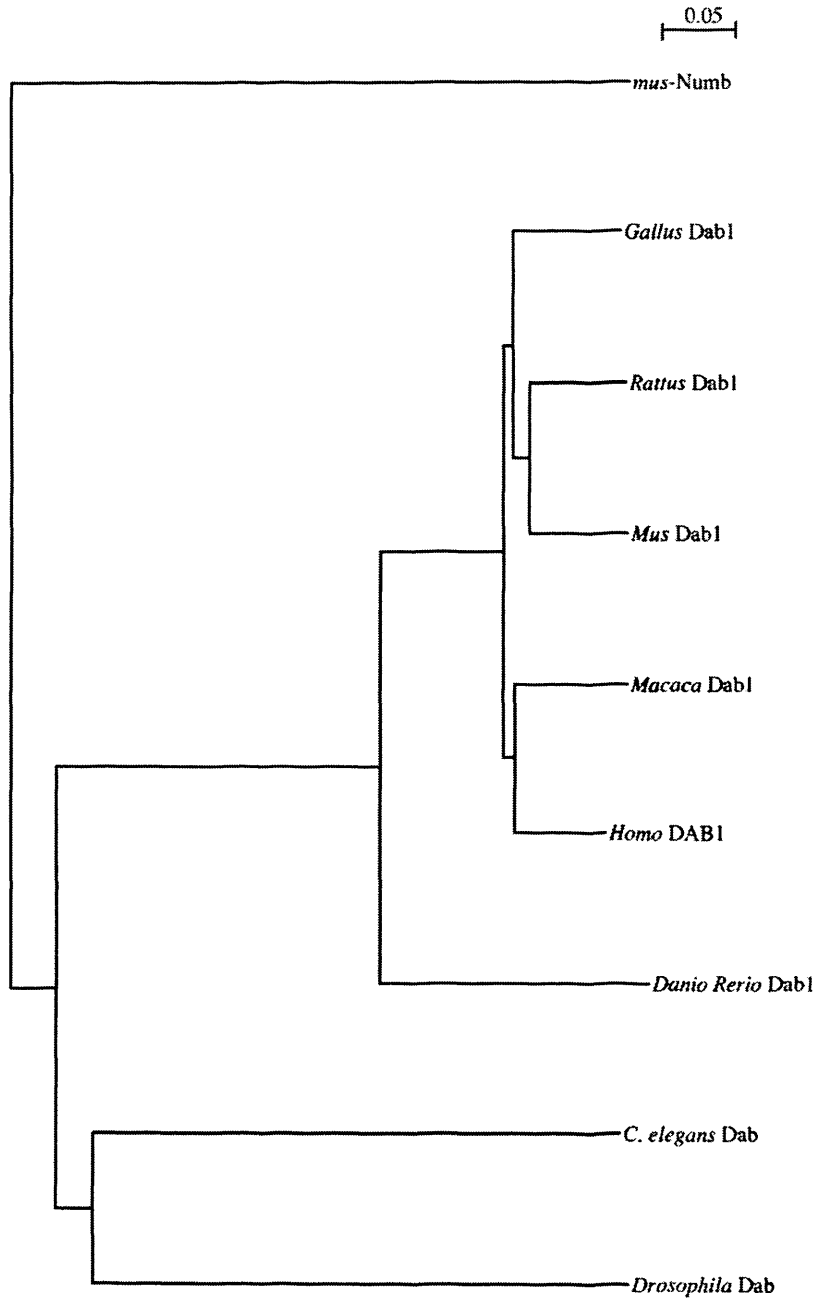


Figure.2. A distance tree of the dabl proteins as in fig. 1.

For the *Drosophila* protein it was used the full-length clone. The distance tree was drawn with the Neighbor-joining program from the Phylip package and rooted on mouse Numb (accession number: Q9QZS3).

4.2.2. Identification of three genomic clones encoding *dab1*

It was possible to screen electronically the zebrafish genomic database at NCBI using stretches of sequences of about 30 nucleotides of zebrafish *dab1_tv1* and three clones covering most of the genomic sequence of *dab1* were identified. The size, coding sequences and contig assembly of these three BAC clones are shown in fig. 3.

Analysis of the sequences of the 3 BAC clones revealed that *dab1_tv1* was encoded by 13 exons, mainly corresponding to those present in mDab1-555 (fig. 3A). However some exons differed or were missing suggesting that *dab1_tv1* was one of several possible combinations of exons transcribed from the zebrafish *dab1* gene. Most of all, the region containing the stretch of tyrosyl residues, known to be important for signaling, differed notably from that of mouse Dab1-555. This was suggestive of the presence of genomic sequences for additional exons encoding the missing tyrosine residues (see below).

The *dab1*-coding region starts in exon 2, which harbors the start codon, and ends in exon 15 (fig. 3 A). The position of the exons in the three BAC clones is shown in fig.3A. Although partially conserved, exon 14 does not contain a stop codon as in the human and in the mouse. The organization exon-intron and the splice junctions followed mostly the GT/AG rules. However intron 9 has a GG/AG junction (table I). The length of the exons is similar to that of the same exons in the other species studied, while introns have variable length (table I).

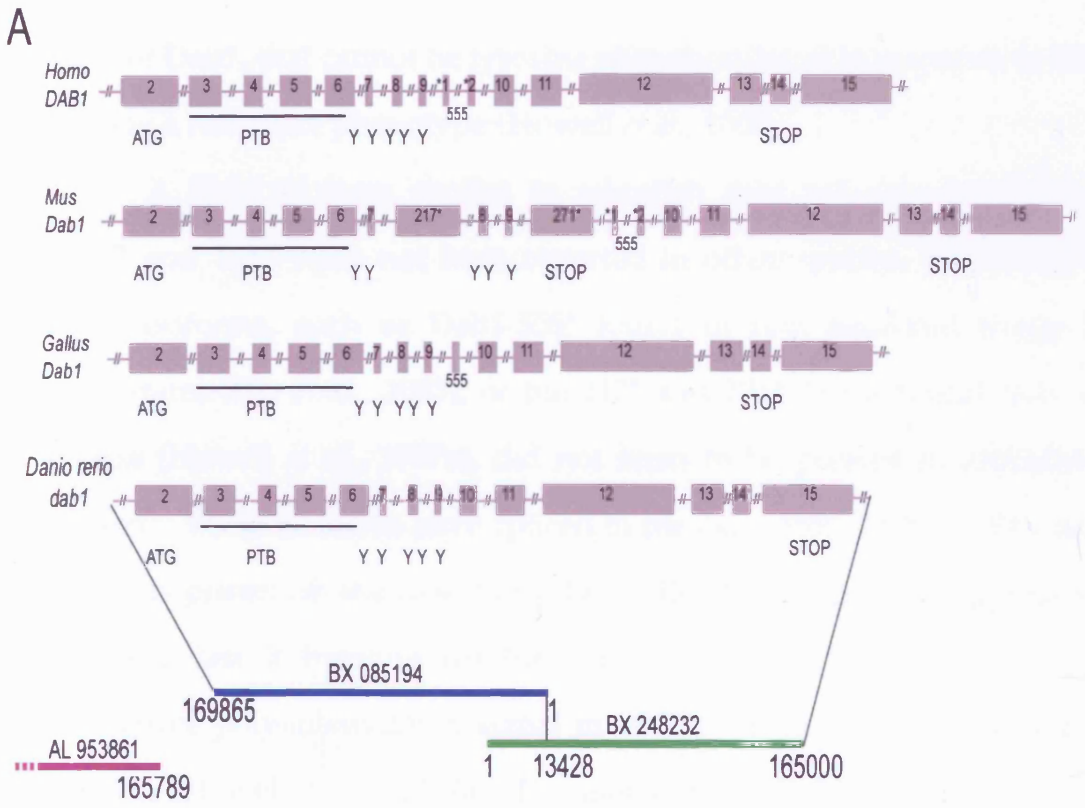
The Protein Interaction/Phosphotyrosine Binding Domain (PI/PTB) of Dab1, which binds the Reelin receptors is encoded by exons 3, 4, 5 and 6, while the phosphorylation domain containing five tyrosine residues is encoded by exons 6 (Tyr^{185}), 7 (Tyr^{198}), 8 (Tyr^{200} and Tyr^{220}) and 9 (Tyr^{232}) similar to mouse and human *Dab1*. The carboxyl-terminal sequence containing a consensus sequence for the serine/threonine kinase Cdk5 was found in exon 13, as in mouse Dab1-555 (fig. 3B).

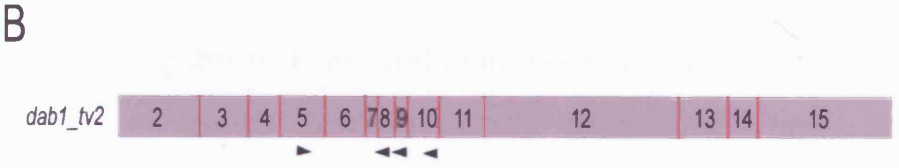
4.2.3. Comparison of alternative splicing event of mouse and zebrafish *dab1* genes

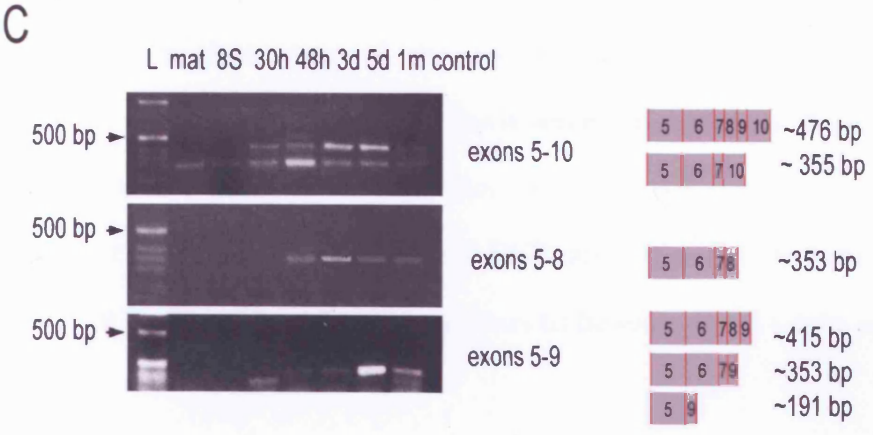
Although the genomic structure of *dab1* is highly conserved among vertebrates, there are differences in the alternative splicing events reported to occur in the various species studied. The zebrafish clone *dab1_tv1* encodes for a protein containing the PTB domain and the serine residue phosphorylated in vitro by Cdk5 (Keshvara *et al.*, 2002) but lacks exons 8 and 9. These two exons encode for tyrosine residues Tyr^{200}, Tyr^{220} and Tyr^{232} that may be important in Reelin singalling. Indeed, mice expressing a mutated

Figure.3. Organization of the *dab1* gene

A) Comparison of human, mouse, chick and zebrafish *dab1* genes with exons (grey blocks) and introns (broken lines). Exons are numbered. The exons encoding for the PTB domain are underlined; the positions of the 5 tyrosines are shown (Y). Start and stop codons are indicated. Below, the genomic clones (zebrafish Bacs), used for reconstruction of the organization of the zebrafish *dab1* gene: Clone CH211-132C16 (violet), clone Dkey 242M13 (blue) and clone CH211-232I5 (green). Orientation and length are shown. B) Position of the primers on *dab1_tv2*. C) Analysis of the RT-PCR products amplified from the sages indicated. Control: no RT. On the right, predicted size of the amplicons. Size of the exons can be found in Table I.


A
Homo
DAB1
ATG
PTB
STOP
Mus
Dab1
Gallus
Dab1
Danio rerio
dab1
BX 085194
169865
BX 248232
AL 953861
165789
13428
165000


B
dab1_tv2


C
L mat 8S 30h 48h 3d 5d 1m control
500 bp
exons 5-10
~476 bp
~ 355 bp
exons 5-8
~353 bp
exons 5-9
~415 bp
~353 bp
~191 bp

4.2.4. Developmental profile of alternative splice forms expressed in zebrafish

To identify expressed *dab1* isoforms in zebrafish and determine their developmental profile were used RT-PCR and *in situ* hybridization analysis with oligos or probes designed to identify specific exons. Firstly it was found that *dab1* is expressed maternally and continues to be expressed before and after gastrulation (fig. 2 C and fig.4 A and B). Interestingly, also mouse *Dab1*-555 is expressed in pre-gastrulating embryos (expression profile of cDNA libraries at NCBI Unigene and Howell *et al.*, 1997a). Second, at least three alternative splice forms of *dab1* were expressed at different stages during zebrafish development (fig. 2 C).

Using primers for exons 5 and 10 (fig. 2 B) that amplify through the zone rich in tyrosines, a single band was amplified until 8 somite (s) stage, whereas from 24-30 hours post fertilization (hpf) a second larger band was also amplified with the same primer pair. Both bands persist until at least 1-month stage (fig. 2 C). Sequence analysis of the two PCR bands showed that the smaller band, expressed throughout development, corresponds to *dab1_tv1*. The larger band contains sequence of two additional exons (exon 8 and 9). Thus a second isoform, *dab1-tv2*, similar to mouse *Dab1-555*, was identified. To better investigate which isoforms were expressed and when, different combinations of reverse primers specific for exon 8 or exon 9 with

the forward primer specific for exon 5 were used. One, two or three bands were obtained when using reverse primers for exon 8, 9 or 10.

Finally, the presence of both exon 8 and 9 or solely of exon 9 sequences in the RT-PCR bands was confirmed by sequence analysis. Thus, a third variant, containing exon 9, but not exon 8, was also identified through RT-PCR.

4.2.5. Occurrence of alternatively spliced Dab1 isoforms in Vertebrates

In order to better characterize *dab1_tv1* and *dab1_tv2* isoforms, their sequences (containing exons 8 and 9) were aligned with the Zebrafish Genome (Zv4, Sanger Institute) using the BLAT tool at UCSC and it was found that both transcripts aligned with the genomic sequence starting from exon 4. Moreover, *dab_tv1* aligned with *dab1_tv2* in exon 5, 6 and 7, skips exon 8 and 9, and then aligned with the last 4 exons of *dab1_tv2*. The last two exons (14 and 15) were supported by an EST belonging to a cDNA library prepared from adult brain.

Furthermore, a reference (RefSeq XM_681536) predicted by NCBI using GNOMON, an automated computational gene prediction method was found throughout BLAST search it and this sequence aligned perfectly with *dab1_tv2* and *dab1_tv1* (fig.3B). The lack of genomic sequences supporting the first 4 exons suggested that the Zv4 assembly did not place them in the right contig. However, 2 of the 3 clones (BX085194 and BX248232) will

appear arranged as described in this Chapter in the next Vega release (http://vega.sanger.ac.uk/Danio_rerio/ personal communication, Dr. Mario Caccamo, Sanger Institute).

The alignment with non-zebrafish mRNAs revealed that mRNAs belonging to different species (Homo, Mus Musculus, Gallus) all coding for different transcripts of *disabled1* were mapped to this zebrafish genomic region.

An investigation into the conservation of the sequences of the zebrafish *dab1_tv2* exons 8 and 9 was carried out in different species, namely, mouse, human, chick and zebrafish to find ESTs that lack these two exons. Using BLAT at UCSC it was found that these two exons are very conserved in all species, with only a few ESTs lacking exon 8 (3 human, 1 mouse and 1 chick). Exon 9 was less conserved at the nucleotide level and aligned perfectly only with the human sequence. However ESTs lacking this exon were not found in any species.

4.2.6. Expression pattern of *dab1* during zebrafish development

RT-PCR assays at several stages showed the presence of *dab1* isoforms from maternal stage to 1-month. Subsequently, *in situ* hybridization assay

permitted to determine the spatial localization of *dab1* transcripts in tissues of the zebrafish embryo.

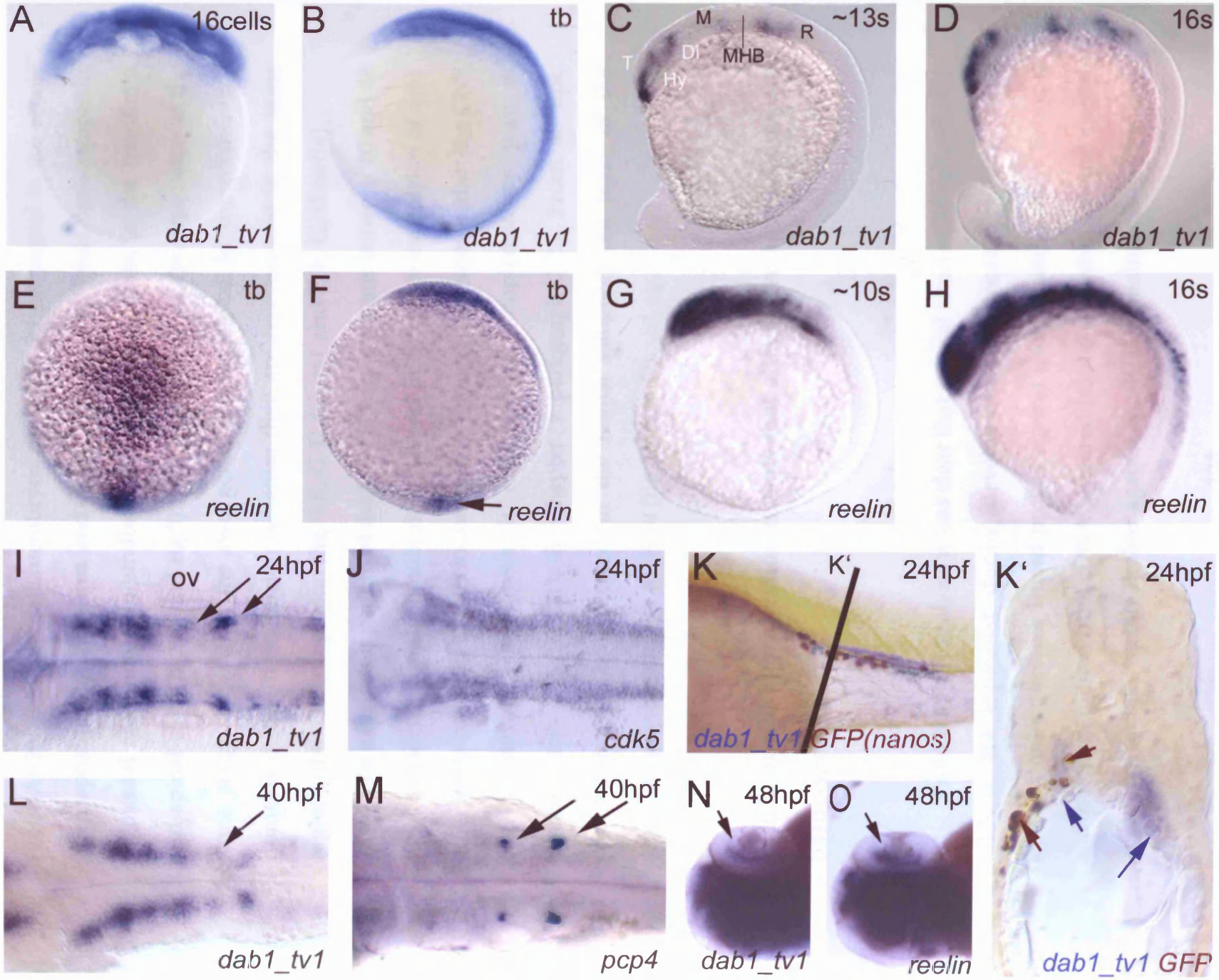
A digoxigenin probe for *dab1_tv1* showed ubiquitous expression from maternal stages to tail bud and then the signal became restricted to CNS regions as development progresses (fig.4 A-D). On the contrary, *reelin* is not expressed maternally but starts to be detected at tail bud stage in the head and the tail (fig.4 E-H). *reelin* and *dab1* signals increased at 24 hpf and reached a peak at 48 hpf, and continue to be expressed until adulthood as in all other vertebrates.

