

# Delta proteins and MAGI proteins: an interaction of Notch ligands with intracellular scaffolding molecules and its significance for zebrafish development

Gavin J. Wright<sup>1,2,\*</sup>, Jonathan D. Leslie<sup>1,\*</sup>, Linda Ariza-McNaughton<sup>1</sup> and Julian Lewis<sup>1,†</sup>

<sup>1</sup>Vertebrate Development Laboratory, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

<sup>2</sup>Vertebrate Functional Proteomics Laboratory, Wellcome Trust Sanger Institute, Cambridge CB10 1SA, UK

\*These authors contributed equally to this work

†Author for correspondence (e-mail: julian.lewis@cancer.org.uk)

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## Summary

Delta proteins activate Notch through a binding reaction that depends on their extracellular domains; but the intracellular (C-terminal) domains of the Deltas also have significant functions. All classes of vertebrates possess a subset of Delta proteins with a conserved ATEV\* motif at their C termini. These ATEV Deltas include Delta1 and Delta4 in mammals and DeltaD and DeltaC in the zebrafish. We show that these Deltas associate with the membrane-associated scaffolding proteins MAGI1, MAGI2 and MAGI3, through a direct interaction between the C termini of the Deltas and a specific PDZ domain (PDZ4) of the MAGIs. In cultured cells and in subsets of cells in the intact zebrafish embryo, DeltaD and MAGI1 are co-localized at the plasma membrane. The interaction and the

co-localization can be abolished by injection of a morpholino that blocks the mRNA splicing reaction that gives DeltaD its terminal valine, on which the interaction depends. Embryos treated in this way appear normal with respect to some known functions of DeltaD as a Notch ligand, including the control of somite segmentation, neurogenesis, and hypochord formation. They do, however, show an anomalous distribution of Rohon-Beard neurons in the dorsal neural tube, suggesting that the Delta-MAGI interaction may play some part in the control of neuron migration.

Key words: DeltaD, DeltaC, MAGI proteins, Notch, PDZ domains, Zebrafish, Morpholino, Rohon-Beard neurons

## Introduction

The Notch cell-to-cell signalling pathway has a central role in animal development, controlling cell diversification and many other processes in a great variety of tissues (Artavanis-Tsakonas et al., 1999; Lewis, 1998). At its core lie the Notch family of receptors and their ligands, the proteins of the Delta/Serrate family. Both the receptors and the ligands are single-pass type I transmembrane glycoproteins, which are thought to interact at sites of cell-cell contact. Signalling is initiated when the extracellular domain of the ligand interacts with that of the receptor, resulting in a proteolytic cleavage that releases an intracellular fragment of Notch, N<sup>ICD</sup>, which translocates to the cell nucleus and regulates transcription of various target genes (Selkoe and Kopan, 2003).

From this perspective, it might seem that the only part of the ligand that really matters is the part that binds to Notch. There are, however, strong grounds for believing that the intracellular domains of Notch ligands are functionally important. The Delta subfamily has attracted most attention in this respect and is our concern in this paper. There are four main lines of evidence, to which the present study adds a fifth.

First, when the intracellular (C-terminal) domain of Delta is truncated, the protein acquires a powerful dominant-negative

effect, blocking Notch signalling in cis (Chitnis et al., 1995; Haddon et al., 1998; Henrique et al., 1997; Sun and Artavanis-Tsakonas, 1996). The cell expressing the truncated Delta protein is thereby made insensitive to Notch-activating signals from its neighbours (Henrique et al., 1997; Sakamoto et al., 2002). It is not clear how the intracellular domain of Delta influences this effect.

Second, the intracellular domain of Delta contains sites for ubiquitination, and this is crucial for Delta function (Itoh et al., 2003; Lai et al., 2001; Pavlopoulos et al., 2001; Yeh et al., 2000). The ubiquitination promotes internalization of Delta, targets Delta for degradation in proteasomes, and, most importantly, is required in order to enable Delta to activate Notch. The effect operates in trans, affecting the ability of the cell to deliver a signal to its neighbours, and it is distinct from the dominant-negative cis effect described above, which is independent of ubiquitination (Itoh et al., 2003).

