Published in final edited form as:

Dev Genes Evol. 2006 June ; 216(6): 291–299. doi:10.1007/s00427-005-0052-5.

Identification of alternatively spliced *dab1* isoforms in zebrafish

Arianna Costagli

Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC1E 6BT, UK

Barbara Felice and Alessandro Guffanti

Ifom, Firc Institute of Molecular Oncology, Via Adamello 16, 20139 Milan, Italy

Stephen W. Wilson

Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC1E 6BT, UK

Marina Mione

Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC1E 6BT, UK

Ifom, Firc Institute of Molecular Oncology, Via Adamello 16, 20139 Milan, Italy

Abstract

We have investigated the genomic organization, the occurrence of alternative splicing and the differential expression of the zebrafish disabled1 (*dab1*) gene. Dab1 is a key effector of the Reelin pathway, which regulates neuronal migration during brain development in vertebrates. The coding region of the zebrafish *dab1* gene spans over 600 kb of genomic DNA and is composed of 15 exons. Alternative splicing in a region enriched for tyrosine residues generates at least three different isoforms. These isoforms are developmentally regulated and show differential tissue expression. Comparison with mouse and human data shows an overall conservation of the genomic organization with different alternative splicing events generating species-specific isoforms. Because these alternative splicing events give rise to isoforms with different numbers of phosphorylatable tyrosines, we speculate that alternative splicing of the *dab1* gene in zebrafish and in other vertebrates regulates the nature of the cellular response to the Reelin signal.

Keywords

Disabled1; Neuronal migration; Transcriptional variants; Reelin; Tyrosine phosphorylation

Introduction

The intracellular adaptor Dab1 is a key regulator of neuronal migration during brain development (Howell et al. 1997b, 1999a; Sheldon et al. 1997). The protein is composed of an N terminus domain of approximately 180 amino acids, named PI/PTB (protein interaction/phosphotyrosine binding domain), followed by a cluster of five tyrosines and a C terminus containing two consensus regions for the serine/threonine kinase Cdk5 (Howell et

© Springer-Verlag 2006

Correspondence to: Marina Mione.

marina.mione@ifom-ico-campus.it Tel.: +39-02-574303229 Fax: +39-02-574303221.

Electronic Supplementary Material Supplementary material is available for this article at <http://dx.doi.org/10.1007/s00427-005-0052-5> and accessible for authorised users

al. 1997a; Keshvara et al. 2002). The PI/PTB domain binds a tetra-amino acid motif, Asn-Pro-X-Tyr (NPXY), present in several transmembrane proteins including members of the low-density lipoprotein receptor and amyloid precursor families (Trommsdorff et al. 1998; Howell et al. 1999b). Two of these lipoprotein receptors function as receptors for Reelin (D'Arcangelo et al. 1999; Hiesberger et al. 1999), a large glycoprotein implicated in the control of neuronal migration (D'Arcangelo et al. 1995). Further confirmation that *Dab1* conveys a Reelin signal in neuronal migration through lipoprotein receptors, came from the observation that mouse mutants for the four genes (*Dab1*, *Reelin*, *ApoER2* and *VLDL*) show the same neuronal migration defects (Goffinet 1979; Howell et al. 1997b; Sheldon et al. 1997; Trommsdorff et al. 1999).

Dab1 is present in the developing central nervous system (CNS), where it can initiate a phosphorylation cascade with multiple targets when phosphorylated on its tyrosine residues (Howell et al. 2000; Ballif et al. 2003). These targets include the Src family kinase Fyn (Arnaud et al. 2003b; Bock and Herz 2003), the PI3K pathway (Bock et al. 2003) and various SH2 domain adaptor proteins, including Nck β (Pramatarova et al. 2003), Crk and CrkL (Ballif et al. 2004; Chen et al. 2004; Huang et al. 2004). Tyrosine phosphorylated *Dab1* is also a target of the proteasome/ubiquitin machinery (Bock et al. 2004).