4.2.6.1. *reelin* and *dab1* are expressed in several areas of the CNS

At around 10-15 somites *dab1_tv1* is expressed in the forebrain, hindbrain and in the tail at the level of the spinal cord, while *reelin* is widely express in almost all CNS regions (fig.4 C, D and G, H).

Figure. 4. Expression patterns of dab_tv1 (A-D, I, K, K'and L), reelin (E-H and O) and cdk5 (J) in zebrafish embryos at several stages of development.

In A lateral view of an embryo of 16 cells stage; *dab1_tv1* is expressed in all the cells. B) At tailbud (tb) stage *dab1_tv1* is expressed all over the embryo (lateral view). At 13-16s *dab1_tv1* is localized in the head at level of the forebrain and hindbrain (lateral view). *reelin* starts to be expressed at tb stages (E dorsal view and F lateral view) in the presuntive head and in the tail (arrow). At 10s *reelin* expression in the tail disappears (G lateral view) and at 16s (F lateral view) is at level of all CNS. In K and K' (trasversal section of K) double *in situ* hybridization for *dab1_tv1* and immuno against construct fused with GFP that stains germs cells (Koprunner *et al.*, 2001)) at 24hpf stage. Germ cells (brown arrows) are surrounded and co localizes with *dab1_tv1* (blue arrows) .At 24 and 40hpf (I and L dorsal views) *dab1_tv1* is at level of the lateral columns of the hindbrain, where *cdk5* (J; 24hpf stages) and *pcp4* (M; 40hpf) are expressed as well. At 48hpf *dab1_tv1* (N) and *reelin* (O) are coexpressed in retina (arrows). Di: diencephalons; Hy: hypothalamus; M: mesencephalon; MHB: mid-hindbrain boundary; T: telencephalon.


A
16cells
dab1_tv1
B
tb
dab1_tv1
C
~13s
M
R
T
Di
MHB
Hy
dab1_tv1
D
16s
dab1_tv1
E
tb
reelin
F
tb
reelin
G
~10s
reelin
H
16s
reelin
I
ov
24hpf
dab1_tv1
J
24hpf
cdk5
K
K'
24hpf
dab1_tv1 GFP(nanos)
K'
24hpf
dab1_tv1 GFP
L
40hpf
dab1_tv1
M
40hpf
pcp4
N
48hpf
dab1_tv1
O
48hpf
reelin

At 20-30 hpf zebrafish embryos showed *dab1_tv1* signal in the whole telencephalon, preoptic area and hypothalamus, epiphysis, mesencephalic tegmental area, midbrain/hindbrain boundary and again in the hindbrain and spinal cord (fig.4D and fig.5). Comparison with *reelin* expression at the same stage shows that the two genes are expressed in complementary domains in the diencephalon, whereas they are co-expressed in the same regions of the telencephalon (fig. 4 B, C, D *dab_tv1* and E, F, G, H *reelin*).

The expression pattern of *dab1_tv1* is very dynamic between 20 and 48 hpf, as evidenced in the hindbrain (fig.4 and fig.5). Here expression of *dab1* at 24 hpf is confined to cells located along two lateral stripes within the CNS (fig.4 I and J) while *reelin* is expressed in complementary way in rhombomeres 2-7 (fig. 2D, E, Chapter 3 paragraph 2.2.). From 48 hpf, *dab1_tv1* mRNA is expressed in the retinal ganglion cell layer of the developing retina, similar to *reelin* (fig.4N and O).

4.2.6.2. *In situ* hybridization reveals that alternative splice forms are tissue-specific

From the PCR analysis it was clear that several alternative spliced *dab1* mRNAs are expressed during development. To localize where these isoforms are expressed, I used probes against exons 8 and 9 only. Exons 8 and 9 were amplified from cDNA prepared from 5-dpf zebrafish and cloned in an appropriate vector. Probes against the PTB domain and C-Terminus

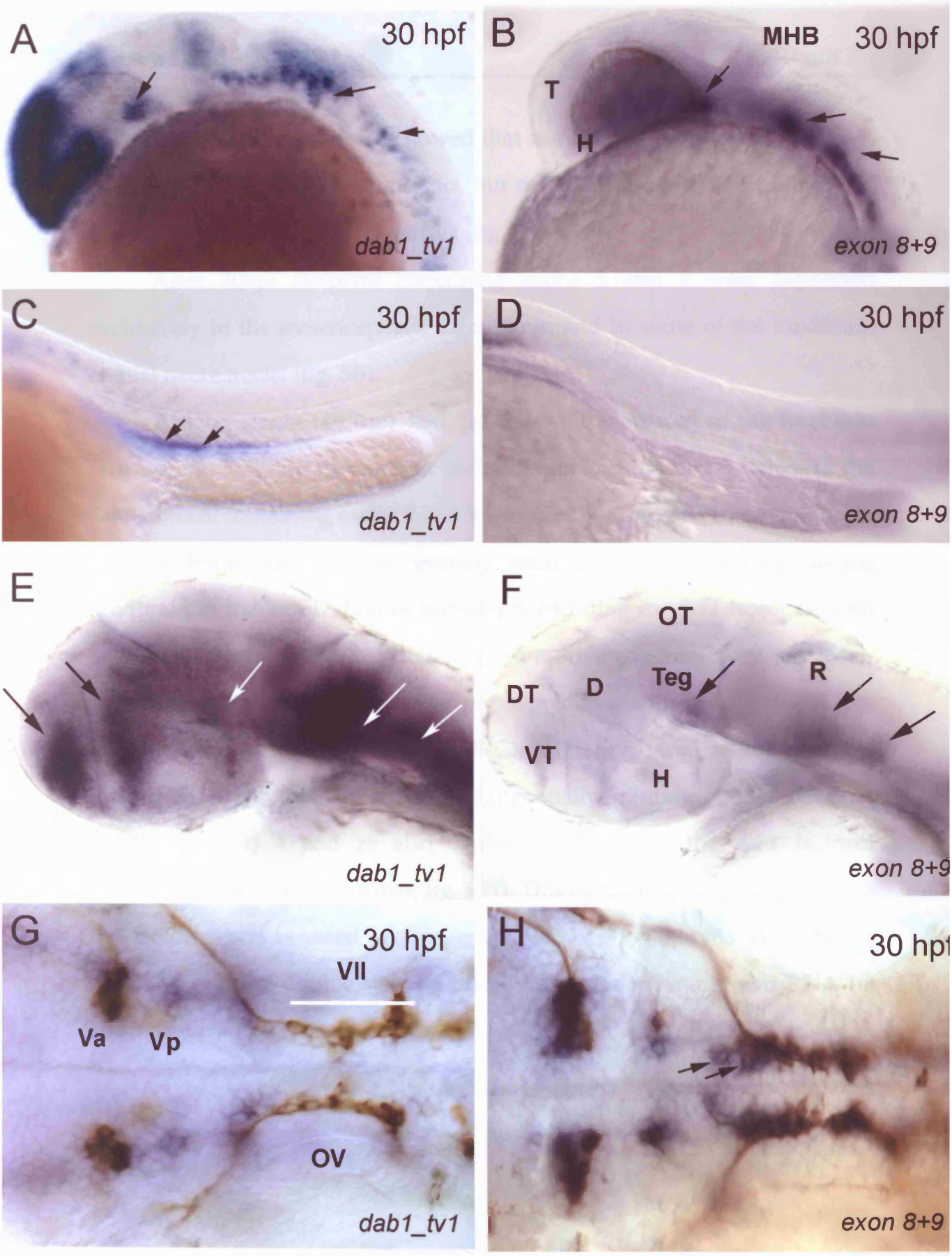
showed an expression pattern identical to the probe against zebrafish *dab1_tv1* described in the previous paragraphs (fig. 5A and C). By contrast, the probe specific for exons 8 and 9 revealed a pattern of expression that differed substantially from the others in spatial and temporal manner (fig. 5D).

First of all the probe recognizing common regions of all *dab1* isoforms revealed a widespread expression pattern at the level of the whole embryos until tailbud stage and later was restricted to the forebrain, midbrain and hindbrain (fig. 4A).

At 24 hpf *dab1_tv1* was expressed in the region of the pronephric ducts (arrows, fig. 4K, K' and fig. 5 C), whereas the *dab1* isoform containing exons 8 and 9 is never expressed in that area (fig. 5 D). It is interesting to note that the pronephric region marked by *dab1_tv1* gives rise to the reproductive organ, but also cells of the blood are generated or migrate here (Willett *et al.*, 1999; Serluca *&* Fishman, 2001). Immunohistochemistry for GFP (expressed under regulative region of the germ cell specific transcript nanos, GFP-*nos*1 3'UTR; Koprunner *et al.*, 2001), followed by *in situ*

Figure. 5. Differential expression of dab1 transcripts.

In situ hybridization analysis of the expression of *dab1_tv1* (A, C, E, G) and *dab1_tv2,* revealed with a cRNA probe for exon 8+9 (B, D, F, H) at the stages indicated (upper right corner). Arrows point to sites of prominent *dab1* expression (see text). G and H are embryos of the *tg(islet1-GFP)* transgenic line, stained for GFP (brown) and Dab1 transcriptional variants as indicated (blue). T: telencephalon; Hy: hypothalamus; MHB: mid-hindbrain boundary; DT: dorsal telencephalon; VT: ventral telencephalon; Di: diencephalons; OT: optic tectum; Teg: tegmentum; R: rhombencephalon; Va: anterior nucleus of trigeminal nerve; Vp: posterior nucleus of trigeminal nerve; VII: nucleus of facial nerve; OV: otic vescicles.


A
30 hpf
dab1_tv1
B
MHB
30 hpf
T
H
exon 8+9
C
30 hpf
dab1_tv1
D
30 hpf
exon 8+9
E
dab1_tv1
F
OT
Teg
R
DT
D
VT
H
exon 8+9
G
30 hpf
VII
Va
Vp
OV
dab1_tv1
H
30 hpf
exon 8+9

hybridization for dab1, showed that some of the germ cells co-express dab1 and GFP-*nos*1 3'UTR construct, but not of all the cells expressing *dab1* expressed GFP-*nos*1 3'UTR construct (fig.4 K, K').

After 30hpf isoforms containing exons 8 and 9 were expressed exclusively in the mesencephalic tegmentum and in some of the hindbrain cranial nerve nuclei (fig. 5B).

At later stages (48 hpf) *dab1_tv1* was still expressed in the forebrain (arrows, fig. 5 E) and throughout the midbrain and hindbrain, whereas the *dab1* isoform containing exons 8 and 9 was faintly expressed in the midbrain and hindbrain (fig. 5 F). Interestingly, there are some differences in the hindbrain in the localization of transcripts encoding for *dab1* isoforms with or without exons 8 and 9. Whereas presumptive pre-migratory and post-migratory facial motor neurons express predominantly *dab1_tv1* (*i.e.* the isoform lacking exons 8 and 9, fig. 5 G), migrating facial motor neurons, located in a paramedian string of tangentially migrating cells between rhombomere (r) 4 and r6 also express *dab1_tv2* (*i.e* the *dab1* isoform containing exon 8 and 9; arrows, fig. 5 H). This differential localization of the two isoforms was assessed in transgenic *tg(islet1-GFP)* embryos (Higashijima *et al.*, 2000), using double staining for GFP (immunostaining) and RNA (*in situ* hybridization).

4.3. Discussion

4.3.1. Comparison of the genomic organization of the *dab1* gene between species

The genomic organization of the *dab1* gene showed an unusual complexity at the level of the coding region, which is about 5.5 kb long and spans more then 1.1 Mbp of DNA in the mouse and human genomes. Similarly in zebrafish, the *dab1* gene spans more than 600 kb (based on the contig reconstructed from the 3 BAC clones) for a coding region of 1.8 kb (including exons 2-15).

The analysis of the sequences of the BAC clones showed that the main functional domains, the location of the exon/intron boundaries and the length of the exons were conserved between fish and human/mouse *dab1* genes. The most important differences between the zebrafish *dab1* gene and that of other species concern the length of the introns. In fact, introns in the *Danio rerio dab1* gene were shorter than in humans and mice (see table I and Bar *et al.* 2003). It was interesting to note that in *Drosophila* the *disabled* gene has even smaller introns and extends over 12 kb of genomic DNA (Gertler *et al.*, 1989), suggesting that the large size of *Dab1* in vertebrates depends on intron extension and is an evolutionary acquisition.

Interestingly, exon 14 did not contain the stop codon as in mouse and human *Dab1* (Bar *et al.*, 2003). The stop codon of zebrafish *dab1* could be found in exon 15. Moreover exons corresponding to mouse 217* and 271* were absent from the zebrafish genomic sequence. In addition sequence coding for exon 555*, which is present in all other vertebrates and is

duplicated in the human and the mouse, was never found. It might be that these exons have been acquired through mammalian specific events (retroviral or retrotransposome insertions) and subserved specific functions that became important during evolution.

4.3.2 Dab1 isoforms might have dominant negative effects and influence positive feedback control

From the analysis of *dab1* expression through *in situ* hybridization it appears that Dab1 isoforms in Vertebrates are sometimes expressed in the same tissue at the same stage of development or in different subpopulations of cells depending of the stage of development (Bar *et al.*, 2003; Katyal & Godbout, 2004).

Of particular interest is *Danio rerio dab1_tv2* identified through RT-PCR and containing exons 8 and 9. This *dab1* isoform appears to be expressed in a spatial and temporal specific manner in subdivisions of the CNS when certain neurons are migrating to reach their final destination.

The presence in *dab1_tv2* of exons 8 and 9, adds three more tyrosines (corresponding to Tyr^{200}, Tyr^{220} and Tyr^{232} in the mouse) in addition to those present in the shorter isoform *dab1_tv1*. *dab1_tv2* isoform is expressed from 24-30 hpf, at the time when facial motor neurons migrating from rhombomere (r) 4 are reaching r6-r7, *i.e.* their final destination (Chandrasekhar *et al.*, 1997).

Two alternative splice forms, chDab1-E and chDab1-L are found in the chick retina (Katyal & Godbout, 2004). ChDab1-E lacks two tyrosines (corresponding to Tyr^{198} and Tyr^{220}), which are usually phosporylated upon activation of the Reelin pathway. This isoform is suggested to be able to block the transduction of the Reelin signal when expressed in some neuronal populations. ChDab1-L, which contains all the five tyrosine, provides a substrate for full phosphorylation upon Reelin signaling. Those neurons expressing chDab1-L are able to grow neurites in response to the Reelin signal (Katyal & Godbout, 2004).

All Dab1 isoforms reported so far have the same N-terminus, which contains the PTB/PI domain. These isoforms could be competing for the same receptors (*i.e.* NPxY motives in the cytoplasmic tail of lipoprotein receptors) but only the isoform with a full complement of tyrosines could transduce the Reelin signal, whereas the other isoforms could work as dominant negative because lacking most of the tyrosine phosphorylation domain and block the receptor interacting domain without signaling. The binding of these presumptive dominant negative isoforms could result not only in the inhibition of the Reelin signaling but also in accumulation of Dab1, because unphosphorylated Dab1 isoforms do not became ubiquitinated and therefore are not degraded via the proteasome pathway (Arnaud *et al.*, 2003a; Morimura *et al.*, 2005).

The unphosphorylated isoforms could accumulate in the cytosol and participate in other pathways. Indeed, it is possible that combinations of different phosphorylation sites can result in the binding and activation of

different targets. In the mouse, alternative splicing also generates isoforms with reduced number of tyrosines. The isoform Dab1-217* has the same complement of tyrosines as Dab1_tv1 but its C-terminus differs substantially due to premature truncation (Howell *et al.*, 1997a). Nevertheless this and other truncated forms (*i.e.* Dab1-271*) are likely to be able to compete with Dab1-555 for the binding to the Reelin receptors without relaying further the Reelin signals, and thus functioning as modulators. The same function could be performed by the isoform Dab1_tv1, since in zebrafish exons corresponding to 217* and 271* have not been found.

In other words the complexity of the *dab1* gene may be an evolutionarily conserved strategy to achieve functional regulation of Dab1 phosphorylation through alternative splicing. Dab1 may play other roles in addition to the regulation of neuronal migration.

4.3.3. Alternatively spliced Dab1 isoforms are expressed inside and outside the CNS

In this preliminary study of *dab1* expression it was shown that the isoform *dab1_tv1* has a maternal expression, whereas at least three isoforms are expressed after 24hpf mainly in the CNS and during adulthood. *reelin,* whose expression pattern from 24hpf is well described in Chapter 3, showed a very early expression starting at tail bud stage. Interestingly, it was not possible to detect any *reelin* expression at earlier stages.

In general, the expression of both *reelin* and *dab1* in zebrafish are comparable to that seen in the other vertebrates (diencephalon, midbrain hindbrain and spinal cord) and their function may be compatible to ones normally attributed to the Reelin/Dab1 pathway in vertebrate CNS. An exception is the expression in telencephalic structures at early stages of development. From somite stages both *reelin* and *dab1* were co-expressed in the region of the neural tube that gives rise to the dorsal telencephalon, while in mammals only Cajal-Retzius cells express Reelin (Alcantara *et al.*, 1998).