Third, recent work has shown that the Notch ligands, like Notch itself, are cleaved, releasing an intracellular fragment. This may have a function in the nucleus as a transcriptional regulator (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six et al., 2003).

The fourth line of evidence for importance of the intracellular domain of Delta comes from sequence

comparisons among the vertebrates: within this group, it is highly conserved (although it shows no detectable conservation between vertebrates and insects). For example, the Delta1 (DLL1) protein of humans has an intracellular domain that is 67% identical to that of chick Delta1, 61% identical to that of *Xenopus* X-Delta1 and 56% identical to that of zebrafish DeltaD. In all vertebrates, and in at least one echinoderm (Sweet et al., 2002), we find a subset of Delta proteins that share a conserved motif – ATEV\* – at their C terminus. This motif fits the consensus for a PDZ-domain-binding protein (Nourry et al., 2003; Songyang et al., 1997). The ATEV subset of Delta proteins includes Delta1 and Delta4 in mammals and birds, Delta1 and Delta2 in *Xenopus*, and DeltaC and DeltaD in zebrafish. From their expression patterns, it seems that the ATEV Deltas have some functions in common that set them apart from other vertebrate Deltas that lack this motif. They are expressed in endothelial cells of blood vessels (Beckers et al., 1999; Mailhos et al., 2001; Shutter et al., 2000; Smithers et al., 2000), in the gut epithelium (Schroder and Gossler, 2002) (M. Skipper, G.J.W., L.A.-M. and C. Crosnier, unpublished), and in the presomitic mesoderm, where they play an essential part in the oscillator mechanism that controls somite segmentation (Davis et al., 2001; Holley et al., 2002; Hrabé de Angelis et al., 1997; Jen et al., 1997; Jiang et al., 2000).

On the other hand, the ATEV motif is not required for the core function of Delta proteins in the central nervous system (CNS) as mediators of lateral inhibition during neurogenesis: all members of the vertebrate Delta family seem to share this activity (e.g. Haddon et al., 1998), regardless of whether or not they possess a terminal ATEV. Moreover, removal of the ATEV is not sufficient to confer dominant-negative activity: in the chick retina, a version of Delta1 with a mild C-terminal truncation, removing the ATEV motif along with 96 adjacent amino acids, still showed normal function, activating Notch and delivering lateral inhibition; only with a more severe truncation, eliminating all but 13 amino acids of the intracellular domain, was a dominant-negative effect seen (Henrique et al., 1997).

What then is the function of the ATEV motif? In this paper we show that it mediates binding to MAGI proteins (MAGUK proteins with inverted domain arrangement) – a subset of the MAGUK (membrane-associated guanylate kinase homolog) protein family – through a specific one of the six PDZ domains that these contain. A similar conclusion has recently been reached independently by another group (Pfister et al., 2003), who have shown that the mouse Delta1 protein binds through its C terminus to Acvrinp-1, also known as MAGI2. Our data demonstrate that in fact all three members of the MAGI protein family can bind ATEV Delta proteins in this way. We show that MAGI1 is widely expressed in the developing zebrafish embryo, and that it co-localizes with DeltaD at a subcellular level if and only if the ATEV motif is intact. We find, furthermore, that disruption of the DeltaD-MAGI interaction leaves Delta-Notch signalling practically unaffected; but it appears to alter the migratory behaviour of some neurons in the embryonic neural tube.

## Materials and methods

### Animals

Zebrafish were maintained at 27.5°C on a 14/10 hour light/dark cycle

and embryos collected from spontaneous spawnings. Staging was according to Kimmel et al. (Kimmel et al., 1995).

### Isolation of binding partners of Delta proteins

We used a modification of the protocol of Hutchings et al. (Hutchings et al., 2003). A peptide corresponding to the C-terminal 27 residues of human Delta1 was chemically synthesized containing an N-terminal biotin and aliphatic spacer. Peptide-saturated streptavidin-coated paramagnetic beads (Dynabeads® M-280, DYNAL®) were added to an adult mouse brain lysate [0.1 g wet weight per ml of WOP-40 buffer (Wright et al., 2000)]. The brains were crudely chopped in buffer and solubilized using a dounce homogenizer at 4°C. Insoluble material was removed by centrifugation at 400 g and the supernatant filtered. The beads were recovered with a magnet after an overnight rotating incubation at 4°C, and washed; associated proteins were eluted by boiling in SDS loading buffer.