The genomic organization of *Dab1* is very complex; analysis of human and mouse *Dab1* genomic sequences revealed that it is composed of 14 exons, spanning over 1.1 Mb and giving rise to at least five alternative tissue-specific splicing events in mouse plus several alternatively spliced 5'-untranslated regions (5'UTRs) with different promoters (Bar et al. 2003). Although alternative splicing contributes significantly to protein diversity in eukaryotes, there have been relatively few studies on the identification, differential expression and functional diversification of alternative spliced isoforms. In this study, we have investigated the occurrence of alternative splicing in the zebrafish *dab1* gene.

During characterisation of a zebrafish *dab1* clone, we found that the predicted amino acid sequence lacked three of the five conserved tyrosine located downstream of the PI/PTB domain. This prompted us to investigate whether this was due to alternative splicing. Therefore, we undertook a study of the genomic structure of *dab1* using the reported organization of human and mouse *Dab1* genes as reference (Bar et al. 2003). Our study demonstrates that the zebrafish *dab1* gene shows a high degree of complexity in its genomic structure, and different isoforms show temporal and tissue-specific expression.

Materials and methods

Cloning

To identify zebrafish *dab1* cDNA clones, we screened a micro-arrayed adult zebrafish brain library distributed by RZPD (library no. 611) with a cDNA fragment corresponding to nt 450–1311 of mouse *Dab1*-555 (GenBank accession no. NM_010014). This fragment was obtained through polymerase chain reaction (PCR) using an E13.5 mouse brain cDNA as template. Hybridisation yielded a number of positive clones, which were analysed for their sequence and expression pattern. In situ hybridisation for two of these clones with identical sequence gave a neuronal specific pattern. Conceptual translation yielded an open reading frame (ORF) of 538 amino acids, similar to that of mouse and human *Dab1*. Screening the available zebrafish expressed sequence tags (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 transcriptional variant 1 of *dab1* (*dab1_tv1*) (see below). However, we predicted and confirmed with reverse transcription-

polymerase chain reaction (RT-PCR) the presence of different isoforms of zebrafish *dab1* on the basis of the analysis of genomic sequence (see below).

Reverse transcription-polymerase chain reaction

Total RNA was extracted using Trizol (Invitrogen) at various stages from pools of approximately 50 zebrafish embryos and larvae at the following stages: 1–32 cells, 8 somites, 30 and 48 hours post-fertilisation (hpf), 3 and 5 days post-fertilisation (dpf). Total RNA was also extracted from three adult brains. 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's instructions.

The following primers were used:

- Forward exon 5: CTACATCGCGAAGGATATCAC
- Reverse exon 10: GGACATGTCTCCAAAAAGCTC
- Reverse exon 8: CTGATATATGCTCTCCTCTGATGG
- Reverse exon 9: TCACTGGATGTCGCTTTGGGA
- Forward exon 8: CATTGTATTTGAGGCGGGACAC

In addition, primers in all other exons (see supplementary Table S1) were used in various combinations to confirm the results presented here.

Fish strains

Danio rerio of wild type and transgenic lines were from the University College London (UCL) Zebrafish Facility. The *transgenic (islet-1-GFP)* line was donated by H. Okamoto (Higashijima et al. 2000).

In situ hybridisation

An antisense probe, which recognises predominantly *dab1_tv1*, was generated by linearisation with *SalI* and in vitro transcription with SP6 in the presence of dig-labelled ribonucleotides (Roche). 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 (Invitrogen), sequenced and linearised using *BamHI*. The sequence of this probe is provided in the supplementary Table S1. In vitro transcription was performed as described. The differential localisation of the two isoforms was assessed in *tg(islet1-GFP)* embryos (Higashijima et al. 2000) using double staining for green fluorescent protein (GFP) (immunostaining) and cRNA (in situ hybridisation).

Sequence and genome alignments

For the identification of the genomic clones containing the *dab1_tv1* sequences and the localisation of the exons, we used the Blast tool from National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/blast>). The database used consisted of Bac clones fully sequenced by the Sanger Institute and deposited in Genbank 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 exon 8 and 9, we used mouse sequences to blast the clone BX248232. The same approach was taken for the missing exons 555*, 217* and 271*. For the comparative genomic analysis, we used the Blat tool (Kent 2002) at UCSC (<http://genome.ucsc.edu/>), 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.