In addition, maternal *reelin* expression was not found suggesting a possible role of *dab1* in at these stages not linked to the Reelin pathway at least in zebrafish. The isoform *dab1_tv1* is also expressed in the hematopoietic region, *i.e.* pronephric ducts and later in blood vessels. In mouse isoforms Dab1-555*, 217* and 271*, containing the tyrosine residues and the PTB domain, are expressed in kidneys, liver and in general in non-neural tissue (Howell *et al.*, 1997a; Bar *et al.*, 2003). These data suggest that in mouse Dab1 isoforms with a reduced complement of phosphorylable tyrosines exert a role different from the regulation of neuronal migration.

Mouse mutants for Dab1 have a phenotype largely restricted to neuronal migration, however interpretation of the mouse mutant phenotype is complicated due to the fact that both the *yotari* and *scrambler* mutants, (Howell *et al.*, 1997b, Sheldon *et al.*, 1997) produce truncated proteins, leaving open the possibility that these truncated forms of Dab1 maintain some functions. Indeed knock in of P45, a mDab1 truncated just at the 3' of

the tyrosine phosphorylation domain, is an hypomorph for some Dab1 functions in hippocampal development (Herrick & Cooper, 2002).

On the other hand, the occurrence of such a rich constellation of alternatively spliced isoforms of Dab1, for which no gene duplication seems to have occurred in fish (present work and Zebrafish Genome Project at www.sanger.ac.uk) fits well with the hypothesis, recently reported by Kopelman *et al.* (2005), that "alternative splicing and gene duplication are inversely correlated evolutionary mechanisms".

In the next chapter I will introduce a preliminary study of the function of Dab1 in some of the regions inside and outside the CNS, where I found it was expressed.

CHAPTER 5

5. Results 3. Dab1 isoforms are involved in neuronal and non-neuronal development

5.1. Introduction

dab1 is a very complex gene and is subject to several alternative-splicing events (Chapter 2 and Bar *et al.,* 2003). Alternatively spliced isoforms are expressed in a temporal and tissue specific manner (Howell *et al.,* 1997; Bar *et al.,* 2003; Katyal & Godbout, 2004). Isoforms of Dab1 are not only expressed in the CNS, but are also found in blood cells, kidney, uterus and in the developing heart (Ikeda & Terashima, 1997; Howell *et al.,* 1997, Smalheiser *et al.,* 2000; Kam *et al.,* 2004; Takahashi *et al.,* 2004). These findings suggest potential roles for Dab1 in both neuronal and non-neuronal tissues.

To investigate the functions of *dab1* in zebrafish, I used morpholino (mo) injections together with over expression of *dab1* mRNAs as approaches for in vivo studies of gene functions.

Although my project focused on the involvement of the Reelin/Dab1 pathway in neuronal migration, I observed morphological changes

subsequent to mo injection also in non-neuronal structures. Here I will present the results concerning the effects of mo injections in the VII nuclus and nerve and a preliminary analysis of the defects induced by mo injections in the reticularspinal neurons, retina and in the jaw development.

5.1.1. Pleiotropic phenotypes in *dab1* morpholino (mo) injected embryos.

Based on *dab1* full-length sequence (see Chapter 2) it was possible to design three different morpholinos: one against the ATG start codon (mo1; fig 1A), a second further upstream and not targeting the ATG (mo2; fig1A) and finally a third mo against a splice donor site (spmo; fig.1A and table I). The first two morpholinos have as target the maternal and zygotic mRNAs (mo2 was designed to compare its effects with those of mo1; Ekker, 2000), while the third morpholino hybridises to a splice donor site in exon 6 of *dab1* precursor mRNA (fig.1B; Draper *et al.*, 2001).

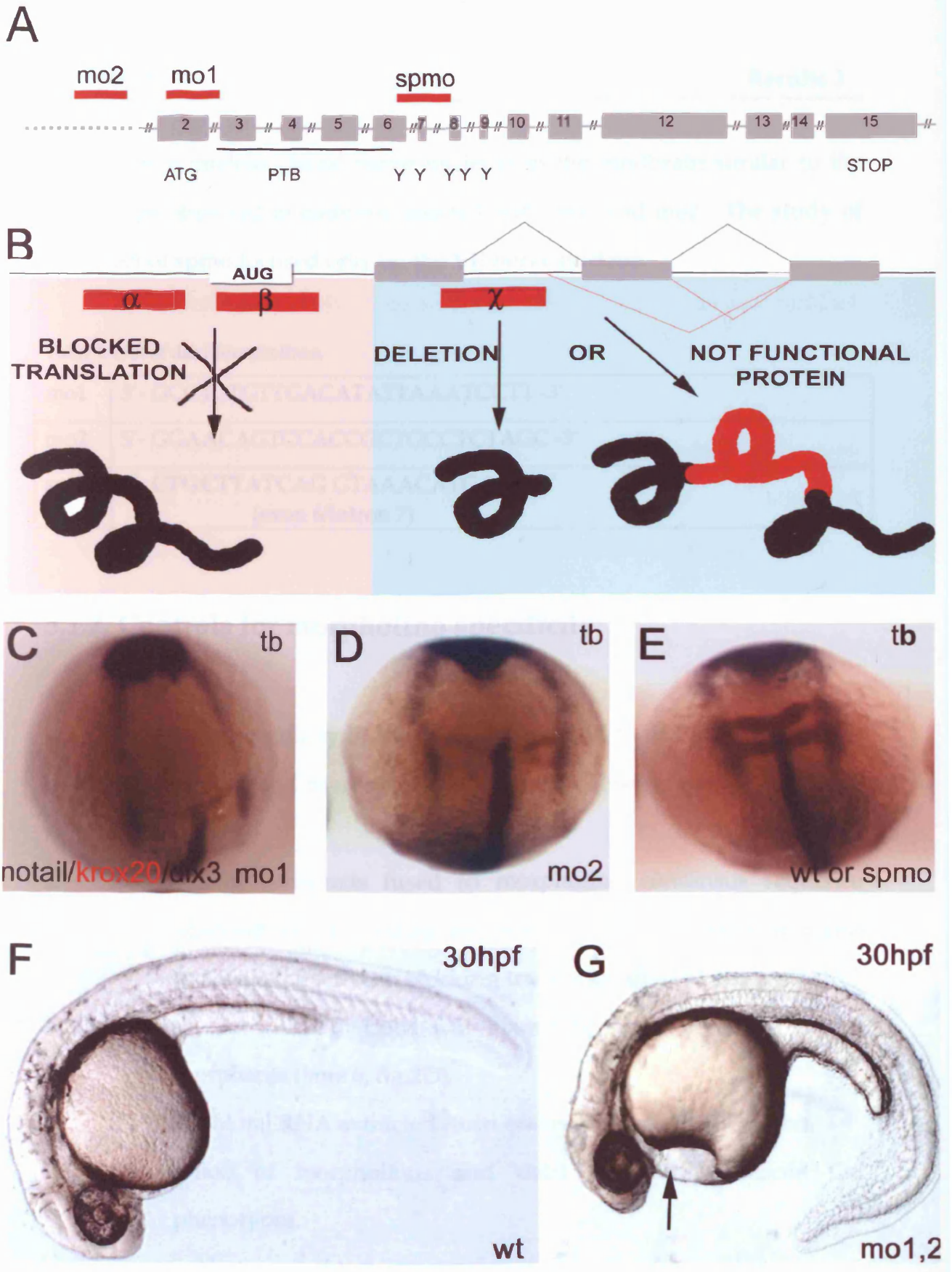
At high doses mo1 and 2 caused the same neuronal phenotype (see below), but they also affected other processes, as the embryos appeared shorter and deformed. Looking at tailbud stage injected embryos, they were found defective in gastrulation. Using a cocktail of probes, it was possible to observe that the notochord was shorter and the neural plate was wider in embryos injected with mo1 and mo2 but not with spmo (fig.1C, D, E). These defects could be due to the knock down of the *dab1* isoforms expressed at early stages including maternal transcripts.

At 24-30 hpf, when dab1 expression starts to increase in the CNS, the injected embryos displayed developmental delays, a curved body, small head and eyes, reduced blood circulation, pericardial oedema and reduced motility (fig 1.G). The injection of mo1 or mo2 at one cell stage gave rise to similar phenotypes but at different concentrations. For example 2 ng of mo2 was sufficient to produce a phenotype similar to that observed following injections of 6 ng of mo1. This suggests that mo2 was more potent then mo1. This may be due to the three-dimensional structures of mo2, resulting in a more stable hybridisation to the target sequence compared to mo1. Alternatively, mo2 may simply be more soluble, diffuse more easily and so be more efficient (see www.gene-tools.com).

The spmo was designed to be effective only on zygotic, prior to-splice, mRNA and to lack a possible effect on maternal mRNA (Draper *et al.*, 2001). The morphants injected with 1-3 ng of spmo were morphologically normal but a more detailed analysis revealed an interesting phenotype at the level

Figure.1. Morhpolinos used to knock down dab1

A) Schematic drawing of dab1 gene structure and morpholinos (mo1 and mo2). Mo2 is targeting a sequence of dab1 that does not contain the ATG. The spmo is against the splice site between exon 6 and intron 7. B) Schema of morpholino antisense activity. In α and β the mos target 5'UTR and ATG and the translation of the mRNA is blocked. In χ the result is an aberrant splicing that give rise to a truncated or not functional protein. Mo1 and mo2 injections (C and D) give rise to defective gastrulation, where the size of the neural plate is abnormal (*dlx3* and *krox-20*) and the notocord is shorter (*notail*). E) Spmo morphants does not show phenotypes at tailbud stage. G) At 24hpf mo1 and mo2 morphants are retarded with a curved body, small head and eyes, reduced blood circulation, pericardial oedema (arrow) and reduced motility, when compared to the wild type (F).


A
mo2
mo1
spmo
2
3
4
5
6
7
8
9
10
11
12
13
14
15
ATG
PTB
Y Y Y Y Y
STOP
B
AUG
α
β
χ
BLOCKED TRANSLATION
DELETION
OR
NOT FUNCTIONAL PROTEIN
C
tb
notail/krox20/dlx3 mo1
D
tb
mo2
E
tb
wt or spmo
F
30hpf
wt
G
30hpf
mo1,2

of VII nerve nucleus (facial nerve nucleus) in the hindbrain similar to the phenotype observed in embryos injected with mo1 and mo2. The study of the effect of spmo focused only on the VII nerve nucleus.

Table I. List of dab1 Morpholinos.

mo1	5′- GCCTCTGTTGACATATTAAATCCTT -3′
mo2	5′- GGAACAGTCCACCGCTGCCTCTAGC -3′
spmo	5′- **CTGCTTATCAG** GTAAACATCACC -3′ (**exon 6**/intron 7)

5.1.2. Controls for morpholino specificity

To test the specificity of the morpholinos and their capacity to block translation/splicing and thereby create a functional knockout, 4 strategies were used.

1. Injections of constructs fused to morpholino consensus sequence upstream of GFP coding sequence (fig.2A-C) to see if mo1 and mo2 were effective in blocking translation of dab1 sequences.
2. Western blot to see if Dab1 was absent or down regulated in the morphants (lane 6, fig.2D).
3. RT-PCR of total RNA extracted from embryos injected with spmo.
4. Co-injection of morpholinos and dab1 mRNAs to rescue the phenotypes.

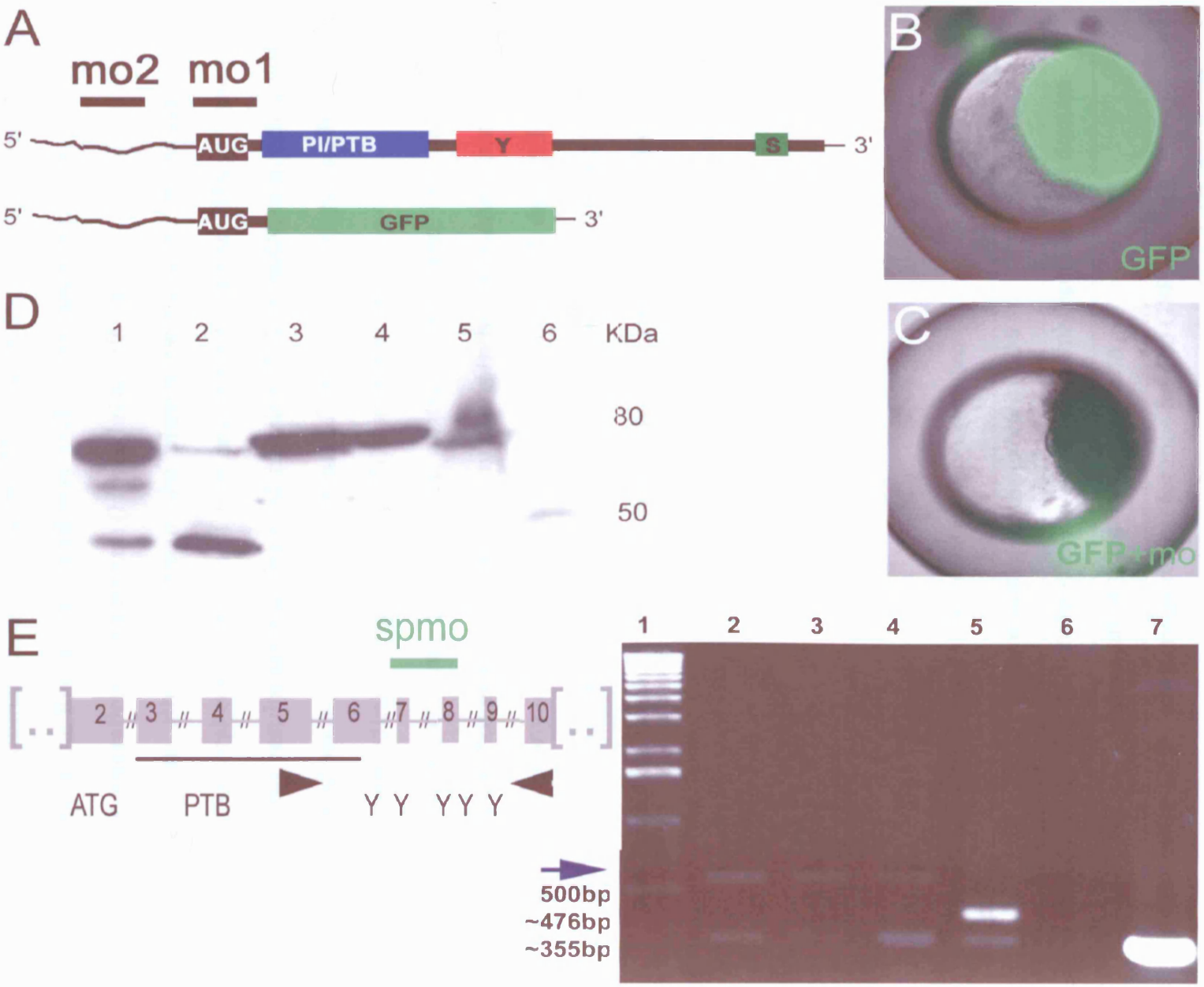
1) Both morpholinos were able to block the expression of GFP from fusion constructs carrying the morpholino consensus sequences upstream of GFP coding sequence (fig.2 A, B and C).

2) Western blot analysis showed that morpholino 1 was able to block the translation of *dab1* (lane 6 fig. 2 D).

3) The splice blocking morpholino (fig. 1 A, B and fig.2 E) was designed to be effective on the precursor mRNA of zygotic dab1 and not on the maternal transcripts already spliced. It was possible to test the ability of morpholino to block the splicing of *dab1* mRNA by performing RT-PCR on total RNA extracted from splice morpholino-injected embryos. The PCR products retain intron 7 that was not spliced out (fig 2 E). Analysis of the sequence of RT-PCR products suggests that the retention of intron 7 causes a premature termination of the protein due to a stop codon present in frame in the intron sequence (Marina Mione personal communication). It is

Figure.2. Efficacy of dab1 morpholinos.

A) Schematic drawing of *dab1* mRNA and location of the sequences targeted by morpholinos (mo1 and mo2). Mo2 is against a sequence of *dab1* that does not contain the ATG. The lower schematic shows the mo consensus sequence fused to the GFP coding sequence. B) Injection of the construct coding for GFP downstream of the mo consensus site (refer to fig.1A) results in GFP expression which is blocked by co-injection of morpholino (mo1, C). D) Western blot analysis of dab1 expression using antibodies directed against the C-terminus. Lane 1) Zebrafish injected with *dab1_tv1* mRNA. Lane 2) Zebrafish injected with *dab1_tv1* mRNA and morpholino 1. Lanes 3-5) Dab1 bands in mouse brain extracts (embryonic, adult, *scrambler* +/- respectively) which have similar electrophoretic mobility to zebrafish Dab1. Lane 6) Disappearance of the 80 KDa band in embryos injected with morpholino 1. E) Schematic representation of the *dab1* gene. The black arrows show the region amplified using primer for exon 5 and exon 10 and containing the site of splicing blocked by spmo. In G, lane1) 1kb ladder; lanes 2, 3, 4) cDNA from 3 pools of embryos injected with spmo; lane 5) not injected embryos; lane 6) negative control; 7) *dab1_tv1* positive control. The lane 2, 3 and 4 show a band higher then the controls (lanes 5). The sequence analysis of the higher band reveals that it contains sequences belonging to intron 7, which was not spliced out.


A
mo2
mo1
5'
AUG
PI/PTB
Y
S
3'
5'
AUG
GFP
3'
B
GFP
C
GFP+mo
D
1
2
3
4
5
6
KDa
80
50
E
spmo
2
3
4
5
6
7
8
9
10
ATG
PTB
Y Y
Y Y
Y
1
2
3
4
5
6
7
500bp
~476bp
~355bp

hypothesized that the new molecule produced lacks most of C-terminus including the domain rich in tyrosines. This region is phosphorylated during activation of the Reelin/Dab1 pathway and it was described to be involved in neuron migration in the mouse (Howell *et al.*, 1997a).

4) In an attempt to rescue the phenotype induced by morpholino injection, several combinations of mos and mRNAs (about 200pg) were co-injected and results are summarized in table II. The mRNAs injected with mo1 or mo2 were (i) *dab1_tv1*, (ii) mouse *Dab555*, (iii) *dab1_tv1* without the region surrounding serine (S^{464}), (iiii) PTB only mRNA.