### Mass spectrometry

Samples were resolved on 4-12% Bis-Tris NuPAGE™ precast gels and stained using SYPRO Orange dye (BIO-RAD) and scanned on a STORM 860 phosphoimager (Molecular Dynamics). Gels were then Coomassie stained and bands excised with a scalpel. Gel slices were destained and digested with 100-200 ng of trypsin for 4 hours at 37°C. Peptides were extracted and analysed by MALDI on a Tof Spec 2E (Micromass).

### Morpholino and mRNA injections

Injected reagents were diluted in Danieau buffer (Nasevicius and Ekker, 2000) containing 0.2% phenol red and 2-4 nl were injected into the yolk of 1- to 4-cell stage embryos. Morpholino sequences (GeneTools) are: for Delta splicing (MO[did-V]): GGTTTTGGACTTACCTCGGTTGCAA, with mismatch control GcTTTTcGACTTAgCTCGcTTcCAA; and for MAGI1 translation blocking (MO[MAGI1]): CACAGAAACAGGTGGCTCCGCTGAC (FITC-labelled). The MAGI-GFP fusion was made in pEGFP-N1 (Clontech) with the introduction of a linker (the protein product contains the sequences VFTP-GGVPRARDPPVAT-MVSK, where the first four and last four amino acids correspond to the C terminus of MAGI1 and N-terminus of EGFP respectively). The coding regions were then subcloned into the pCS2+ vector and mRNA was made using the mMessage mMachine kit (Ambion). For immunohistochemistry, embryos were dechorionated, fixed and stained with zdd2 anti-DeltaD monoclonal antibody and Alexa594-conjugated secondary antibody (Molecular Probes) as previously described (Itoh et al., 2003). MAGI1-EGFP was detected with Alexa488-conjugated rabbit anti-GFP antibody (Molecular Probes). Stained embryos were flat-mounted on slides in Citifluor mounting medium (Citifluor) and examined with a Zeiss LSM510 confocal microscope.

For analysis of phenotypes in the living state, embryos were anaesthetized, mounted in 3% methyl cellulose, and photographed on a Leitz Diaplan microscope.

### PDZ domain binding biochemistry

Sequences (available on request) corresponding to the six individual PDZ domains of zebrafish MAGI1 were amplified and cloned in-frame with a C-terminal 6 histidine tag in pET-23b and expressed in the *E. coli* strain BL21(DE3)pLysS. Proteins were purified using Nickel-NTA Agarose (Qiagen) using minor modifications to the manufacturer's protocols and stored at -70°C.

Biotinylated peptides corresponding to the C-terminal 27 or 26 amino acids of zebrafish DeltaC and D were synthesized as above, both with, and without, the C-terminal valine residue. 200 µl of streptavidin-coated Sepharose (Amersham Biosciences) was saturated with peptides corresponding to zebrafish DeltaC or D and divided equally into six aliquots; 12 µg of purified zFMAGI1 PDZ domains were added per tube and incubated with rotation at 4°C for 2 hours,

washed and eluted by adding SDS-PAGE loading buffer and heating to 50°C for 10 minutes. Eluates were resolved by SDS-PAGE on 4-12% NuPAGE gels using MES running buffer. Gels were stained with SYPRO orange as above.

### In situ hybridization

Wholemount in situ hybridization followed the protocol of Thisse (Westerfield, 2000) or of Ariza-McNaughton and Krumlauf (Ariza-McNaughton and Krumlauf, 2002). DIG-labelled antisense probes for zebrafish MAGI corresponded to nucleotides 3018 to 3530 of the *magil* cDNA sequence. Probes for *islet1* (Inoue et al., 1994), *col2a1* (Yan et al., 1995) and *myoD* (Weinberg et al., 1996) were as previously described.

### PCR and cloning procedures

Total RNA was extracted from embryos using Trizol (GIBCO-BRL) according to the manufacturer's instructions and cDNA made according to Wright et al. (Wright et al., 2000). The primers used to detect the splicing forms of zebrafish *deltaD* were AGCTGAAG-CAGGAGGACTTG (sense) and CTTCAAGTTGAGAACCAGCT-CATT (antisense), usually for 30 PCR cycles. The altered splice forms were characterized by cloning and sequencing.