Results

Cloning of zebrafish *Dab1*

We identified a zebrafish *dab1* cDNA clone through library screening. This clone encodes for a protein of 538 amino acids showing high similarity to other Disabled 1 proteins (Fig. S1) and was named *Danio rerio disabled1, transcriptional variant 1* (*dab1_tv1*, GenBank accession no. DQ 166810). RT-PCR analysis revealed the existence of a second transcriptional variant (*dab1_tv2*, Genbank accession no. DQ166811). Comparison of the longest isoforms of *Dab1* from several species yielded the phylogenetic tree shown in Fig. 1.

Next we analysed the genomic structure of the zebrafish *dab1* gene using the genomic sequence provided by the Sanger Institute and validated the predicted exon/intron structure with RT-PCR and in situ hybridisation.

Identification of three genomic clones encoding *dab1*

We used stretches of sequences of about 30 nucleotides of zebrafish *dab1_tv1* to electronically screen the zebrafish genomic database at NCBI. Three clones covering most of the genomic sequence of *dab1* were identified. The size, coding sequences and contig assembly of these Bac clones are shown in Fig. 2a. Analysis of the sequences of the three Bac clones revealed that *dab1_tv1* is encoded by 13 exons, mainly corresponding to those present in mouse *Dab1(555)* (Fig. 2a). However, some exons differ or are missing suggesting that *dab1_tv1* is 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 prompted us to search the genomic sequence for additional exons encoding the missing tyrosine residues (see below).

Genomic organisation of the coding region

The *dab1* coding region starts in exon 2, which harbors the start codon, and ends in exon 15 (Fig. 2a). The position of the exons in the three Bac clones is shown in Fig. 2a. Although partially conserved, exon 14 does not contain a stop codon as in human and in mouse. The organisation of exon–intron and the splice junctions follow mostly the GT/AG rules; however, intron 9 has a GG/AG junction (Table 1). The length of the exons is similar to that of the same exons in the other species studied, whilst introns have variable lengths (Table 1).

PI/PTB of *dab1*, which binds Reelin receptors, is encoded by exons 3, 4, 5 and 6, whilst the phosphorylation domain containing five tyrosine residues is encoded by exons 6 (Tyr¹⁸⁵), 7 (Tyr¹⁹⁸), 8 (Tyr²⁰⁰ and Tyr²²⁰) and 9 (Tyr²³²) similar to mouse and human *Dab1*. The carboxyl-terminal sequence containing a consensus sequence for the serine/threonine kinase Cdk5 is found in exon 13, as in mouse *Dab1-555* (Fig. 2b).

Comparison of the alternative splicing events of mouse and zebrafish *dab1* genes

Although the genomic structures of fish, avian and mammalian *dab1* genes are very similar, 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 tyrosine residues Tyr²⁰⁰, Tyr²²⁰ and Tyr²³² that may be important in Reelin signalling. Indeed, mice expressing a mutated form of *Dab1*,

which cannot be tyrosine-phosphorylated in response to Reelin, display a reeler-like phenotype (Howell et al. 2000). A *Dab1* isoform similar to zebrafish *dab1_tv1*, i.e. containing only Tyr¹⁸⁵ and Tyr¹⁹⁸, has not been reported in other species. Moreover, other *Dab1* isoforms such as *Dab1-555** found in non-neuronal tissue in mammals (Bar et al. 2003) or the *217** and *271** forms found only in mouse (Howell et al. 1997a) do not seem to be present in zebrafish (see below). These isoforms have spliced in the exon *555**, *217** or *271**, none of which are present in the main form *Dab1-555*. For example in mouse *Dab1-217**, the last three tyrosine residues are missing but the presence of an alternative polyadenylation signal in intron 7 results in a truncated *Dab1* isoform (Howell et al. 1997a). The mouse form *Dab1-271** is prematurely truncated, all the five tyrosine residues are present, but part of the C terminus is missing due to a stop codon inside exon *271** (Howell et al. 1997a).