The Dab1_tv1 can rescue most of the phenotypes caused by the morpholinos. For example, Mauthner neurons (reticular spinal neurons) are normal in many embryos injected with *dab1* mo1 and *dab1_tv1* mRNA (fig8C.). It should be noted that the rescue could be due to the fact that mo1 may hybridize to *dab1_tv1* mRNA and so the two injected molecules could

Table II. List mRNA injected in the control assay for morpholino

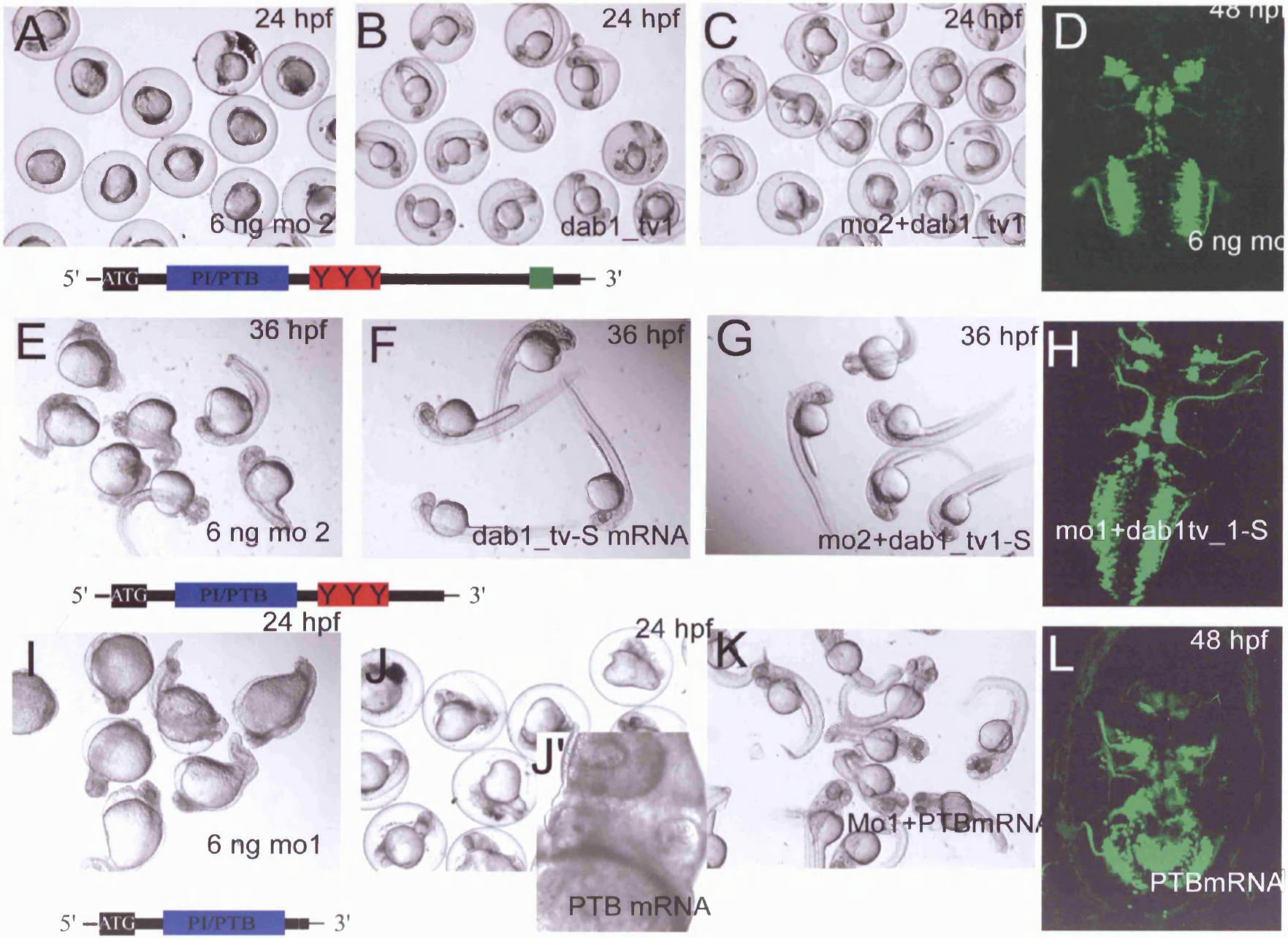
dab1_tv1	isoform lacking of the exons 8 and 9
dab1_tv1-S	isoform lacking the exons 8,9 and phosphorabe Ser domain
dab1 PTB	domain of receptors interaction
consensus+GFP	consensus region for mos fuse to GFP see fig.2

neutralize each other. However, mo2 is against the 5'UTR and cannot block the translation of injected *dab1_tv1* mRNA, which does not contain upstream sequences target of mo2. The injection of *dab1_tv1* mRNA enhanced the survival of the embryos injected with mo2, but did not provide a complete rescue and for example the migration of VIIn was only partially rescued (fig.6 B).

Interestingly, mRNA from mouse *Dab1* (m*Dab555*), containing all the 5 phosphorylable tyrosines, can rescue phenotypes for both morpholinos (Marina personal comunication). The third rescue construct has a C-terminus truncation that removes a region containing serine (S^{464}) and which is known as "Cdk5 phosphorylation domain" (Keshvara *et al.*, 2002).

Figure. 3. Rescues of morpholino injections.

In A injection of a very high dose of mo2, the embryos do not gastrulate and die at early stages. B) Injection of *dab1_tv1* mRNA, the embryos are morphologically normal. C) Co-injection of 6ng of mo2 and *dab1_tv1* mRNA, the embryos can gastrulate normally. The pictures D, H and L are related to paragraph 5.1.3. D) Injection of 6ng mo2 in an embryo from *islet-1 GFP line*. The VIIn does not migrate. E) Injection of 2ng mo2, the phenotype is less severe then in A. F) Injection of *dab1_tv1-S* mRNA (terminally truncated). The embryos are normal. G) Co-injection mo2 and *dab1_tv1-S*. The embryos look normal. H) Co-injection of mo1 + *dab1_tv1-S* in an embryo from islet1-GFP line see paragraph 5.1.3. The migration of VIIn is rescued by *dab1_tv1-S*. I) Injection of 6 ng of mo1. The embryos show several defects but the phenotype is less severe then in A. J) Injection of PTB mRNA. The embryo shows several defect and cyclopia in 30% of the case (higher magnification of a cyclopic embryo in J'. K) The co-injection of mo1 with PTB mRNA does not rescue morpholino phenotype. L) Injection of PTB mRNA in *islet1* GFP line. The embryos present cyclopia but the VIIn can still migrate see paragraph 5.1.3.


A
24 hpf
6 ng mo 2
B
24 hpf
dab1_tv1
C
24 hpf
mo2+dab1_tv1
D
6 ng mo
5' ATG PI/PTB Y Y Y 3'
E
36 hpf
6 ng mo 2
F
36 hpf
dab1_tv-S mRNA
G
36 hpf
mo2+dab1_tv1-S
H
mo1+dab1tv_1-S
5' ATG PI/PTB Y Y Y 3'
24 hpf
I
6 ng mo1
24 hpf
J
J'
PTB mRNA
K
Mo1+PTBmRNA
L
48 hpf
PTBmRNA
5' ATG PI/PTB 3'

The construct without S^{464} is able to ameliorate the migration and nerve growth (and all other defects) of the VII nerve induced by *dab1* mo2, exactly as *dab1_tv1* does, suggesting that the S^{464} domain is not required for the functions of Dab1 in nerve growth (fig. 10).

A construct encoding for *dab1* PTB alone lacking the tyrosine domain, which is necessary to convey the *reelin* signal caused a phenotype due to overexpression that precludes the analysis of any possible rescue effects (fig. 10).

In conclusion, the most efficient rescue for both morpholinos was through using the m*Dab1-555* construct, carrying all tyrosines, while mo1 was rescued by *dab1_tv1* that contains the consensus region for mo1 binding.

Table III. Co-injections of morpholinos and dab1 mRNAs

Co-injection	*dab1_tv1*	*Dab1-555*	*dab1_tv1-S*^{464}	*dab1 PTB*
mo 1	Rescue of all phenotypes	Rescue of all phenotypes	Rescue of all phenotypes	No rescue
mo 2	Partial rescue (nVII axon growth, Mauthner n)	Rescue of all phenotypes	Partial rescue (nVII axon growth, Mauthner n)	No rescue

5.1.3. The VII nucleus and nerve show defects due to injection of morpholinos

5.1.3.1. The cranial neurons of the hindbrain are mis-positioned in embryos injected with morpholinos 1 or 2 or splice morpholino

In *yotari* and *scrambler* mice, mutant for *dab1,* certain hindbrain motoneuron nuclei do not migrate properly (Martin, 1981; Goffinet, 1984; Sheldon et al, 1997; Howell *et al,* 1997; Fujimoto *et al.,* 1998; Ohshima *et al.,* 2002; Rossel *et al.,* 2005) indicating that Dab1 is involved in neuronal migration in the hindbrain.

To study the migration of branchiomotor neurons in zebrafish hindbrain, embryos of the *tg(islet1- GFP)* line were injected at one cell with morpholinos to induce a loss of function of Dab1. The migration of facial motor neurons from rhombomeres (r) 4 to 7 is a stereotyped tangential migration that has been described in all vertebrates studied (with the exception of the chick, Studer, 2001) and recorded in *tg(islet1-GFP)* zebrafish transgenic embryos (Higashijima *et al.,* 2000.)

Neurons of the facial nerve nucleus begin their rostro-caudal migration from r4 just adjacent to the midline at around 18 hpf (Chandrasekhar *et al.,* 1997). Three hours later, the first neurons reach r6 and start to move laterally (fig.3 A′-A‴ and movie 1). This process continues through the following hours and by 40 hpf each fluorescent nucleus is composed of 16-20 cells located paramedially in r5-r7. By 72 hours there are

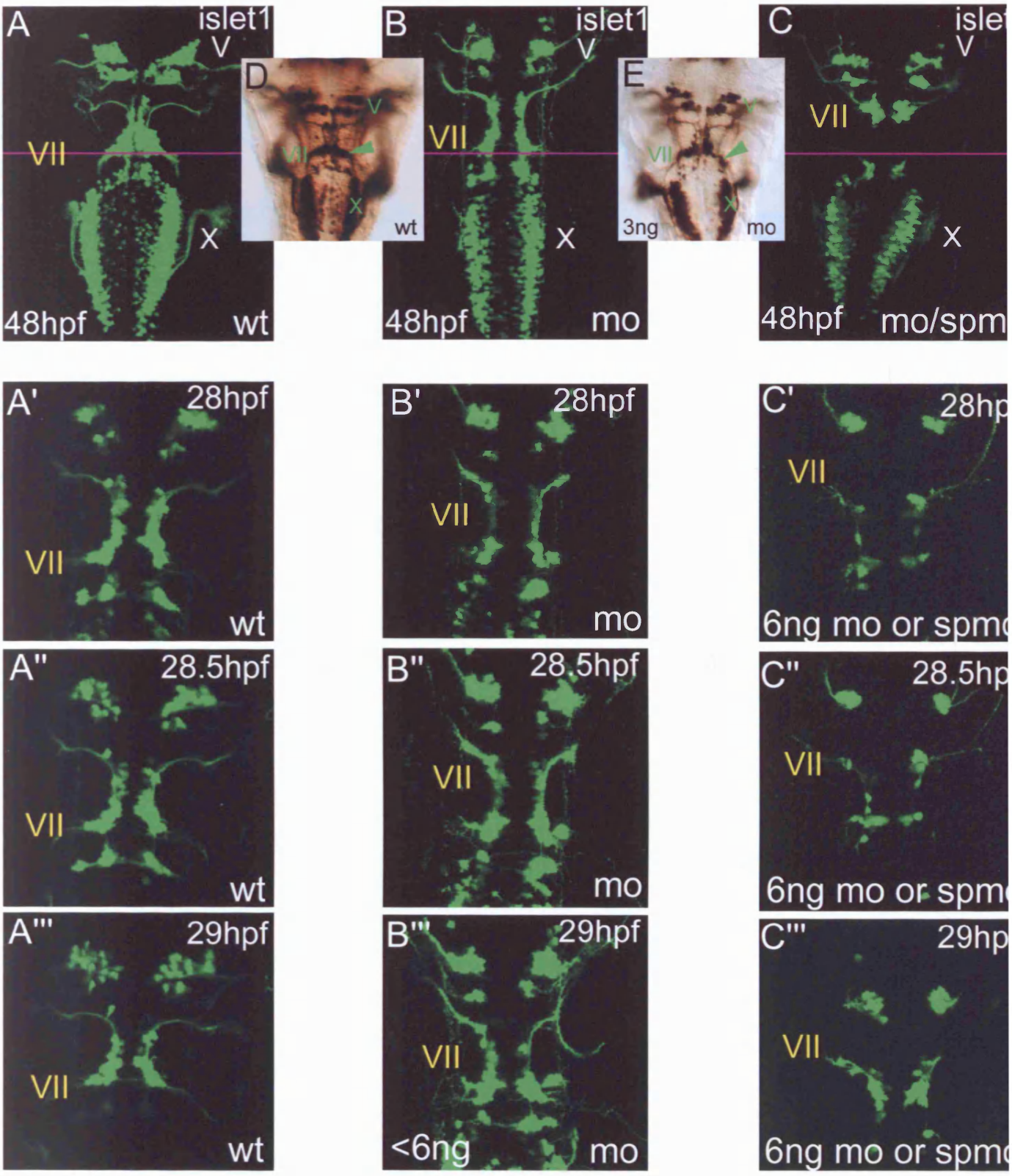
28-32 fluorescent cells in each nucleus and at 5 days there are 30-33 fluorescent cells per nucleus, located in r6-r7 and extending laterally to form a triangular shaped structure. In the *tg(islet1-GFP)* line it was possible to visualize *in vivo* the severe defect of the nucleus in the morphants (movie 2 and 3).

The analysis of 48hpf *tg(islet1-GFP)* morphants revealed abnormal positioning of neurons of the facial nucleus (VII). In less severely affected mo1 and mo2 morphants the facial motor neurons successfully completed their tangential rostro-caudal migration but the shape of the nucleus was laterally truncated (fig. 3.B and E), suggesting that the neurons did not move laterally correctly even if they did reach their r6/7 destination. In the more severe phenotypes the VII nerve nucleus does exist, but it does not migrate to the proper location in rhombomere 6-7 (fig.4C).

In mo1-injected embryos, the number of neurons that forms the facial nucleus at 5 dpf was reduced: 20-22 cells for embryos injected with 6 ng of mo1. However, no reduction of cell number was present in embryos injected

Figure.4. Dorsal view of cranial nerve nuclei (V, VII, X) in the hindbrain of embryos imaged using the confocal (A-A''', B-B''', C-C''') and compound (D, E) microscopes.

In A a control embryo of the tg(GFP-islet-1) where the trigeminal (V), the facial (VII) and the glossofaringeus (X) nuclei are fluorescent. In B, embryo injected with a low dose of mo1 or mo2 (<6ng mo1 or <2ng mo2). The VIIn migration is only partially migrated. In C embryo injected with a high dose of mo1 and mo2 (>6ng mo1 and >2ng mo2). The VIIn did not migrate at all. In D, immunohistochemistry for islet1 on a control embryo from GFP-islet1 line to show the anatomy of the hindbrain at 48hpf stage. In E embryo injected with 3 ng mo1. The left panel shows a normal VII nucleus, while the right panel shows a VIIn truncated laterally as result of a low dose of morpholino injection. A'-A'''stacked pictures at three different time points of movie 1: control embryos of the *tg(islet-1 GFP)* line. B'-B''' stacked pictures from movie 2: embryos from *tg(islet-1 GFP)* injected with <6 ng of mo1. C'-C''' stacked pictures from movie 3: embryos from *tg(islet-1 GFP)* injected with 6 ng of mo1 (or spm).