The full-length zebrafish *magil* cDNA was cloned by both 5' and 3' RACE using SMART RACE kit (Clontech) following the manufacturer's protocols using 24 hour zebrafish cDNA and oligonucleotides based on the zebrafish EST sequence number AW078333. The full-length zebrafish *magil* cDNA has been submitted to GenBank (Accession number AY465352).

### Cell culture, transfection, and immunocytochemistry

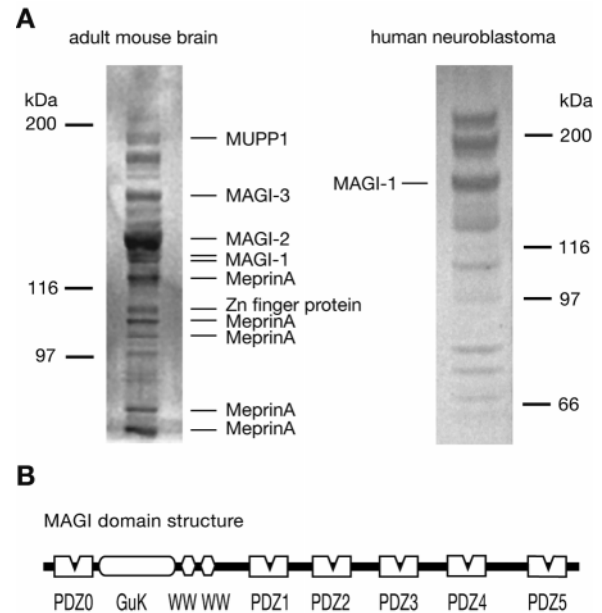
HEK293T cells grown on coverslips were transiently transfected with 1 µg of plasmid DNA per well in a 24-well plate using Superfect Transfection Reagent (Qiagen). Twenty-four hours after transfection, cells were rinsed in PBS and fixed in 4% formaldehyde in PBS for 15 minutes at room temperature. Fixed cells were permeabilized in 0.2% Triton X-100 in PBS for 15 minutes at room temperature, rinsed in PBS, and incubated in blocking solution (1% BSA in PBS) for 30 minutes at room temperature. Cells were then stained with zdd2 antibody, essentially as in Itoh et al. (Itoh et al., 2003). Coverslips were mounted on slides in Citifluor (Citifluor) and examined with a Zeiss LSM510 confocal microscope.

## Results

### The C terminus of Delta1 binds MAGI proteins

To identify proteins that might interact with the intracellular domain of Delta in vertebrates, we used a biochemical purification strategy. A synthetic 27-amino-acid peptide corresponding to the C terminus of human Delta1 was used to purify Delta-binding proteins from lysates of either adult mouse brain or the human neuroblastoma cell line NB100, as described in Materials and methods. Bound proteins were resolved by SDS-PAGE and identified by tryptic peptide mass spectrometry. The mouse brain lysate gave the largest number of clearly identifiable bands (Fig. 1A). The most prominent and most reproducible of these corresponded to MAGI2 (MAGI paralog 2); bands corresponding to the closely related proteins MAGI1 and MAGI3 were also present. Other bands in the mouse brain preparation were identified as MPDZ/MUPP1 (multi PDZ protein), meprin A, and a putative zinc-finger protein. MAGI1 was also identified in the NB100 lysate, where it gave the most prominent band.

We have chosen to focus on the interaction with the MAGI proteins. This protein family, corresponding (in mammals) to



**Fig. 1.** Biochemical purification and identification of proteins that interact with a peptide corresponding to the C terminus of an ATEV Delta. (A) Proteins purified using a C-terminal peptide of human Delta1 from lysates prepared from either an adult mouse whole brain (left panel) or the human neuroblastoma NB100 cell line (right panel) were resolved by SDS-PAGE under reducing conditions and identified by tryptic peptide mass spectrometry. Proteins that could be confidently identified have been labelled. (B) Domain structure of the MAGI family of proteins.