A careful analysis of the sequence of the third zebrafish Bac clone using mouse sequences encoding for exons *217**, *555** and *271**, as in silico probes, shows that the zebrafish gene probably lacks these three exons that should be located between exon 8 and 10. Between exons 9 and 10, there is a long intronic sequence rich in AT repeats, which could harbor a stop codon, but with extensive RT-PCR analysis, we have not found any alternative splice form lacking the C terminus (data not shown).

Developmental profile of alternative splice forms expressed in zebrafish

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

Using primers for exons 5 and 10 (Fig. 2b) that amplify through the zone rich in tyrosines, we obtained a single band until 8 somite (s) stage, whereas from 24–30 hpf, a second larger band can also be amplified with the same primer pair; both bands persist until at least 1 month stage (Fig. 2c). 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 (exons 8 and 9). Thus a second isoform, *dab1_tv2* similar to mouse *Dab1-555*, was identified. To better investigate which isoforms are expressed and when, we used different combinations of reverse primers specific for exon 8 or 9 with the forward primer specific for exon 5. One, two or three bands were obtained when using reverse primers for exon 8, 9 or 10. The presence of both exons 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.

Occurrence of alternatively spliced Dab1 isoforms in vertebrates

To check whether we had identified the correct *dab1* gene and all its transcriptional variants reported in public databases, we aligned the sequence of *dab1_tv1* and *dab1_tv2* with the Zebrafish Genome (Zv4, Sanger Institute) using the BLAT tool at UCSC (Karolchik et al. 2003). Both transcripts align with the genomic sequence starting from exon 4. Moreover, *dab_tv1* aligns with *dab1_tv2* in exons 5, 6 and 7, skips exons 8 and 9 and then aligns with the last four exons of *dab1_tv2*. The last two exons (14 and 15) are supported by EST belonging to a cDNA library prepared from adult brain. Furthermore, we found a reference sequence (RefSeq XM_681536) predicted by NCBI using GNOMON, an automated computational gene prediction method, that aligns perfectly with *dab1_tv2* and *dab1_tv1*

(data not shown). The lack of genomic sequences supporting the first four exons suggests that the Zv4 assembly did not place them in the right contig. However, two of the three Bac clones are placed in the last release of the zebrafish genome assembly performed by the Sanger Institute (Zv5, <http://www.sanger.ac.uk>) and all the three clones will appear arranged as described in this paper in the next Vega release (http://vega.sanger.ac.uk/Danio_rerio/ personal communication, Dr. Mario Caccamo, Sanger Institute).

We investigated the conservation of the sequences of the zebrafish *dab1_tv2* exons 8 and 9 in different species: mouse, human, chick and zebrafish and looked for ESTs that lack these two exons. We found that these two exons are highly conserved in all species, with only a few ESTs lacking exon 8 (3 human, 1 mouse and 1 chick). Exon 9 is less conserved at the nucleotide level and aligns perfectly only with the human sequence; however, we could not find any ESTs lacking this exon in any species (data not shown, please contact the corresponding author for BLAT alignment).

In situ hybridisation reveals that alternative splice forms are tissue-specific

From the PCR analysis, it is clear that several alternative spliced *dab1* mRNAs are expressed during development. To localise where these isoforms are expressed, we used two different probes: one against *dab1_tv1* and one against exons 8 and 9 only. Probes against the PTB domain and C terminus domain show an expression pattern identical to the zebrafish *dab1_tv1* probe (Fig. 3a,c). By contrast, the probe specific for exons 8 and 9 revealed a pattern of expression that differed substantially both temporally and spatially from the others (Fig. 3d).