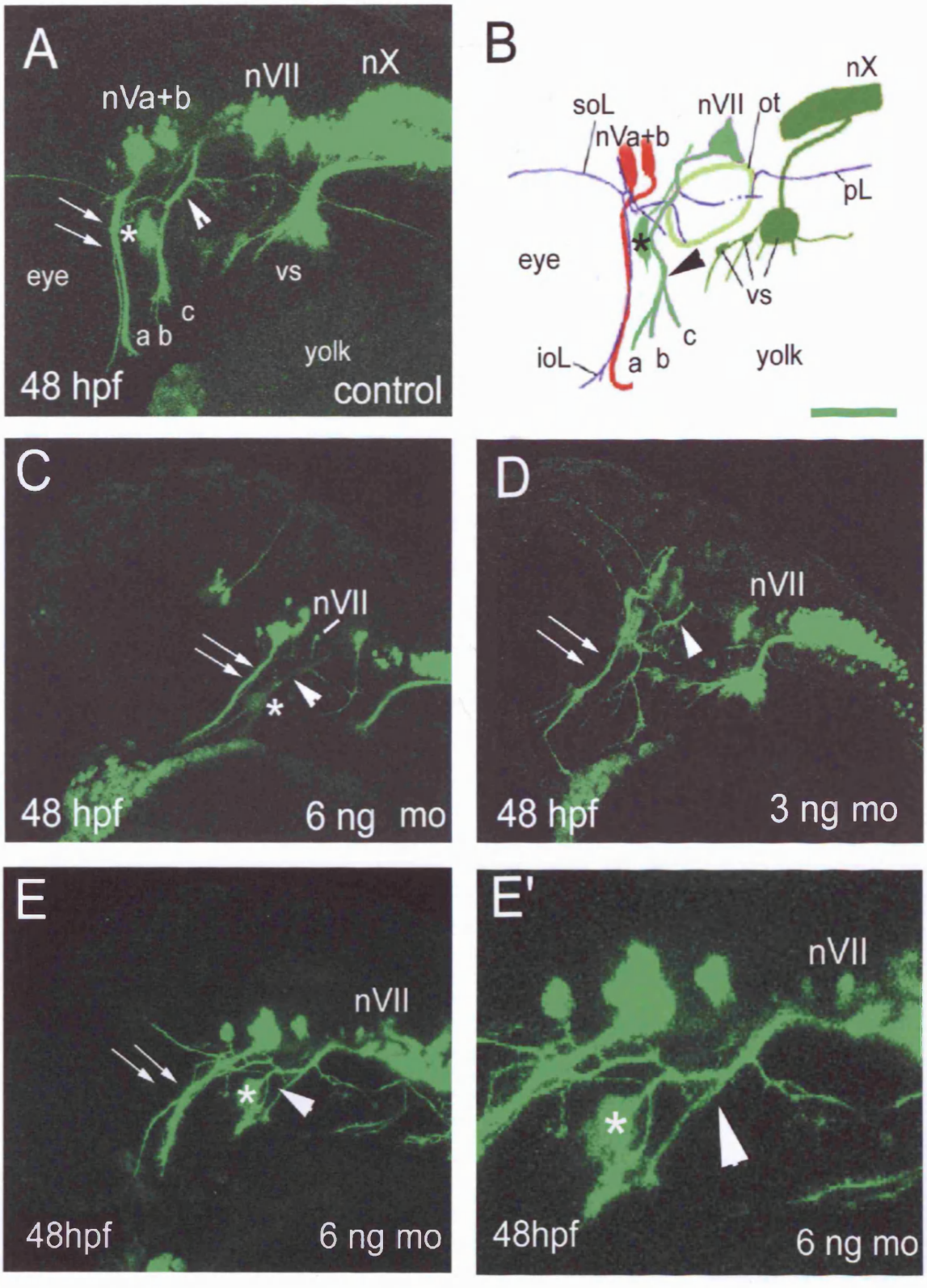

A
islet1
V
VII
X
48hpf
wt
D
V
VII
X
wt
B
islet1
V
VII
X
48hpf
mo
E
V
VII
X
3ng
mo
C
isle
V
VII
X
48hpf
mo/spm
A'
28hpf
VII
wt
B'
28hpf
VII
mo
C'
28hp
VII
6ng mo or spm
A''
28.5hpf
VII
wt
B''
28.5hpf
VII
mo
C''
28.5hp
VII
6ng mo or spm
A'''
29hpf
VII
wt
B'''
29hpf
VII
<6ng
mo
C'''
29hp
VII
6ng mo or spm

with about 3 ng of mo1 (fig. 2B). At high dose of morpholino the facial motor neurons did not migrate from r4-r5 (fig. 4C), while in embryos injected with lower doses of mo1 or mo2 the facial motor neurons were located throughout r4 to r6-7 and clustered near the midline instead of extending laterally (fig. 4B, E). This suggests that the delay in migration caused by morpholino injection eventually results in misplaced facial motor neurons along their migration path and that the severity of the phenotype depends on the dose of the morpholino 1 or 2 injected. On the contrary, spmo injection gave rise to a cleaner phenotype, where the VIIn did not migrate at all and a reduction of neuron number was never observed (fig. 4C).

These observations showed that, in zebrafish, the loss of function of *dab1* caused by the three morpholinos gave rise to a defective development and migration of the facial (VII) nerve nucleus in the hindbrain.

Figure.5. Lateral view of 48 hpf *tg(islet1-GFP)* embryos imaged using the confocal microscope.

A) Lateral view of a control embryos of the *tg(islet1-GFP)*. The two arrows indicate the trigeminal and infraorbital lateral (ioL) nerves and the asterisk indicates the facial sensory ganglion. B) Scheme of the principal nerves labelled by GFP in *tg(islet1-GFP)*. Trigemino nuclei (nVa+b), facial nucleo (nVII), peripheral projection of facial sensory gaglion cells otic capsule (ot), vagus (X), vagus sensory ganglion lateral line (vs), supraorbital lateral line (soL), postirior lateral laine (pL). Modified from Higashijima et al., 2000. C-E') Lateral view of embryos injected with morpholino 1. The facial nerves (arrow) in C, E and E' are thinner then the control (in green in the scheme in B). In D the facial is defasciculated at level of the facial sensory gaglion. Scale bar, 100 μm.


A
nVa+b
nVII
nX
eye
vs
a b
c
yolk
48 hpf
control
B
nX
soL
nVII
ot
nVa+b
pL
eye
vs
c
ioL
a b
yolk
C
nVII
48 hpf
6 ng mo
D
nVII
48 hpf
3 ng mo
E
nVII
48hpf
6 ng mo
E'
nVII
48hpf
6 ng mo

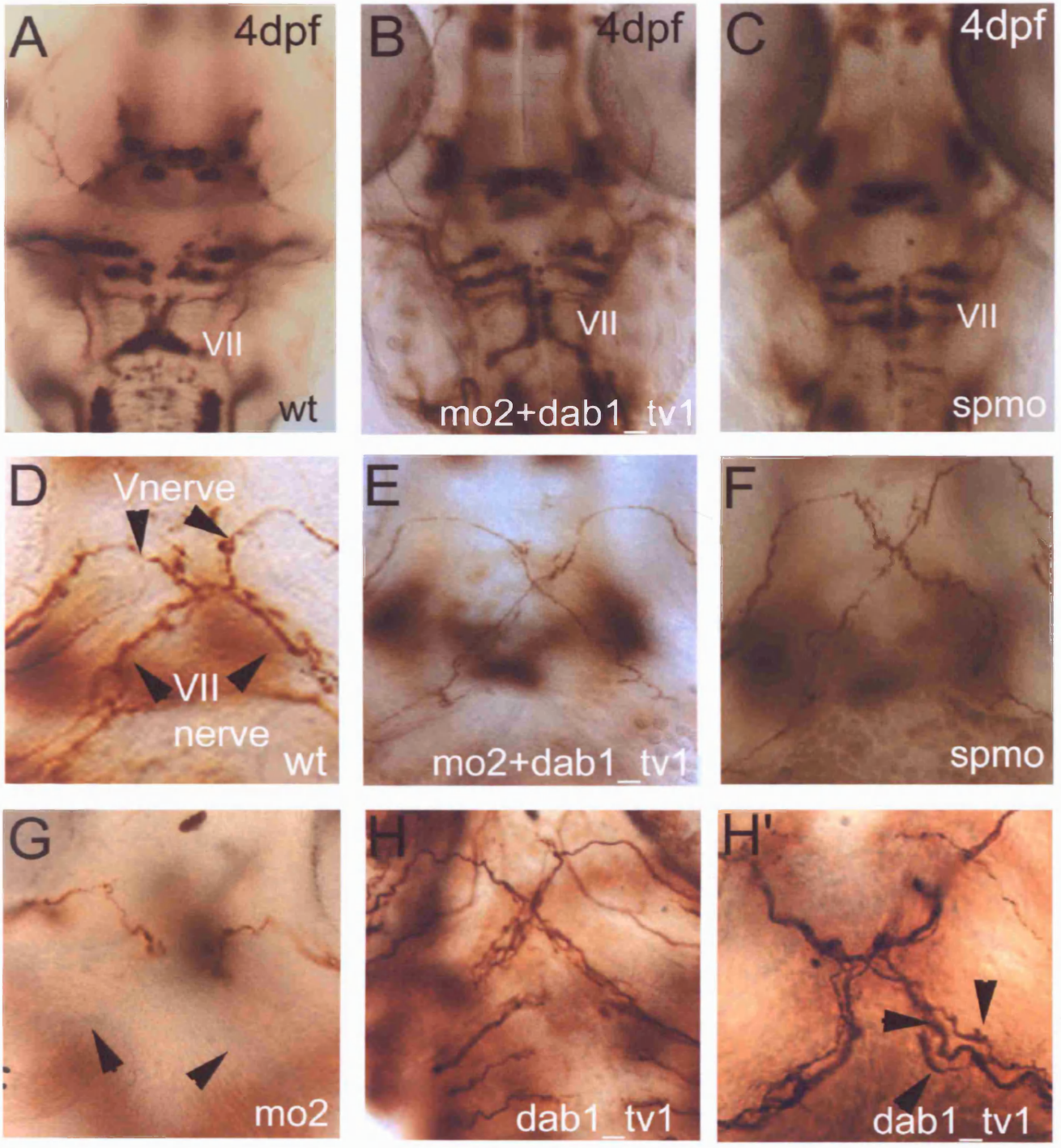
5.1.3.2 Abnormal branching of axons in embryos injected with morpholinos 1 or 2, but not with spmo

Through the visualizion of the axonal projections of VII neurons, using both confocal microscopy and immunohistochemistry for GFP in 48 hpf and 5 dpf islet1-GFP transgenic zebrafish, it was possible to identify defects in nerve projections, branching and fasciculation in *dab1* morphants (fig.5 and 6).

The most frequent abnormality was a marked reduction in length and thickness of the facial nerve, often accompanied by defasciculation of the nerve at level of the facial sensory ganglion (fig. 5). However, even for high doses of morpholino the facial nerve almost always exited correctly at r4 and then defasciculated into several axon fascicles at the level of the facial sensory ganglion (fig. 5). At 48hpf the extension of the facial nerve in mo1-injected embryos was still defective and in some cases the axons of the facial nerve were found not to have grown properly and not to innervate the jaw

Figure.6. Dorsal (A, B, C) and ventral (E, F, G, H, H') view of the head of 4hpf embryos of the *tg(islet-1-GFP)* line stained for GFP (A, B, C E, F, G, H, H').

A) Control. (B) Embryos c-injected with mo2 and *dab1_tv1* mRNA showing a partial rescue. (C) Embryos injected with spmo showing the complete absence of VIIn migration. (D) Ventral view of a control embryo where arrows indicate the V and VII nerves. E) Ventral view of an embryo injected as shown. The co-injection of mo2 and *dab1_tv1* rescues completely the facial nerve (but not the migration of the VIIn nucleus; B). (F) Ventral view of a morphant injected with spmo, the nerve is normal. G) Ventral view of an embryo injected or with mo1 or mo2, the VII nerve is absent. H and H') injection of *dab1_tv1* mRNA induces overgrowth of the VII nerve.


A
4dpf
VII
wt
B
4dpf
VII
mo2+dab1_tv1
C
4dpf
VII
spmo
D
Vnerve
VII
nerve
wt
E
mo2+dab1_tv1
F
spmo
G
mo2
H
dab1_tv1
H'
dab1_tv1

even at 5 dpf, while the nearby trigeminal motor nerves were perfectly normal (fig.6G).

On the contrary, the splice morpholino did not cause this severe phenotype: cells number, axons projection and axon extension by the VII nerve were normal, but the VII nerve nucleus failed to migrate in almost 100% of the cases (fig. 6C and F).

These findings are quite surprising and suggest a function for Dab1 in VII nerve growth that has not been observed in *dab1* mouse mutants (Rossel *et al.*, 2005).

5.1.2. Preliminary observation of additional phenotypes resulting from the injection of mo1 and mo2

5.1.2.1. Reticulospinal neurons show several defects in embryos injected with morpholinos 1 or 2

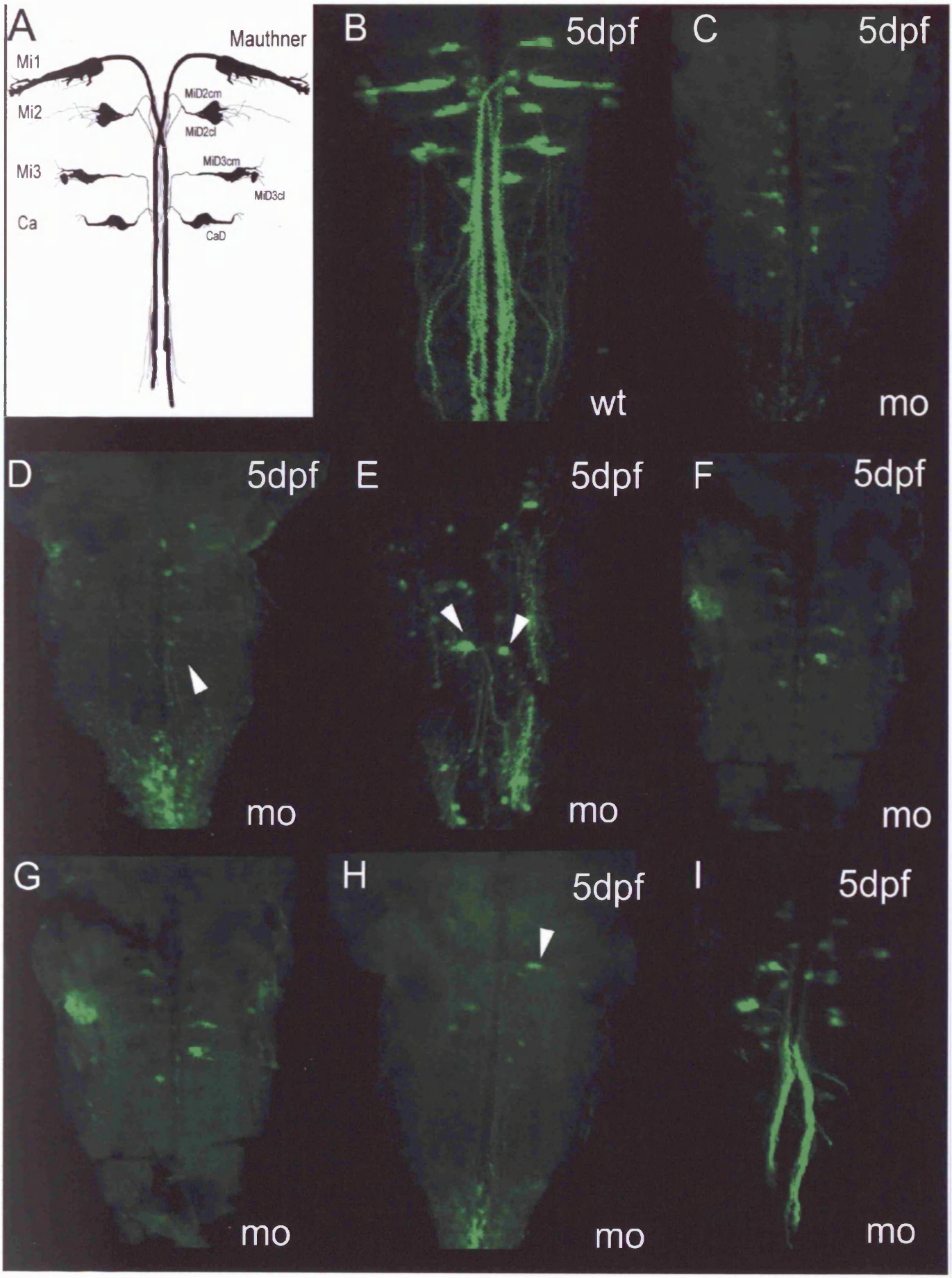
At 40hpf, when zebrafish hindbrain is still organized in rhombomeres, it is possible to visualize the developing reticular spinal neurons, using the marker *pcp4a* (Mione *et al.*, 2006). This population of neurons is organized bilaterally to the midline and conveys motor stimuli to the spinal cord. At this stage *reelin* expression is localized at level of rhombomeres 2-7 (Chapter 3) while *dab1* expression is restricted to cell populations of two lateral columns of the hindbrain where the marker pcp4a is also expressed (fig. 4 C,

D in Chapter 4). After 40hpf stage, the *dab1* expression becomes stronger and the pattern changes as the neural tube develops and by 48hpf is widespread throughout the hindbrain.

Because of the peculiar expression pattern of *dab1* in the hindbrain I looked at the effects of injection of *dab1* morpholino 1 or 2 on reticulospinal neurons, and I found that they were seriously affected, in particular Mauthner neurons (fig.7 and fig.8). Mauthner neurons are two large neurons present only in fish and tadpoles and together with all the reticular spinal neurons project their long axons into the spinal cord. Retrograde labelling with fluorescent dye (fig.7), immunohistochemistry performed with the 3A10 antibody (specific for Mauthner neurons. See fig. 8 A, B) at 40 hpf and *in situ* hybridisation for the *calcium binding peptide pcp4a*, all showed that the segmental array of reticulospinal neurons was affected in *dab1* mo injected embryos. Reticulospinal neurons were reduced in number and misplaced, Mauthner neurons were unhealthy showing a somas rounded in shape (fig.7 E and F) instead of elongated (see scheme fig. 7A and control 7B) and in general reticular spinal neurons axon bundles were thinner, misguided or absent (fig. 7 and fig. 8 B-F).

Figure.7. Fluorescent retrograde labelling of the reticulospinal neurons at 5dpf.

A) Scheme of the reticular spinal neurons that project controlaterally in Zebrafish hindbrain modified from Metcalfe et al. 1986. (B) Wild type embryo. C-I) dab1 mo2 injected embryos. The reticulospinal neurons appear round in shape and reduced in number and difficult to identify. In D reticular neurons are visible only in half of the embryos; in C, D, E and F cell bodies are clearly stained but the axons are very thin and misguided. In E) Mauthner neurons (arrows) are visible and show misguided axons. In H, only one Mauthern neuron is present (arrow).