three distinct genes, is widely expressed; MAGI2 is especially plentiful in the adult central nervous system. This may account for its prominence in our purification assay using mouse brain. The domain structure of the MAGI proteins is shown in Fig. 1B: it includes a non-catalytic guanylate kinase homology region, two WW (protein interaction) domains, and six PDZ domains, numbered from 0 to 5, typical of scaffold proteins that hold the multiple components of signalling complexes by their C-terminal tails and keep them together at the plasma membrane. Because of their many interactions, the MAGI proteins have been given many different names (see Data S1 in supplementary material). In particular, the human MAGI1 protein is also known as BAIAP1 (BAI1-associated protein), because it binds BAI1 (brain angiogenesis inhibitor 1). BAI1, like Delta1, is a transmembrane protein with an intracellular C terminus ending in TEV. This strongly suggests that MAGI1, and perhaps the other MAGI proteins, contain a PDZ domain that specifically recognizes proteins with a terminal TEV and is thus responsible for the binding of Delta1.

Regarding the other Delta-binding proteins we identified, MUPP1, like the MAGI proteins, is a membrane-associated scaffold protein, containing no less than 13 PDZ domains; it too may recognize the C-terminal ATEV of Delta1. Meprin A is more puzzling: meprins are transmembrane or secreted proteins that normally act extracellularly as metalloproteinases. In our assay, they may have been detected because they bound to the Delta1 C-terminal peptide as a (non-physiological) substrate, or because they were attached to it indirectly via some scaffold protein such as MAGI1/2/3 or MUPP1. The zinc

**Table 1. Homology relationships between zebrafish and human MAGI proteins, as reflected in the percentage amino-acid sequence identity for PDZ domain 4**

	zf MAGI1 PDZ4 (ENSDARG00000003169)	zf MAGI2A PDZ4 (ENSDARG00000010164)	zf MAGI2B PDZ4 (ENSDARG00000018908)	zf MAGI3 PDZ4 (ENSDARG00000004394)
Hs MAGI1 PDZ4 (BAIAP1)	99	66	67	61
Hs MAGI2 PDZ4 (AIP1)	61	84	86	63
Hs MAGI3 PDZ4	56	67	63	78

These data are from BLAST alignment of human MAGI sequences with transcribed sequences from the Ensembl zebrafish database. The ENSDARG numbers are Ensembl gene ID numbers.

finger protein we detected is one for which no function is yet known.

### The MAGI protein family is highly conserved between fish and mammals

For further analysis of the interactions of Delta proteins with MAGI proteins, we have used the zebrafish. A BLAST search for counterparts of human *MAGI1*, -2 and -3 in the Ensembl zebrafish cDNA database reveals one *MAGI1* ortholog, two *MAGI2* orthologs, and one *MAGI3* ortholog. We shall call the corresponding four zebrafish genes *magi1*, *magi2a*, *magi2b*, and *magi3*. The orthology relationships can be inferred from the percent amino-acid identity when corresponding domains are compared; Table 1 shows values based on PDZ domain 4 (PDZ4). From the sequence comparisons, it seems that the ancestral *MAGI1*, -2 and -3 genes must have diverged from one another before the divergence of the fish and tetrapod lineages, while divergence of the zebrafish *magi2a* and *magi2b* genes may have occurred after this.

Having identified a part of the *magi1* coding sequence in the zebrafish EST database, we were able to clone the entire cDNA sequence. The length of the corresponding MAGI1 protein (1247 amino acids) is similar to that of human MAGI1 (1256 amino acids), and the two proteins are 71% identical in sequence overall, with the same domain structure. The degree of conservation of individual domains is remarkably high: for example, the PDZ4 domains of human and zebrafish MAGI1 are identical in 101 out of 102 amino acids. We have used *magi1* as representative of the MAGI family for further analysis of Delta-MAGI interactions.