First of all, the probe recognizing common regions of all *dab1* isoforms reveals a widespread expression pattern at the level of the forebrain, midbrain and hindbrain (Fig. 3a). In contrast isoforms containing exons 8 + 9 are initially expressed exclusively in the mesencephalic tegmentum and in some of the hindbrain cranial nerve nuclei (Fig. 3b). It is interesting to note that outside the brain, *dab1_tv1* is expressed beneath the notochord, possibly in cells of the pronephric ducts (arrows, Fig. 3c), whereas the *dab1* isoform containing exons 8 and 9 is never expressed in that area (Fig. 3d). At later stages (48 hpf), *dab1_tv1* is still expressed in the forebrain (arrows, Fig. 3e) and throughout the midbrain and hindbrain, whereas the *dab1* isoform containing exons 8 and 9 is faintly expressed in the midbrain and hindbrain (Fig. 3f). At 5 and 14 dpf, the expression of *dab1* isoforms with or without exons 8 and 9 in the telencephalon and cerebellum is comparable (data not shown). Interestingly, there are some differences in the hindbrain in the localisation of transcripts encoding for the two *dab1* isoforms. Whereas presumptive pre-migratory and post-migratory facial motor neurons express predominantly *dab1_tv1* (i.e. the isoform lacking exons 8 and 9, Fig. 3g), 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. 3h).

Discussion

Comparison of the genomic organisation of the *dab1* gene between species

The genomic organisation of the mammalian *dab1* gene shows an unusual complexity at the level of the coding region, which is about 5.5 kb long (including exons 1–14) and spans more than 1.1 Mb of DNA in the mouse and human genomes. Similarly in zebrafish, the *dab1* gene spans more than 600 kb for a coding region of at least 1.8 kb (including exons 2–15). Analysis of the sequences of the Bac clones shows that the main functional domains, the location of exon/intron boundaries and the length of the exons are conserved between fish and human/mouse *dab1* genes. The most significant differences between the zebrafish

dab1 gene and that of other species are that introns in the *Danio rerio dab1* gene are shorter than in human and mouse (see Table 1 and Bar et al. 2003). It is 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, we found that exon 14 does not contain the stop codon as in mouse and human *Dab1* (Bar et al. 2003). The stop codon of zebrafish *dab1* can be found in exon 15. Moreover, exons corresponding to mouse 217* and 271* are absent from the zebrafish genomic sequence; in addition, we could not find any sequence coding for exon 555*, which is present in all other vertebrates analysed and is duplicated in human and mouse. It might be that these exons have been acquired through mammalian specific events (retroviral or retrotransposome insertions) and sub-serve specific functions that became important during evolution. One possibility, which will be discussed at the end of the next paragraph, is that the mouse isoforms *Dab1-217** and *Dab1-271** exert a function similar to that of zebrafish *dab1_tv1* (i.e. the isoform lacking exons 8 and 9).

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

From the analysis of *dab1* expression through in situ hybridisation, 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 on the stage of development (Bar et al. 2003; Katyal and Godbout 2004).

Of particular interest is *dab1_tv2* containing exons 8 and 9, which 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. We think that the presence of exons 8 and 9, which contain three more tyrosines (corresponding to Tyr¹⁹⁸, Tyr²²⁰ and Tyr²³² in mouse) in addition to those present in *dab1_tv1*, may be crucial for the development of hindbrain neurons. This isoform is expressed from 24–30 hpf, at the time when facial motor neurons migrating from r4 are reaching their final destination in r6–r7 (Chandrasekhar et al. 1997). Two alternative splice forms, chDab1-E and chDab1-L, were found in the chick retina (Katyal and Godbout 2004). ChDab1-E lacks two tyrosine (corresponding to Tyr¹⁹⁸ and Tyr²²⁰), which are usually phosphorylated 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 contains all the five tyrosine and thus provides a substrate for full phosphorylation upon Reelin signaling. The data suggest that only those neurons expressing chDab1-L are able to grow neurites in response to the Reelin signal (Katyal and Godbout 2004). By analogy, *dab1_tv2* may be fully functional, whereas *dab_tv1* may antagonise such function.