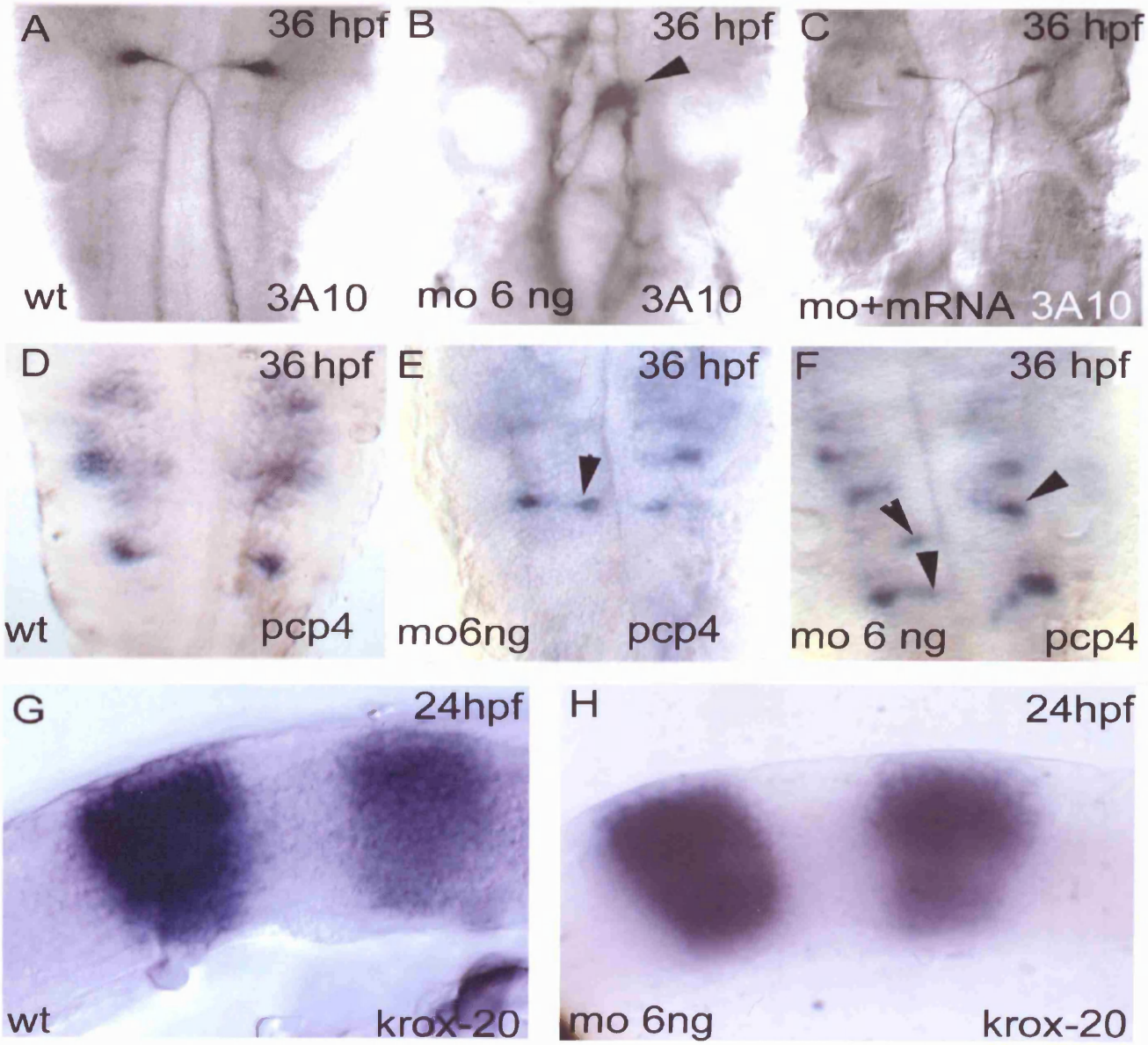

A
Mauthner
Mi1
Mi2
Mi3
Ca
MiD2cm
MiD2cl
MiD3cm
MiD3cl
CaD
B
5dpf
wt
C
5dpf
mo
D
5dpf
mo
E
5dpf
mo
F
5dpf
mo
G
mo
H
5dpf
mo
I
5dpf
mo

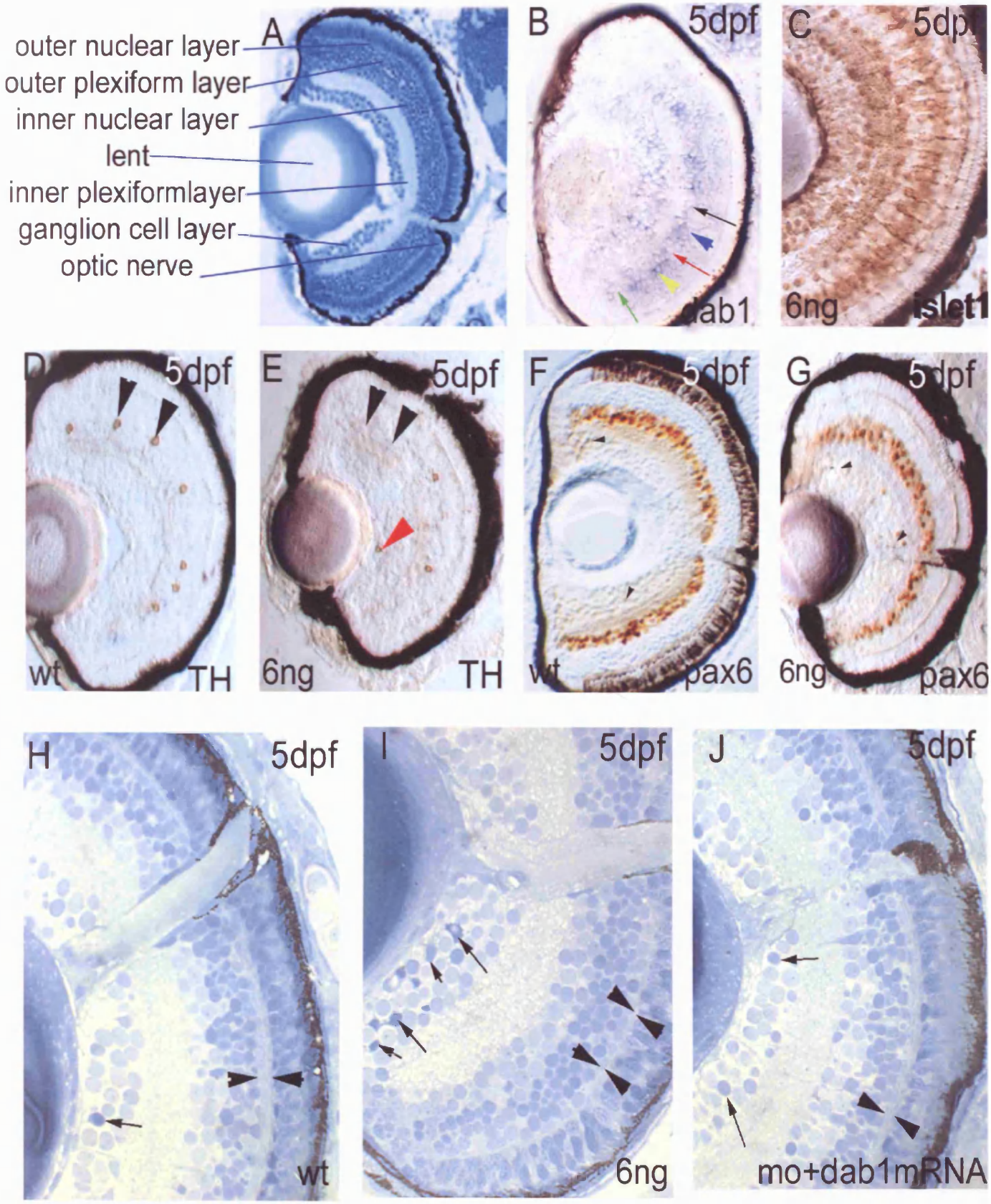
Moreover, the Mauthner neurons, which are normally located one on each side in ventral r4, were sometimes found either on the same side or near the midline in morpholino injected embryos (fig.8B). The axons of Mauthner neurons were found to be dramatically altered in morphants. In wild type embryos these large axons cross the midline at the level of r4 and advance in a straight path along the spinal cord without branching. In *dab1* mo injected embryos, however, Mauthner axons displayed a range of defects from complete absence to pathfinding defects and abnormal branching (see below table IV).

In order to ascertain that the defects in the localization and projections of reticulospinal neurons in *dab1* mo injected embryos are due to abnormal migration/axon growth and not to abnormal hindbrain patterning resulting in mis-specification of cell types, I performed an *in situ* hybridization for a segmental marker (*i.e.* rhombomere identity marker), *krox20*. Krox20, which is expressed in r3 and r5 at 24hpf, did not show altered expression in *dab1* mo injected embryos (fig.8H). This confirms the hypothesis that the defect

Figure.8. Reticulospinal neurons phenotypes caused by dab1 mos.

Immunohistochemistry performed with 3A10 antibody (A-C) to label Mauthner neurons at 36 hpf. *In situ* hybridization for the calcium binding peptide pcp4 (D-F) at 36 hpf and for the rhombomere 3 and 5 using the marker *krox-20* at 24hpf (G, H). Compare wild type embryos (A, D, G) with mo1 injected embryos (B, E, H) and mo1 plus *dab1* mRNA injected embryos (C, F). The Mauthner neurons are on the same side or near the midline in embryos injected with morpholino 1 (arrow; B), but the co-injection of morpholino plus dab1 mRNA can partially rescue the phenotype (C). In general reticulospinal neurons are duplicated or miss-positioned (arrows) in injected embryos (B, E). (G, H) *krox-20* expression appears normal in injected embryos.

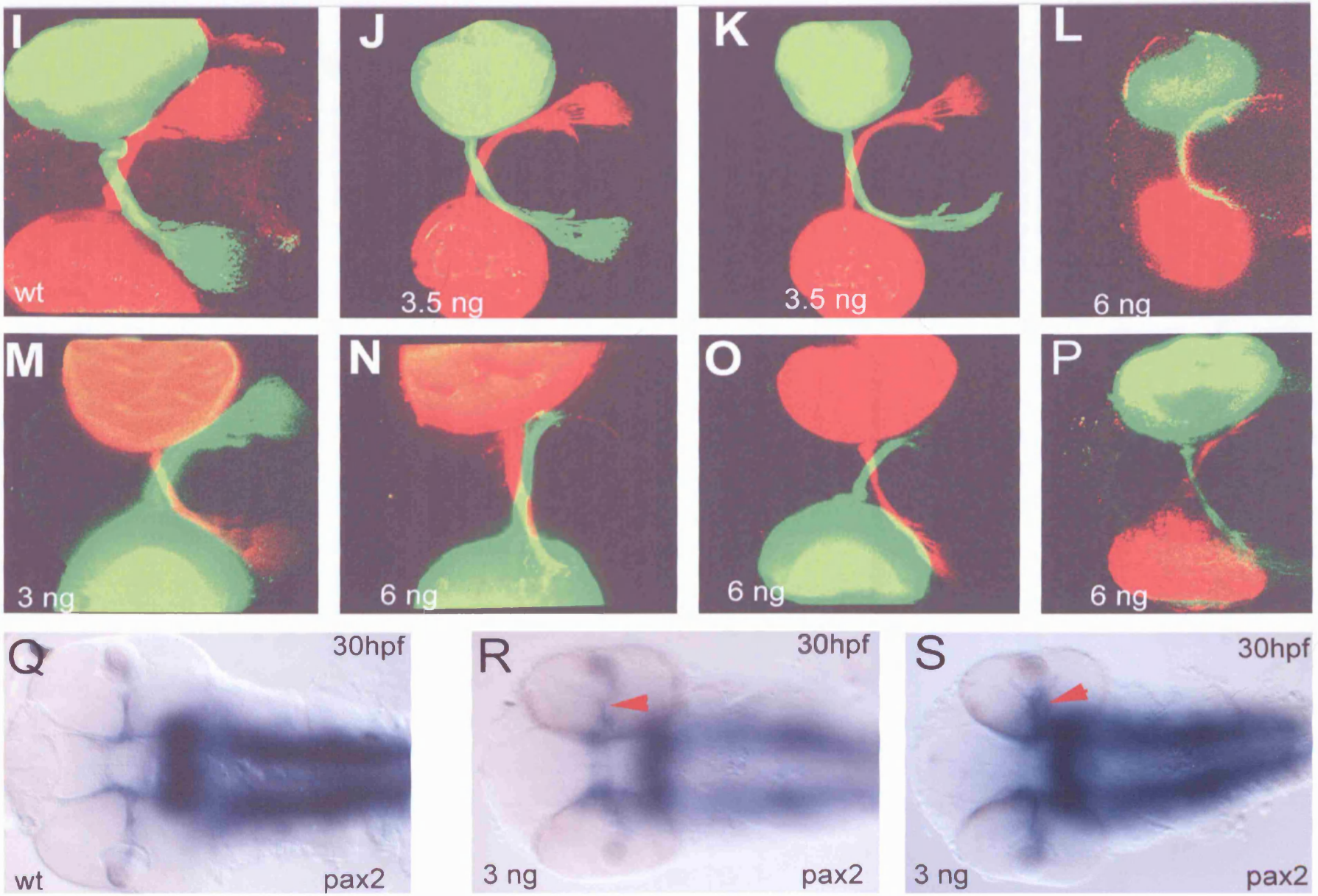

A
36 hpf
wt
3A10
B
36 hpf
mo 6 ng
3A10
C
36 hpf
mo+mRNA 3A10
D
36 hpf
wt
pcp4
E
36 hpf
mo6ng
pcp4
F
36 hpf
mo 6 ng
pcp4
G
24hpf
wt
krox-20
H
24hpf
mo 6ng
krox-20


outer nuclear layer
outer plexiform layer
inner nuclear layer
lent
inner plexiformlayer
ganglion cell layer
optic nerve
A
B
5dpf
dab1
C
5dpf
6ng
islet1
D
5dpf
wt
TH
E
5dpf
6ng
TH
F
5dpf
wt
pax6
G
5dpf
6ng
pax6
H
5dpf
wt
I
5dpf
6ng
J
5dpf
mo+dab1mRNA

Over 90% of the embryos injected with *dab1* mos displayed smaller eyes, which showed also an incomplete closure of the optic fissure (coloboma; see arrows fig.9bR and S). This was also revealed by *in situ* hybridization for *pax2.1*, a probe that labels the optic stalk (fig. 9b R, S). Then further investigations were carried out to verify whether the ordered architecture of the zebrafish retina was altered as a result of the loss of function of *dab1*. To identify defects in retinal morphogenesis, sections of zebrafish retina were labelled with several antibodies: Pax6 as a marker of amacrine cells, TH as a marker of dopaminergic interneurons and Islet1 for the general architecture (fig. 9a A-E). Despite the size reduction, no changes in retinal architecture or significant losses of cell types were observed, except for the dopaminergic amacrine cells. At 5 dpf, dopaminergic amacrine cells were reduced to less than one third of the normal complement and sometimes displaced (fig. 9a E). The size reduction of the eye may be in part due to reduction of the plexiform caused by a substantial reduction in neurites (see arrows pointing at each other in fig. 9a F, G) a phenotype already described in *scrambler* mice (Rice *et al.,* 2001). A range of defects in the optic nerve of morpholino-injected embryos was found when DiI and DiO anterograde tracing was used at 4dpf (table V; fig. 9b I-P).

Figure.9b. The effect of dab1 knock down on the zebrafish optic nerve.

I-P) DiI and DiO anterograde tracing at 4dpf imaged using the confocal microscope. In the mo injected embryos the optic nerve is thinner (J-P) and in severe cases does not reach the tectum (L, N, O, P). Q-S) Dorsal view of the head of embryos of 30hpf stained for *pax2.1*. In Q, the wild type embryo shows the optical nerves crossing the midline. In R and S embryos injected with 3 ng of mo1 show disorganization of the optical nerves that do not reach the midline. Coloboma is also present (arrows).


I
wt
J
3.5 ng
K
3.5 ng
L
6 ng
M
3 ng
N
6 ng
O
6 ng
P
6 ng
Q
30hpf
wt
pax2
R
30hpf
3 ng
pax2
S
30hpf
3 ng
pax2

These defects are summarized in table V and consisted of thin optic nerves, inability to reach the target and defasciculation. The defects found in the optic nerve could be related to transient expression of *dab1* in retinal ganglion cells seen up to 5 dpf (see fig. 9aA) or possibly, to a strong expression of *dab1* in the pre-optic area at 24-30 hpf (see Chapter4).

In conclusion, Dab1 is involved in retinal development and in zebrafish loss of function of *dab1* determine miss positioning of dopaminergic amacrine cells, reduction of the size of the retina and defective optic nerve growth. These phenotypes could be rescued with the injection of *dab1* mRNA suggesting that they are specific to the abrogation of Dab1 function.

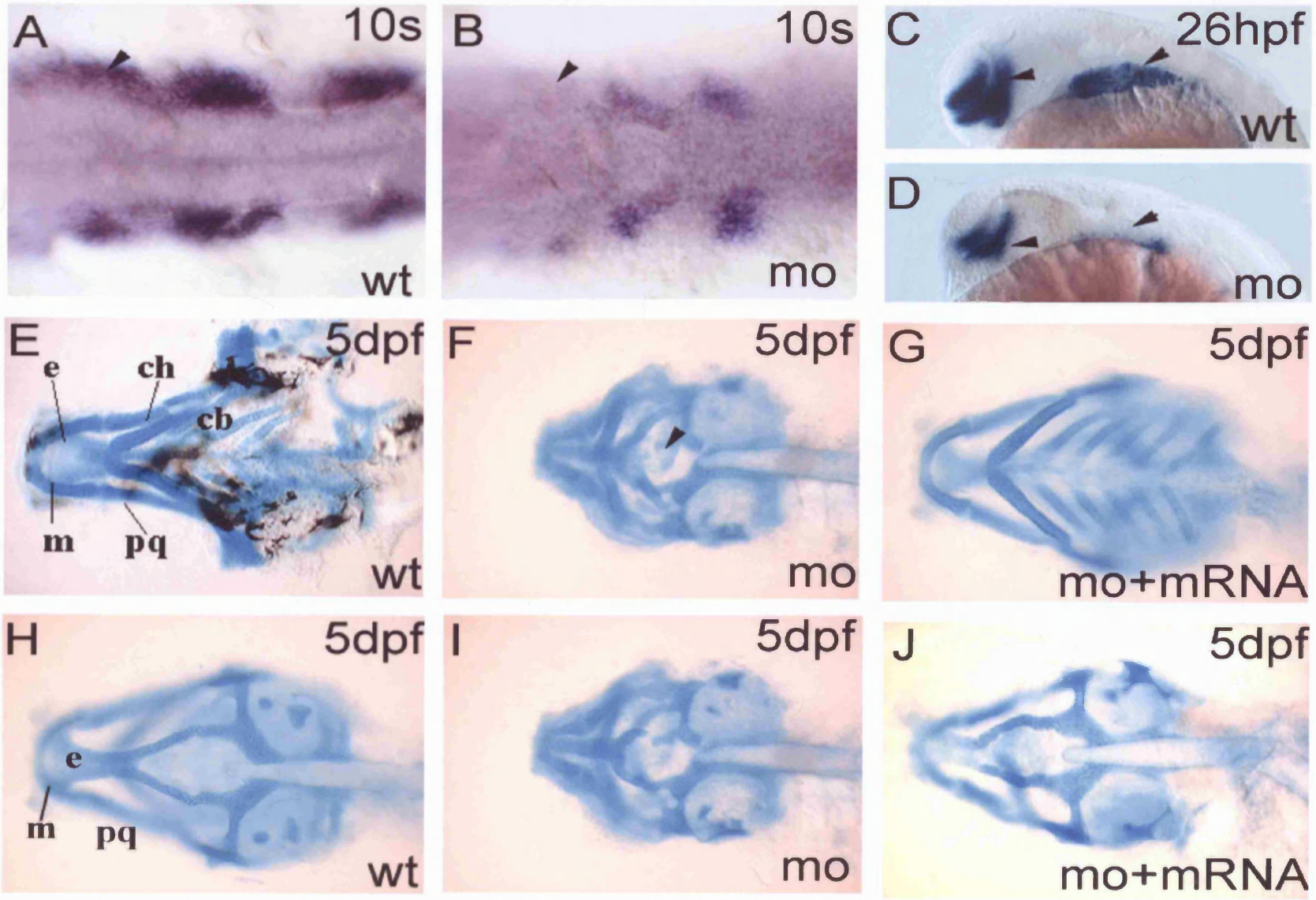
Table V. Abnormalities in morphants optic nerve.