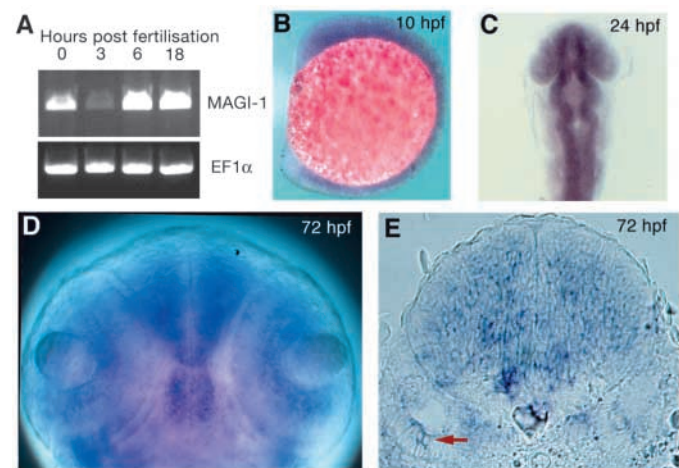
### *magi1* is widely expressed in the developing zebrafish, becoming most plentiful in the CNS

As a first step towards analysis of the Delta-MAGI interaction in vivo, we examined the expression of *magi1*, to see whether it overlaps with that of *deltaC* and *deltaD*. We used RT-PCR to assess the time course of early expression of *magi1* (Fig. 2A). The message is present already in the egg at 0 hours post-fertilization (hpf), has largely disappeared by 3 hpf, but is plentiful again by 6 hpf. This presumably reflects degradation of maternal message followed by a delay of a few hours before zygotic transcripts accumulate. Since the primary transcript is very long (>160 kb), there will presumably be an interval of 2 hours or more (assuming transcription at 1.2 kb/minute) from the initiation of zygotic transcription at 2.5-3 hpf until mature mRNA reaches the cytoplasm. Transcription of *deltaC* and *deltaD* begins to be seen by in situ hybridization at 5-6 hpf (Haddon et al., 1998; Smithers et al., 2000). Thus MAGI1 becomes available to interact with the Delta proteins at about the time of their first appearance.

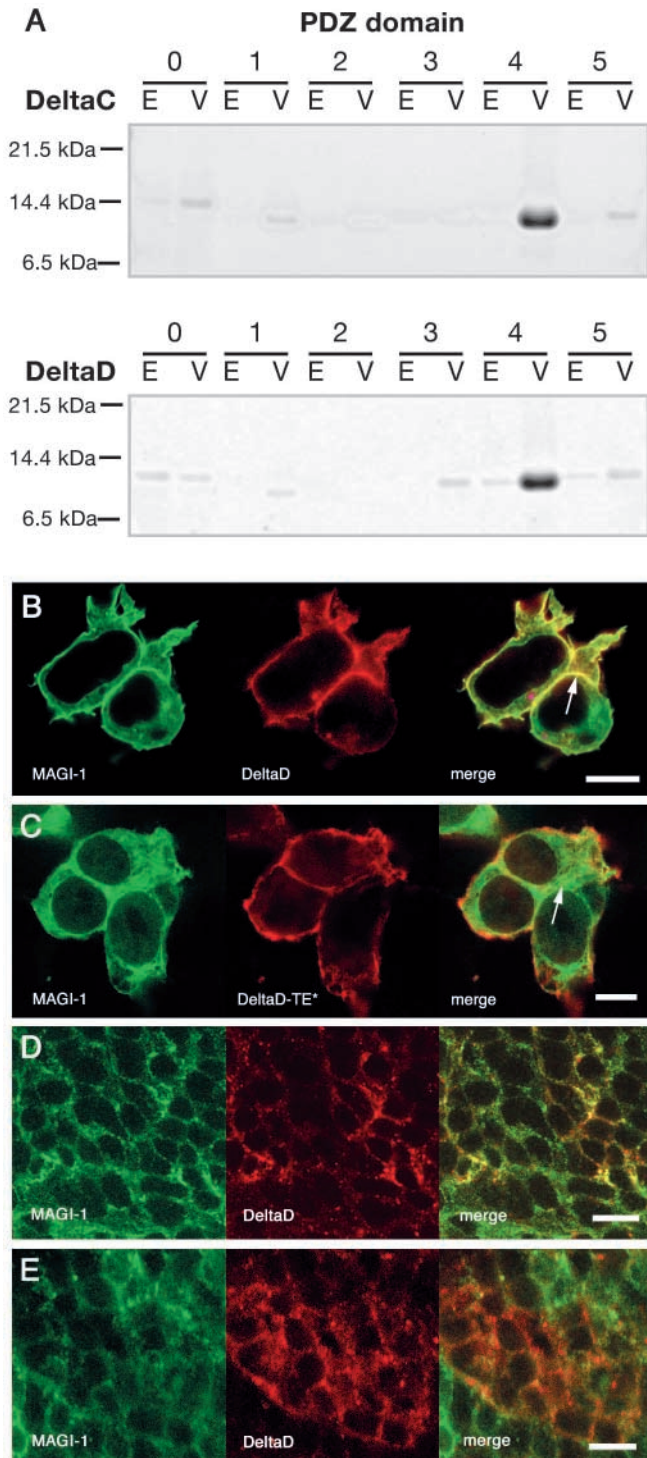
Judged by in situ hybridization (Fig. 2B), *magi1* seems to be expressed ubiquitously up to 24 hpf. By 48 hpf, expression is seen more in the CNS than elsewhere, and by 72 hpf this heightened expression in the CNS is striking (Fig. 2D,E). Expression in the CNS is, however, evident even at early embryonic stages, while the neural tube is forming and the first neurons are being born – in other words, at the time when *delta* genes are being expressed and playing their part in regulating neurogenesis. Expression is seen also in other sites where Delta genes are expressed, such as the hair cells of the inner ear (Fig. 2E). Our further experiments imply that interaction between Delta and MAGI proteins indeed occurs and is functionally important.