All *Dab1* isoforms reported so far have the same N terminus, which contains the PTB/PI domain. These data suggest that all 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, while the other isoforms could work as dominant negative because they lack most of the tyrosine phosphorylation domain, i.e. block the receptor interacting domain without signalling. 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 become 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 mouse, alternative splicing also generates isoforms with reduced number of tyrosines. *Dab1-217** has the same complement of tyrosines as *Danio rerio 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 able to compete with *Dab1-555* for the binding to the Reelin receptors without relaying further the Reelin signals, 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. In mouse, the isoform *Dab1-555**, containing the tyrosine residues and the PTB domain, is expressed in kidneys, liver and generally in non-neural tissue (Howell et al. 1997a). These data suggest that in mouse at least one *Dab1* isoform exerts a role different than the regulation of neuronal migration. In zebrafish, *dab1_tv1* is expressed in the pronephric ducts, blood vessels and branchial arches (AC, SWW, MM, unpublished data). Mouse mutants for *Dab1* have a phenotype largely restricted to neuronal migration; however interpretation of the mouse mutant phenotype is complicated because 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 m*Dab1* truncated just at the 3' of the tyrosine phosphorylation domain is a hypomorph for some *Dab1* functions in hippocampal development (Herrick and 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 <http://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”.

Acknowledgments

We would like to thank the UCL Zebrafish Facility and the UCL Zebrafish Group for their help throughout this work. We are grateful to Dr. Mario Caccamo, Wellcome Trust Sanger Institute, for helping us with the genomic data. The project was supported by Wellcome Trust grants to Steve Wilson.

References

- Arnaud L, Ballif BA, Cooper JA. Regulation of protein tyrosine kinase signaling by substrate degradation during brain development. *Mol Cell Biol.* 2003a; 23(24):9293–9302. [PubMed: 14645539]
- Arnaud L, Ballif BA, Forster E, Cooper JA. Fyn tyrosine kinase is a critical regulator of disabled-1 during brain development. *Curr Biol.* 2003b; 13(1):9–17. [PubMed: 12526739]
- Ballif BA, Arnaud L, Cooper JA. Tyrosine phosphorylation of Disabled-1 is essential for Reelin-stimulated activation of Akt and Src family kinases. *Brain Res Mol Brain Res.* 2003; 117(2):152–159. [PubMed: 14559149]
- Ballif BA, Arnaud L, Arthur WT, Guris D, Imamoto A, Cooper JA. Activation of a *Dab1/CrkL/C3G/Rap1* pathway in Reelin-stimulated neurons. *Curr Biol.* 2004; 14(7):606–610. [PubMed: 15062102]
- Bar I, Tissir F, Lambert de Rouvroit C, De Backer O, Goffinet AM. The gene encoding disabled-1 (DAB1), the intracellular adaptor of the Reelin pathway, reveals unusual complexity in human and mouse. *J Biol Chem.* 2003; 278(8):5802–5812. [PubMed: 12446734]
- Bock HH, Herz J. Reelin activates SRC family tyrosine kinases in neurons. *Curr Biol.* 2003; 13(1):18–26. [PubMed: 12526740]