Thin optic nerve	Target innervation	Defasciculation	Total
17	11	2	24

5.1.2.3. Neural crest migration and jaw development are affected in mos 1 or 2 morphants

In *dab1* mouse mutants there have been no descriptions of any defects of the neural crest and jaw. On the contrary, zebrafish *dab1* morphants displayed an unusual shape of the mouth visible at 4-5 dpf (fig.10F, I).

Alcian blue staining (Fisher & Halpern 1999) of the cartilages of the jaw showed that some of them were missing and others were reduced in size


A
10s
wt
B
10s
mo
C
26hpf
wt
D
mo
E
5dpf
e
ch
cb
m
pq
wt
F
5dpf
mo
G
5dpf
mo+mRNA
H
5dpf
e
m
pq
wt
I
5dpf
mo
J
5dpf
mo+mRNA

pattern of *dlx2* (a marker of the neural crest at stages when the migration starts) in morpholino-injected and control embryos. In wild type embryos, at 13 somites *dlx2* was expressed at the level of the rhombomeres 3-5 in three bilateral groups along the neural tube (fig.10 A, B).

The morphants showed a reduction or absence of *dlx2* expression at 13 s and at 24hpf stage at the level of the future arches and at the level of the hypothalamus in the forebrain (fig.10D). Finally, experiments, where *dab1_tv1* mRNA was co-injected with mo1 and 2, showed that indeed mRNA of *dab1_tv1* rescues morpholino phenotype (fig.10G, J). This suggests that the morpholino acts during early events when the neural crest migrates from the hindbrain (*i.e.* around 20-24 hpf). In zebrafish, the effect of antisense morpholinos 1 and 2 is a reduced size of the jaw and the absence of the 5 branchial arches, forming the gills, in most cases.

5.2. Discussion

The data presented in this chapter show that loss of *dab1* function affects the development of several regions of zebrafish embryos. In the hindbrain, migration and axon extension of the VII nerve nucleus are abrogated, reticulospinal neurons are misplaced and Mauthner neurons display defective axon positioning. The retina showed a reduced size, its dopaminergic amacrine cells were reduced in number, misplaced and the extension of the optic nerve was affected. Surprisingly morpholino

phenotypes were also evident in non-neuronal tissues including jaw cartilages, heart and blood circulation.

2.1. Dab1 plays a key role in the migration of facial branchiomotor neurons

Loss of *dab1* function affects the migration of the VII nerve nucleus. The formation of the facial branchiomotor nucleus is a complex event that can be divided into several steps that differ in mice and in chicks. In the mouse, the VIIn neurons leave the ventricular zone of r4 and migrate tangentially towards r6. Then, the migrating cells follow the glial processes and migrate radially towards the pia within r6. In chicks the migration starts and terminates within r4 (Studer, 2001). The specification of the facial neurons requires an intact rhombomere 4, and is affected in the absence of hindbrain segmentation (Cooper *et al.*, 2003; Deflorian *et al.*, 2003; Miller *et al.*, 2004).

At the molecular level, the VIIn migration requires the downregulation of *kreisler/val* in rhombomere 5 (Theil *et al.*, 2002) and the sequential activation of combinations of membrane associated molecules, including Tag-1, Ret and Cadh-8 in mouse (Garel *et al.*, 2000), and in zebrafish it is impaired in *trilobite* (*tri*) mutants, lacking the transmembrane protein Strabismus/Van Gogh (Jessen *et al.*, 2002). Several reports suggested an involvement of the Reelin/Dab1 pathway in this migration and the *tri* phenotype points to a contribution of the non-canonical Wnt pathway.

Defects in the architecture of the facial nerve nucleus in *reeler* were reported (Goffinet, 1984; Terashima *et al.*, 1993) and the main Reelin effector, Dab1, is normally expressed in the facial nerve nucleus (Carrol *et al.*, 2001; Chapter 4). Ohshima *et al.*, 2002, reported that in Cdk5 knockout mice the tangential migration of facial branchiomotor neurons from r4 to r6 does not take place and that in *yotary*, and even more dramatically in *yotary/p35* (p35 is an activator of Cdk5) knockout, the final radial migration in r6 is affected. Recently, it was shown that in *reeler* and *scrambler* the facial nucleus is disorganized and does not migrate properly (Rossell *et al.*, 2005).

In the most severe morphants phenotypes, the neurons do not migrate from r4 at all. This phenotype resample what it has been shown recenlty in the zebrafish mutants for *off-limits/frizzled3a* (*olt/fz3a*) and *off-road/celsr2* (*ord/celsr2*), where the VII neurons are not able to start the tangential migration to reach rombomere 6-7 (Wada *et al.*, 2006).

On the contrary in *reeler* and *scrambler/yotari* mice the nVII neuron population starts the tangential migration from r4 and reaches r6, but then they are not able to migrate radially within r6 toward the pial surface. The authors suggest that, as in the case of the olfactory bulb (Hack *et al.*, 2002), the switch from tangential to radial migration is affected, so nVII neurons are spread in r6 and not localized in the right position to form the VII nucleus (Rossel *et al.*, 2005).

Since Dab1 is phosphorylated in vivo and in vitro by CDK5 on S^{464} (Keshwara *et al.*, 2002), I investigated whether it is through this mechanism that *dab1* regulates the migration of facial motor neurons. The ability of a truncated Dab1 protein lacking a conserved domain containing S^{464} to rescue

the migration of facial motor neurons was comparable to that of full length *dab1_tv1* suggesting that *cdk5* is unlikely to play an important role in Dab1 phosphorylation in this context.

Another important difference between the zebrafish and the mouse is the localization of Reelin during VIIn migration. In mouse Reelin is absent from the route taken by the migrating neurons and presents a strong expression in r6-7, where the neurons terminate the migration (Ashwell & Watson, 1983; Auclair *et al.*, 1996; Studer *et al.*, 1996; Garel *et al.*, 2000). In zebrafish, *reelin* is expressed uniformly from r2 to r7 before the VII has started to migrate and even stronger at 48hpf when the migration has terminated (see Chapter3).

These findings suggest that in zebrafish, the migration of VIIn might be regulated in a different manner compared to mouse and the possibility that other pathways are involved cannot be excluded. Moreover the study of the localization of Reelin receptors in zebrafish is needed to get a more complete picture.

5.2.2. Alternative splice forms of *dab1* are involved in migration and in axon extension of the facial nerve nucleus

The ability to knockdown *dab1* function by splice morpholino injection permitted functional analysis of the tyrosine domains of Dab1. In fact, the spmo is designed against a site of splicing between exon 6 and intron 7 (splice donor site) and it determined the production of a new *dab1* mRNA (+

intron 7) that retains only Tyr^{185}. This "artificial" isoform was lacking of 4 tyrosines and is supposed to take the place of the other Dab1 isoforms in morphants.

In addition, using the splice blocking morpholino it was possible to avoid phenotypes caused at early stages by mo1 and mo2, which may affect the translation of maternal *dab1* transcripts. The resulting morphant is morphologically normal but exhibits a failure of nVII neuron migration (100% of morphants). On the contrary mo1 and mo2, which are against all Dab1 isoforms, produced phenotypes involving both migration and axon pathfinding. Moreover, the overexpression of Dab1_tv1 isoform gave rise to axon defasciculation but the nerves reach always the final target. These findings suggest that isoforms containing the complete set of tyrosines, for example Dab1_tv2, are involved in migration, while the isoforms as Dab1_tv1 are involved in axon development and might have maternal functions that are need to investigate more further. Anyway, both isoforms have as target the cytoskeleton of the cells, but the result depends on the set of tyrosines present.

The spmo gave rise to a molecule where most all the C-terminus, including Tyr^{198}, Tyr^{200}, $Tyr^{202}$and Tyr^{232}, is ablated. *Yotary* and *scrambler* present respectively a truncation and an insertion in Dab1 just at level of the tyrosine domain (Sheldon *et al.*, 1997; Howell *et al.*, 1997). Interestingly, both mutants present defects in nVII migration, but a defective facial nerve was never described. The *reeler* VIIn phenotype is identical to *scrambler* and *yotari*, with a normal facial nerve.

Although more studies are needed to better understand the phenotype found in zebrafish morphants, it is without doubt that the phosphorylation domain of Dab1 is important for the nVII migration.

5.2.3. Development of reticulospinal neurons is affected in morpholino injected embryos

The reticulospinal neurons are born between 7 and 28 hpf in the ventral hindbrain and have a segmental identity (Metcalfe *et al.*, 1986; Mendelson, 1986a). By 5 days Mauthner cells are found in the ventral hindbrain at level of r4, either due to passive displacement (Mendelson, 1986b) or to active radial migration. There are not mammalian homologues of Mauthner neurons and therefore no previous data on the effects of *dab1* or the loss of function of *reelin* on their position.

In zebrafish, events that affect the segmental identity of rhombomeres invariably change the number of reticulospinal neurons and sometimes also their location. This indicates that rhombomere identity dictates the identity of reticulospinal neurons (Maves *et al.*, 2002; McClintock *et al.*, 2002). During reticulospinal neuron development, *reelin* is highly expressed in rhombomeres 2-7 and *dab1* is localized in the lateral columns of neurons of the hindbrain that also includes reticulospinal neurons (chapter2-3).

In *dab1* morphants the segmental identity of rhombomeres is not altered, as shown by the correct expression of *krox20* (fig 8G, H), however there are defects in the location of reticular spinal neurons (Fig 8 B, D-F),

suggesting that these neurons might undergo radial migration and that this migration relies on Dab1. Alternatively, it might be a cell non-autonomous effect of the loss of function *dab1*, *i.e.* the neurons that surround the reticular spinal neurons may be abnormal and/or create an abnormal environment.

Finally, it is possible that the primary defect of reticular spinal neurons (missing *dab1* activity) is in their axons and that in the absence of correct projections the soma of reticular spinal neurons is less stabilized in its position and may be passively displaced at different ventro-dorsal levels or even controlaterally displaced. Midline signals guide the growth cones in the spinal cord (Charron *et al.*, 2003; Bernhardt *et al.*, 1992; Serafini *et al.*, 1994) and this is generally believed to occur through Netrins, Slits and other signals (General Introduction).

The data presented in this thesis are compatible with the suggestion that Reelin could be a signal of positional information for the descending axons of the reticulospinal neurons, which occupy either the middle or the lateral longitudinal fascicles on the two sides of the Reelin positive cell columns of the hindbrain (see Chapter 3).

5.2.4. Loss of *dab1* functions affects the development of retinal circuitries and the retinal neurons

In spite of the laminar expression of both *dab1* and *reelin* in the developing retina, extensive studies have failed to reveal a role of this

pathway in the organization of the ordered retinal cytoarchitecture (Katyal & Godbout 2004). However, the activity of the Reelin pathway seems to be necessary for the patterning of synaptic connectivity in the retina, since both *reeler* and *scrambler* mice show an attenuation of photoreceptor retinal responses (Rice *et al.*, 2001). This defect is associated with a decrease in rod bipolar cell density and an abnormal distribution of processes in the inner plexiform layer.

The injections of *dab1* mos in zebrafish did not affect retinal cytoarchitecture but caused a significant decrease in the number of amacrine dopaminergic cells and a reduction of the thickness of the inner plexiform layer. This phenotype is reminiscent of the retinal phenotype in *scrambler* and *reeler* mice. Rice *et al.* demonstrated that the glycinergic amacrine cells are reduced (they did not assess dopaminergic neurons) and showed that the remaining amacrine cells have reduced dendrites. Together these result in a thinner plexiform layer, a phenotype, which may also be present in zebrafish (see arrows pointing at each other in fig. 9a F, G).

In conclusion, the function of Dab1 in the retina could be to allow the correct development of retinal circuitries, through neurite formation and specification or correct migration of interneurons. This suggestion is further supported by the finding that *dab1* is only transiently expressed in zebrafish retinal ganglion cells. Knock down of *dab1* through morpholino does not affect RGC (Retinal Ganglional Cells) number or position, but severely affects the development of the optic nerve.

5.2.5. Retinal axons requires *dab1* for extension and path finding

As shown in figure 9b, the abrogation of *dab1* functions with mos affected the growth of retinal axons. Reelin is expressed in the preoptic area just above the chiasmatic plate at the time when retinal axons are pioneering this path (Chapter3 and Costagli *et al.*, 2002). This region also expresses other regulators of optic nerve growth and path finding, including Slit2, Ephrin-A5 and Eph receptors (Wong *et al.*, 2004; Knoll &Drescher 2004; Frisen *et al.*, 1998). It is interesting that all these signalling pathways rely on tyrosine kinase activity within the growth cones of retinal axons. Here integration of multiple signals results in the correct path finding for these axons (Wong *et al.*, 2004; Knoll & Drescher, 2004).

The function of the Reelin/Dab1 pathway in the growth of the optic nerve and of other nerves could be that of promoting individual axonal growth and growth cone exploration and allowing for correct selection of paths and exposure to guidance cues. For example, in the developing *reeler* hippocampus, entorhinal axons form abnormally thick bundles and undergo both targeting errors and decrease in collateral branching (Del Rio *et al.*, 1997; Borrell *et al.*, 1999). In the presence of *dab1* mos retinal axons may reduce their growth rate, their exploratory behaviour and finally undergo errors in path finding in reaching their targets in the optic tectum.

All three systems affected by *dab1* abrogation described here, facial nerve, reticulospinal neurons axon and optic nerve, have in common defects in axonal growth, in path finding and branching. These abnormalities might be due to a "weakness" of the growing axon. The cytoskeletal network may be impaired by the absence or severe reduction of an important mediator of the extracellular signals to the cytoskeleton. As a result of that the axons undergo multiple errors including abnormal branching, premature termination, abnormal path and target choice.

5.2.6. The roles of the functional domains of *dab1* are still unknown

The complex structure of *dab1* gene results in a fine regulation of the transcripts during development. Since all *dab1* transcripts seem to include the PTB domain, they should retain the ability to bind the NPxY motif of the lipoprotein receptors. However, the intracellur response depends on the type of isoforms, *i.e.* if they are or not carrying the tyrosines phosphorylable by the Reelin signal.

I have tested the hypothesis of a dominant-negative effect of Dab1-PTB domain in live zebrafish embryos by injecting mRNA encoding for this domain alone. Although these injections caused a range of defects they never caused defects attributable to a blockage of *dab1* function in neuronal migration, namely they did not phenocopy the injections of *dab1*

morpholinos. However, the severity of the phenotype due to overexpression in some cases precluded the analysis of facial nerve growth.

Overexpression of *dab1_tv1* mRNA showed axon defasciculation supporting the evidence that differential expressions of *dab1* isoforms are involved in axon development. In fact, the isoform *dab1_tv1* is expressed in VIIn neurons before and after the migration (see Chapter 4) when the neurons are still extending their axons.

At the moment a homologue of *dab1_tv1* has not been characterized in other vertebrates and so it could be specific to zebrafish. By contrast the isoform *dab1_tv2* is identical in exon organization to *mDab1-555* and the homology with the mouse and human protein domains is high. For these reasons, Dab1_tv2 might be subjected to ubiquitination after phosphorylation similar to Dab1-555 (Suetsugu *et al.*, 2004; Park *et al.*, 2003), while it is not predictable what the targets of the phosphorylated *dab_tv1* isoform are and whether undergoes ubiquitination.

Published *in vitro* data (Ohkubo et al, 2003; Beffert et al, 2002) and unpublished *in vivo* data (personal communication Marina Mione) show that Dab1_tv2 regulates phosphorylation of AKT through PI3K. The Phosphatidylinositol 3-Kinase can be activated by Dab1 and is involved in cytoskletal reorganization and in particular in the phosphorylation of several cytoskeletal proteins (Bock *et al.*, 2003).

Although the functions of the main Dab1 domains are known, the role of the C-terminus is not understood and it is not known whether it influences the others domains. Further studies on the various isoforms of

Dab1 are needed to clarify the activities performed by *dab1* and which are the pathways where these Dab1 isoforms are involved.

5.2.7. Jaw phenotypes resulting from injections of mo1 and 2 suggest alternative roles for *dab1*.

The injection of mo1 and 2 gave rise to phenotypes at level of the cartilages of the jaw. The preliminary data concerning the function of *dab1* in non-neuronal tissues presented here are, to the best of my knowledge, the first shown. In mouse *dab1* mutants a connection of Dab1 to neural crest migration has not been described.

As previously mentioned, mo1 and 2 affect the translation of maternal and zygotic transcripts and at high concentration the embryos die. In mice the knockout of *dab2* results in embryonic lethality as the endoderm migration is affected (Yang *et al.*, 2002). As shown in Chapter 3, *dab1* is maternally expressed as dab2 and the two genes share a high homology in their PTB domain (Yun *et al.*, 2003). Thus, they may be redundant in some functions during very early development.

In the case of the jaw phenotype it is relevant to say that the cartilage derivatives in zebrafish give rise to a structure functionally and structurally not found in mammals. In mice the arches (at stage of pharyngula, when the morphologies of embryos of diverse vertebrates are comparable Gould, 1977) melt together to form the larynx, a structure that completely differs

from branchial arches in fish. Anyway defects at level of the larynx have never been reported in *Dab1* mouse mutants.

In general, the phenotypes I have seen may be due to a different function of *dab1* in zebrafish compared to mouse. The loss of function obtained in the morphants (at least at very early stages) may be more severe than in mouse where the mutation is not null (Dab1 is truncated in *yotari* and a low level of normal *dab1* transcript plus a longer form of ~7kb are present in *scrambler*; Sheldon *et al.*, 1997 and Howell *et al.*, 1997). In addition to that, redundancy between different *dab* genes may differ between species.