### The C termini of DeltaC and DeltaD bind directly and specifically to PDZ4 of MAGI1

Previous studies (see Data S1 in supplementary material) have shown that the different PDZ (and other) domains in MAGI proteins selectively bind different partners. To understand the place that DeltaC and DeltaD might have in protein complexes



**Fig. 2.** Expression pattern of *magi1* in the zebrafish embryo. (A) Early stages analysed by RT-PCR (data replicated in three separate experiments). Maternal mRNA present in the 0 hpf egg has largely disappeared by 3 hpf, with fresh (zygotically synthesized) transcripts appearing by 6 hpf. EF1 $\alpha$  was used as a positive control. (B-E) In situ hybridization patterns. (B) Lateral view at bud stage (10 hpf), showing diffuse expression. (C) Dorsal view at 24 hpf, showing expression still diffuse but strongest in the neural tube. (D) Dorsal view of the head of a whole mount at 72 hpf, showing expression in the retinae and, most strongly, in the diencephalon and telencephalon. (E) Transverse section of embryo stained as a whole mount at 72 hpf, showing expression in the hindbrain and in sensory hair cells in the ear (red arrow).



held together by MAGI1, and to test whether the Delta proteins indeed bind to MAGI1 directly, we examined the binding of artificially synthesized fragments of the proteins *in vitro*. Peptides corresponding to the C-terminal 26 or 27 amino acids of DeltaC and DeltaD were synthesized chemically. We prepared two variants of each – one with and the other, as a control, without the terminal valine. We also prepared individual His-tagged proteins corresponding to each of the six PDZ domains (~100 amino acids) of zebrafish MAGI1. The Delta peptides, as before, were prepared with an N-terminal

**Fig. 3.** Zebrafish MAGI1 binds directly and specifically to zebrafish DeltaC and DeltaD. (A) Beads were coated with peptides corresponding to the C-terminal 27 or 26 amino acids of DeltaC (top panel) or DeltaD (bottom panel) either with the terminal valine (V) or without (E). The beads were then incubated with purified proteins corresponding to each of the six individual PDZ domains of zebrafish MAGI1 (0 to 5). Beads were then washed and bound proteins eluted and resolved by SDS-PAGE under reducing conditions. Both DeltaC and DeltaD peptides that terminated in the ATEV motif interacted directly and selectively with the PDZ4 domain alone, while control peptides that lacked the terminal valine (ending –ATE) showed no binding to any PDZ domain. (B,C) HEK293T cells cotransfected with plasmids coding for MAGI1-EGFP (green) and either full-length DeltaD (red in B) or DeltaD lacking its terminal valine (DeltaD-TE\*; red in C). Both forms of DeltaD were detected with zdd2 anti-DeltaD monoclonal antibody. DeltaD, but not DeltaD-TE\*, recruits MAGI1 to the plasma membrane [compare regions indicated by arrows in (B) and (C)]. 56 out of 59 cells transfected with DeltaD showed MAGI1 membrane recruitment, but only 1 out of 37 transfected with DeltaD-TE\* did so. (D,E) Confocal sections of somite cells in 10–14 somite stage zebrafish embryos injected with 20 pg mRNA encoding for MAGI1-EGFP at the two- to four-cell stage, either (D) alone or (E) with coinjection of 5 ng MO[dld-V]. MAGI1-EGFP was detected with an anti-GFP antibody and endogenous DeltaD was detected with zdd2. Note co-localization of DeltaD and MAGI1 in (D) but not (E). Scale bars: 10  $\mu$ m.