- Bock HH, Jossin Y, Liu P, Forster E, May P, Goffinet AM, Herz J. Phosphatidylinositol 3-kinase interacts with the adaptor protein Dab1 in response to Reelin signaling and is required for normal cortical lamination. *J Biol Chem.* 2003; 278(40):38772–38779. [PubMed: 12882964]
- Bock HH, Jossin Y, May P, Bergner O, Herz J. Apolipoprotein E receptors are required for reelin-induced proteasomal degradation of the neuronal adaptor protein Disabled-1. *J Biol Chem.* 2004; 279(32):33471–33479. [PubMed: 15175346]
- Chandrasekhar A, Moens CB, Warren JT Jr, Kimmel CB, Kuwada JY. Development of branchiomotor neurons in zebrafish. *Development.* 1997; 124(13):2633–2644. [PubMed: 9217005]
- Chen K, Ochalski PG, Tran TS, Sahir N, Schubert M, Pramatarova A, Howell BW. Interaction between Dab1 and CrkII is promoted by Reelin signaling. *J Cell Sci.* 2004; 117:4527–4536. [PubMed: 15316068]
- D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T. A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature.* 1995; 374(6524):719–723. [PubMed: 7715726]
- D'Arcangelo G, Homayouni R, Keshvara L, Rice DS, Sheldon M, Curran T. Reelin is a ligand for lipoprotein receptors. *Neuron.* 1999; 24(2):471–479. [PubMed: 10571240]
- Gertler FB, Bennett RL, Clark MJ, Hoffmann FM. *Drosophila* abl tyrosine kinase in embryonic CNS axons: a role in axonogenesis is revealed through dosage-sensitive interactions with disabled. *Cell.* 1989; 58(1):103–113. [PubMed: 2502313]
- Goffinet AM. An early development defect in the cerebral cortex of the reeler mouse. A morphological study leading to a hypothesis concerning the action of the mutant gene. *Anat Embryol (Berl).* 1979; 157(2):205–216. [PubMed: 517767]
- Herrick TM, Cooper JA. A hypomorphic allele of dab1 reveals regional differences in reelin-Dab1 signaling during brain development. *Development.* 2002; 129(3):787–796. [PubMed: 11830577]
- Hiesberger T, Trommsdorff M, Howell BW, Goffinet A, Mumby MC, Cooper JA, Herz J. Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. *Neuron.* 1999; 24(2):481–489. [PubMed: 10571241]
- Higashijima S, Hotta Y, Okamoto H. Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *J Neurosci.* 2000; 20(1):206–218. [PubMed: 10627598]
- Howell BW, Gertler FB, Cooper JA. Mouse disabled (mDab1): a Src binding protein implicated in neuronal development. *EMBO J.* 1997a; 16(1):121–132. [PubMed: 9009273]
- Howell BW, Hawkes R, Soriano P, Cooper JA. Neuronal position in the developing brain is regulated by mouse disabled-1. *Nature.* 1997b; 389(6652):733–737. [PubMed: 9338785]
- Howell BW, Herrick TM, Cooper JA. Reelin-induced tyrosine phosphorylation of disabled 1 during neuronal positioning. *Genes Dev.* 1999a; 13(6):643–648. [PubMed: 10090720]
- Howell BW, Lanier LM, Frank R, Gertler FB, Cooper JA. The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids. *Mol Cell Biol.* 1999b; 19(7):5179–5188. [PubMed: 10373567]
- Howell BW, Herrick TM, Hildebrand JD, Zhang Y, Cooper JA. Dab1 tyrosine phosphorylation sites relay positional signals during mouse brain development. *Curr Biol.* 2000; 10(15):877–885. [PubMed: 10959835]
- Huang Y, Magdaleno S, Hopkins R, Slaughter C, Curran T, Keshvara L. Tyrosine phosphorylated disabled 1 recruits Crk family adapter proteins. *Biochem Biophys Res Commun.* 2004; 318:204–212. [PubMed: 15110774]
- Karolchik D, Baertsch R, Diekhans M, Furey TS, Hinrichs A, Lu YT, Roskin KM, Schwartz M, Sugnet CW, Thomas DJ, Weber RJ, Haussler D, Kent WJ. The UCSC genome browser database. *Nucleic Acids Res.* 2003; 31:51–54. [PubMed: 12519945]
- Katyal S, Godbout R. Alternative splicing modulates Disabled-1 (Dab1) function in the developing chick retina. *EMBO J.* 2004; 23(8):1878–1888. [PubMed: 15057276]
- Kent WJ. BLAT-the BLAST-like alignment tool. *Genome Res.* 2002; 12(4):656–664. [PubMed: 11932250]