5.2.8. Conclusion

Based on the findings presented in this chapter, I propose that Dab1 is an adaptor protein regulating neuronal movements and correct axonal extension.

In addition, my work suggests that the signals that regulate the activation of Dab1 during axonal growth and pathfinding rely on phosphorylation of a group of tyrosines proven to be activated by Reelin signaling. Given the expression pattern of both *reelin* and *dab1* during axonal growth in zebrafish, I believe that Reelin may represent one of the signals that activate Dab1 phosphorylation in the CNS of zebrafish as in the other Vertebrates, whereas outside the CNS, Dab1 may be activated by other pathways.

Chapter 6

6. General discussion

6.1. Summary of the work carried out

In this work I present a preliminary study of Reelin/Dab1 pathway in zebrafish mainly focused upon analysis of neuronal migration. I started by describing the expression pattern of *Danio rerio* Reelin and its main intracellular effector Dab1 in the developing zebrafish and in particular at level of the CNS.

The use of a sequence of 30 aa probe of mouse *Dab1* permitted the screen of an adult zebrafish brain cDNA library and the cloning of the full-length of the isoform *dab1_tv1*, while of *reelin* only a short sequence was cloned. Access to an online database of zebrafish genomic sequences allowed the study at genomic level of *dab1* and the identification of at least three isoforms. Then, making use of the general techniques applicable in zebrafish model, it was possible to start a study of the functions of two Dab1 isoforms functions at level of CNS and jaw development.

6.2. Zebrafish Reelin and Dab1 show an expression pattern comparable but not identical to other vertebrates

Both *reelin* and *dab1* displayed a highly dynamic expression patterns from the early stages to 48hpf and continue to be expressed in adult brain of zebrafish (for Dab1 Marina personal communication).

reelin and *disabled1* expression from 5pdf has revealed that the distribution of the transcript in various CNS structures, except the telencephalon, resembles that described in other species (Bar & Goffinet, 2000). In zebrafish the telencephalon is not laminated and *reelin* signal is diffuse over its dorsal part, while *dab1* is expressed manly in the ventral part. This remarkable difference in the expression of *reelin* in the telencephalic region may be attributed to the different morphogenetic mechanisms that lead to the formation of zebrafish-everted brain. This theory is supported by the observation of the expression of *reelin* in *Xenopus laevis,* where *reelin* expression is less localized but still comparable to the other land vertebrates (Chapter3 paragraph 2.6). In fact, the telencephalon in amphibian evaginates, but no migration or laminar organization of dorsal telencephalic cells have been reported to occur.

On the contrary, mammalian cortex presents a well-known laminate structure and neurons are organized in an "inside to outside" order reflecting the timing of their birth and patterns of migration. How the transition from non-laminar pallium to laminated mammalian cortex

occurred during evolution is unknown. However, it is interesting to speculate that changes in Reelin expression and function may have contributed to this evolution. In mammalian dorsal telencephalon and olfactory bulb, Reelin was found to control radial migration (D'Arcangelo & Curran, 1998; Hack *et al.*, 2002). In particular, the localization of Reelin solely in the marginal zone orchestrates the formation of the layers of the cortex. The mammalian cortex might derive from a single-layer of ancestral amniote cortex common to reptiles, amphibians and birds, where Reelin changed from a more diffuse signal to a more localized at level of the marginal zone (Bar *et al.*, 2000; Aboitiz *et al.*, 2001; Tissir *et al.*, 2002). Certainly the roles for Reelin in the fish and mammalian telencephalon are likely to be very different as the radial migration patterns that require Reelin activity simply do not occur in the fish telencephalon.

Another peculiarity of zebrafish telencephalon, and in general of all teleosts, is the absence of *reelin* expression in the olfactory bulb, while *dab1* expression is present as in all the other vertebrates.

Zebrafish mesencephalon is more developed in size (in proportion) then in mammals and presents a laminated structure. Both zebrafish mesencephalic structures, the optic tectum and the torus longitudinalis, show the expression of *reelin* to be localized in two well distinct layers. In *reeler* mice the homologue of the optic tectum, the superior colliculus, is disorganized (Frost *et al.*, 1986). These findings are suggestive of a function of zebrafish *reelin* in the complex organization of the region of the midbrain and the localized expression of *reelin* could have the same evolutionary

meaning of the well-described *reelin* signal in the marginal zone of mammalian neural cortex.

To the other hand, in the eye, where the layered structure of the retina is not related to Reelin localization (Rice *et al.*, 2001), zebrafish Reelin and Dab1 could retain the function of control of circuitry formation. Extremely important is the evidence shown here in Dab1 morphants, where the reduction of the plexiform layer (layer of the nerve fibers inside the retina) was comparable to what has found in mice (Rice *et al.*, 2001). In addition, in chicks the formation of the retina circuitry was found related to the differential expression of two *dab1* isoforms, namely Chdab1-E and Chdab1-L. The Chdab1E is expressed in undifferentiated and dividing neurons, while Chdab1-L is expressed in differentiated neurons, *i.e.* neurons able to form neurites and synaptic connections. This finding and present data are the first showing the function of Dab1 isoforms potentially expressed in temporal and tissue specific manner.

6.3. Alternatively spliced *dab1* isoforms in zebrafish add a new level of complexity in Reelin/ Dab1 pathway

The study of *Danio rerio dab1* gene confirmed a complex genomic organization previously shown in the mouse and humans (Bar *et al.*, 2002) and now, with the present work, corroborated in teleosts.

In addition, the phenotypes of the morphants at the level of the brain, jaw, blood circulation and heart are suggestive of *dab1* functions difficult to explain without taking alternative splicing and multifunctional isoforms into consideration (Chapter 2 and Bar I *et al.*, 2003). It has been shown that modular exons are strongly associated with tissue-specific regulation of alternative splicing (Xu *et al.*, 2002; Yeo *et al.*, 2004; Xing & Lee 2005).

At 24-30 hpf the isoforms *dab1_tv1* and *dab1_tv2* are co-expressed in the forebrain, but they show a differential expression in the hindbrain. The isoform *dab1_tv1* is localized in two bilateral stripes of the hindbrain, while *dab1_tv2* isoform is expressed in *reelin* positive region at level of the migrating neurons of the facial nucleus. The presence of two isoforms at level of the non-migrating (isoform *dab1_tv1*) and migrating (*dab1_tv2*) neurons of the VII was never described in the other vertebrates and the *dab1_tv1* it-self was cloned only in zebrafish so far.

Moreover, at 24hpf stage of development only *dab1_tv1* was in the region above the yolk extension, where reproductive organs, pronephric ducts and blood vessels originate. Interestingly, Dab2 that retains a PTB domain almost identical to Dab1 PTB domain (Yun *et al.*, 2003) was seen involved in early stages of development. As both genes are maternally expressed, their functions could be redundant in non-neuronal tissue.

In mice several isoforms were cloned and one in particular is not expressed in neuronal tissue. The isoforms Dab1-271* and Dab1-217* are shorter then the Dab1-555 (Dab1_tv2 is the zebrafish homologue) and the isoform Dab1-555* is expressed in kidneys, liver and in general in non-neural tissue (Howell *et al.*, 1997a).

Taking all this evidence together, it is possible to speculate on roles for Dab1 that may be not only in neural tissue but also participate in other tissues and may not involve the Reelin pathway. On the other hand, the discovery of alternative splicing of Dab1 adds a new level of complexity to the Reelin pathway, so that the study of the several isoforms of Dab1 is becoming an obligatory way to the general comprehension of this pathway.

6.4. Disabled1 is part of the machinery that regulates neuronal migration and axonal growth

Disabled was first identified as an enhancer of *abl* phenotype in *drosophila,* where the reduction of Dab levels in *abl*-/- background caused severe defects in the organization of the nerve cord, the major axon tracts in this species (Gertler *et al.*, 1989). I have investigated the role of *dab1* in neuronal migration and axonal growth in zebrafish in three different contexts, namely the Mauther neurons and their axons, facial nucleus and facial axons and the retina and optic nerve. All cases displayed severe defects in both cell localization and axonal growth in *dab1* morphants, showing *dab1* implication in both migration and axonal growth.

The role of Dab1 in vertebrates has been studied using the mouse mutant *scrambler* and *yotary* that carry mutations in the *dab1* gene (Sheldon *et al.*, 1997 and Howell *et al.*, 1997b), and in mouse *Dab1* knockout (Howell *et al.*, 2000). While migration defects of neuronal cell groups have been

extensively reported, connectivity or projection defects have been often ruled out.

Dab1 is a partner of non-receptor tyrosine kinases Fyn and Src (Arnaud, *et al.*, 2003b; Kuo *et al.*, 2005) and probably sits at the core of several pathways involved in converting extracellular signals into cytoskeletal changes.

6.5. Reelin is not a localized signal in the zebrafish hindbrain and potentially controls migration of the VII nucleus through differential expression of *dab1* isoforms

Reelin is a secreted protein and it is thought to act in a graded way. In the mammalian cortex and in the olfactory bulb, it Reelin is produced by a single layer of peripheral cells (Ogawa *et al.*, 1995; Hack et al 2002) towards which the neurons migrate. In mouse hindbrain *reelin* displays a localized and strong expression in r6 to control the migration of the VIIn at the final position (Ashwell & Watson, 1983; Auclair *et al.*, 1996; Studer *et al.*, 1996; Garel *et al.*, 2000; Rossel et al., 2005). On the contrary, in the zebrafish hindbrain *reelin* is expressed throughout rhombomeres 2-7 and in the forebrain, it is expressed by the entire dorsal telencephalon instead of a single layer, as in reptiles and mammals (Chapter 3).

The presence of *reelin* expressing cells below and above Dab1 positive cells is a common event during migration, and has been observed in the telencephalon of reptiles, birds (Goffinet *et al.*, 1999; Bernier *et al.*, 2000; Tissir & Goffinet, 2003) and amphibia (Chapter 3). Although it is still unknown whether Reelin represents a repulsive (or stop) or attractive signal for migrating cells, it is clear that in radial migration the source of Reelin must be separated from the responsive cells expressing Dab1 and able to activate specific receptors (Bar *et al.*, 2000). On the contrary, in the zebrafish hindbrain, *reelin* is expressed in 6 rombomeres including rombomeres 4, 5 and 6, where the neurons of the VII are migrating. Therefore, it is not a localized source of Reelin that leads neuron migration but it is the expression of at least two different isoforms of Dab1 that determines the correct positioning of the nucleus. In this case, Reelin is not acting as a repulsive or an attractive signal but rather a permissive signal.

Anyway it cannot be excluded the possibility that in zebrafish Reelin is not involved in nVII migration. The *dab1* morphants showed a defect first of all in the tangential migration rather then in the radial migration. As Reelin was seen involved mainly in radial migration, it can be take in consideration the possibility of another signaling molecule to be involved in Dab1 phosphorilation in zebrafish nVII.

To ascertain the function of Reelin in this migration more studies are needed and for example, it is necessary to look at the expressions pattern of the receptors involved in the Reelin pathway but that, at the best of my knowledge, have not been cloned in zebrafish yet.

6.6. *reelin* and *dab1* function in the young/adult brain of all vertebrates

Both Reelin and Dab1 are still expressed in the CNS of young and adult zebrafish. The absence of *reelin* in dividing cells (BrdU lebeling; Chapter 3) indicates an association between *reelin* and differentiating neurons. Reelin may be important for the migration of newly born neurons, for the formation of the nuclei, but also for the growth of their dendrites and axons (Chitnis *et al.*, 1990; Wilson *et al.*, 1990; Ross *et al.*, 1992).

The complexes formed by Reelin and its receptors are thought to promote the stabilization of dendritic spine microtubules through Dab1 phosphorylation and its interactions with various cytoskeletal components (Feng *et al.*, 2001; Stockinger *et al.*, 2000). In addition, Reelin, (through an Apoer2/Vldlr-Dab1-dependent pathway), can positively regulate the long-term potentation (LTP) in mouse hippocampal slices and modulate synaptic plasticity and learning in the adult mouse brain (Weeber *et al.*, 2002). In fact, Reelin can enhance NMDA receptor activity and alter the gating of the channel (Chen *et al.*, 2005).

In zebrafish, *reelin* (and *dab1* as well) is expressed at 5 dpf when the neuron migration and morphogentic movements have reached to completion. At the same stage both *reelin* and *dab1* may be involved in formation of the circuitry of the retina (Chapter 5 and Rice *et al.*, 2001). Moreover, *reelin* is highly expressed in the regions of dendritic spine remodelling of type I neurons in the optic tectum (Chapter 3).

Finally, the observed expression of *reelin* and *dab1* at 1-month stage and later confirms the hypotheses that the Reelin/Dab1 pathway may be involved in zebrafish in dendrite maintenance and remodeling once the migration of neurons in CNS structure is complete.

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Appendix

Molecular Biology Techniques and solutions preparation

Mainly all the solutions and molecular techniques follow the protocols of "Molecular cloning, a laboratory manual" by Sambrook, Fritsch and Maniatis.

Measurement of DNA/RNA concentration

DNA and RNA concentration were quantified by optical densitometry (OD) using a spectrophotometer. The OD was measured at 260nm for DNA (OD=1 equates to 50μg/ml) or RNA (40μg/ml) following calibration with distilled water. The concentration was then calculated based on dilution rates with water.

Bacterial Plasmid DNA extraction

Plasmid DNA was introduced into DH5α strains of E.coli using a 30 second heat shock at 42°C. After overnight growth at 37°C on LB Agar plates with 100μg/ml ampicillin (Sigma), single colonies were picked and

innoculated into mini cultures of 2-3ml of LB with 100μg/ml ampicillin and grown overnight. Plasmid DNA was prepared from individual cultures using Qiagen miniprep kits (Qiagen), and eluted from the column with 10mM Tris-Cl, pH 8.5 according to manufacturers' instructions or by alkaline lysis as described in Sambrook et al. 1989. Larger scale midi- and maxipreps of plasmid DNA were performed using Qiagen kits with standard protocols.

DNA Manipulation

Restriction enzyme digestion was performed using enzymes from Promega in an appropriate buffer with 2-5 units of enzyme per 1ug DNA at the recommended temperature. Protein removal was carried out by phenol/chloroform extraction to purify DNA preparations.

Concentration or purification of nucleic acids was performed using 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol, incubated at -80^{0}C for at least 30 minutes.

For plasmid transformation, circular DNA or ligated DNA was added into competent cells by incubation for 30 minutes on ice, 30 second heat shock at 42^{0}C and further 2 minutes on ice. The cells were then incubated in LB media for 30 minutes at 37^{0}C followed by plating on the appropriate antibiotic-containing LB agar plates.

Ligation reactions

Intermolecular ligations were performed in small volumes; usually 20µl for approximately 20ng of vector DNA with 50 ng of insert DNA. Ligation reaction was carried out overnight at 14^0C with T4 DNA ligase (Invitrogen) and the ligation buffer provided. Sticky end ligation was performed where possible either using enzymes giving compatible ends or digesting primer-engineered restriction enzyme sites on the ends of PCR amplified DNA.

Transformation of the ligated plasmids was performed as described above and colonies containing the ligation product were detected by PCR selection. The direction of insertion was then tested by restriction digest mapping or by PCR.

Polymerase Chain Reaction (PCR)

PCR reactions were carried out according to standard protocols using Applied Biosystems 9700 (Gene Amp) thermocycling machine. Taq-DNA polymerase, Promega was used for ordinary molecular characterisations. Pfu DNA polymerase (Stratagene) was used for amplifying sequences to be cloned into expression vectors for RNA injection studies because it has a very low error rate. Annealing

temperature specific to each primer was optimised with test reactions prior to use.

Manufacturer's instructions were followed to vary the length of the various cycles within the PCR programme according to the enzymes used.

Agarose Gel Electrophoresis and DNA Extraction from Agarose

Separation of DNA (and RNA) fragments was performed by agarose gel electrophoresis in TAE buffer (40mM Tris-acetate, 1mM EDTA). The agarose was dissolved in 1xTAE and ethidium bromide (10 mg/ml) was added for visualisation of DNA or RNA under ultraviolet light. The concentration of agarose ranged between 0.8 and 2% depending to the size of DNA fragment to be run. The DNA samples were loaded by mixing with loading buffer. A DNA ladder (1kb size standard) was run alongside the DNA. Extraction of DNA fragments from the agarose gel was performed using the QiaexII gel extraction kit (Qiagen) according to manufacturer's instructions.

Preparation of digoxigenin and fluorescein RNA probes

Antisense probes for *dab1_tv1* and *reelin* were generated by linearization with SalI and in vitro transcription with SP6 in the presence

of dig-labelled ribonucleotides (Roche), whereas the *Xenopus* reelin plasmid was linearized with NOTI and transcribed with T3-RNA polymerase.

To generate the template for exon 8+9 we used RT-PCR on total RNA extracted from 5 dpf zebrafish larvae. The primers used were forward exon 8 and reverse exon 9. The PCR product was cloned in PCR2.1 using Topo, TA cloning Kit (Invtrogen), sequenced and linearized using BamHI.

Riboprobes were purified using quick spin columns (Roche) and stored in 50% formamide at –70°C. *Islet1* (Inoue, 1994), *eom* (Mione et al., 2001), *nk2.1b* (Rohr et al., 2001), *Krox-20* (Oxtoby, 1993), RNA probes were synthetised using a fluorescein labelling kit (Roche, UK) for two colour *in situ* hybridization.

Plastic Sections protocol

Embryos should be fixed and in 70% glycerol. After being washed 3X10 minutes in H_2O, the embryos were washed into ethanol (30% ethanol/70% H_2O, 50% ethanol, 70% ethanol, 95% ethanol and finally 100% ethanol 2 minutes each step). Activation of solution A (BDH): 0.225g benzoyl peroxide to 25 ml of A solution under the fium hood. Incubation in 1:1 mix of A:100% ethanol 1hr at RT or O/N at 4^0C in an eppendorf tube. The solution A:100% ethanol was replaced by fresh activated A solution and then with A+B solution. The solution A+B was prepared adding 160µl

B into 4ml activation solution A in falcon tube. About 400 μl A+B solution was used for each eppendorf tube containing samples. The samples were placed into wells, 2 each wells. The A+B solution was replaced with fresh A+B, the embryos re-orientated under dissection microscope putting at ends of wells facing out wards. The wells were placed in a box and N_2 gas was added. After sealing the lid, the samples were left in the box with N_2 O/N, RT. Sections were attached to a support and cut at microtome.