- Keshvara L, Magdaleno S, Benhayon D, Curran T. Cyclin-dependent kinase 5 phosphorylates disabled 1 independently of Reelin signaling. *J Neurosci.* 2002; 22(12):4869–4877. [PubMed: 12077184]
- Kopelman NM, Lancet D, Yanai I. Alternative splicing and gene duplication are inversely correlated evolutionary mechanisms. *Nat Genet.* 2005; 37(6):588–589. [PubMed: 15895079]
- Morimura T, Hattori M, Ogawa M, Mikoshiba K. Disabled1 regulates the intracellular trafficking of reelin receptors. *J Biol Chem.* 2005; 280(17):16901–16908. [PubMed: 15718228]
- Pramatarova A, Ochalski PG, Chen K, Gropman A, Myers S, Min KT, Howell BW. Nck beta interacts with tyrosinephosphorylated disabled 1 and redistributes in Reelin-stimulated neurons. *Mol Cell Biol.* 2003; 23(20):7210–7221. [PubMed: 14517291]
- Sheldon M, Rice DS, D'Arcangelo G, Yoneshima H, Nakajima K, Mikoshiba K, Howell BW, Cooper JA, Goldowitz D, Curran T. Scrambler and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice. *Nature.* 1997; 389(6652):730–733. [PubMed: 9338784]
- Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, Nimpf J, Hammer RE, Richardson JA, Herz J. Reeler/disabled-like disruption of neuronal migration in knockout mice lacking the VDL receptor and ApoE receptor 2. *Cell.* 1999; 97:689–701. [PubMed: 10380922]

Fig. 1.

A distance tree of the *Dab1* proteins from human, primate, mouse, rat, chick, worm, fly (truncated at AA 693) and zebrafish. 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 DQ166811 (*dab1_tv2*). For the *Drosophila* protein, we 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)

Fig. 2. 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 five tyrosines are shown (Y). Start and stop codons are indicated. **b** RT-PCR analysis of *dab1* expression in zebrafish. Position of the primers on *dab1_tv2* is shown. Orientation and length of genomic clones (zebrafish Bacs) used for reconstruction of the organization of the zebrafish *dab1* gene. Clone CH211-132C16 is in violet, clone Dkey 242M13 is in blue and clone CH211-232I5 is in green. Accession numbers are indicated. **c** Analysis of the RT-PCR products amplified from the stages indicated. L=dna ladder ; mat=maternal Control: no RT. On the right, predicted size of the amplicons. Size of the exons can be found in Table 1

Fig. 3.

Differential expression of *dab1* transcripts. In situ hybridisation analysis of the expression of *dab1_tv1* (a, c, e and g) and *dab1_tv2* revealed with a cRNA probe for exon 8+9 (b, d, f and 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, H hypothalamus, MHB mid-hindbrain boundary, DT dorsal telencephalon, VT ventral telencephalon, D diencephalon, 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 vesicles

Table 1Size and boundary nucleotide sequence of exons and introns of the zebrafish *dab1* gene

Exon number	Exon size (nt)	5' junction	Intron size (nt)	3' junction
2 (start codon)	202	R G AGAGGG _{GT}	75392	Q D AGCAGGAC
3	137	L K CTCAGG _{GT}	10290	G I AGGGAATC
4	99	S G TCGGGG _{GT}	26003	V L AGGTACTT
5	132	Q S CAGTCT _{GT}	2835	A E AGGCTGAG
6	120	Y ¹⁸⁵ Q TATCAG _{GT}	3727	T I AGACCATT
7	39	Y ¹⁹⁸ Q TATCAG _{GT}	3670	Y ²⁰⁰ I AGTACATT
217* Not found				
8	62	Y ²²⁰ Q TACCAG _{GT}	10226	V P AGGCTCCT
9	59	V S GTGAGT _{GG}	10007	N I AGAATATC
271* Not found				
555*1 Not found				
555*2 Not found				
10	64	P S CCCTCG _{GT}	1549	T P AGACTCCA
11	100	S G TCGCCA _{GT}	9163	Y V AGTATGTG
12	608	S P TCCCCA _{GT}	6884	P T AGCCCACC
13	128	A A GCTGCT _{GT}	8668	G D AGGGAGAC
14	76	P Q CCTCAG _{GT}	14955	G K AGGGGAAG
15 (stop)				