biotin to allow coupling to streptavidin-coated beads, and the individual PDZ domains were then assayed for their ability to bind to these. As shown in Fig. 3A, the C termini of DeltaC and DeltaD both bind selectively to PDZ4, with only a faint trace of binding to other PDZ domains. No binding is seen when the Delta peptides lack their terminal valine. We conclude that PDZ4 selectively and directly binds the Delta proteins through a typical PDZ-domain interaction that depends on the terminal valines of these proteins. Since PDZ4 is conserved and distinctive in all members of the MAGI protein family, it is likely that in all of them it is responsible for binding the ATEV Deltas in the same valine-dependent way.

### DeltaD and MAGI1 interact in living cells

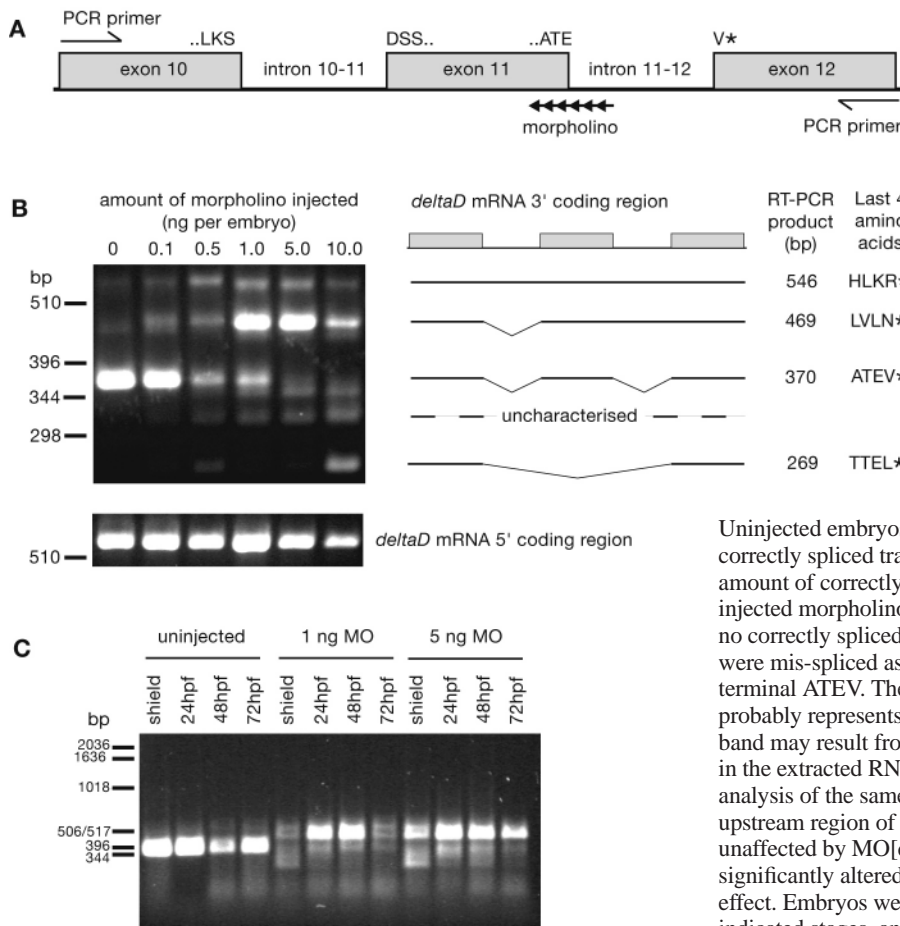
For a further test of the Delta-MAGI interaction, we performed cotransfection experiments in HEK293T cells. We constructed expression plasmids containing cDNA coding (1) for MAGI1, tagged at its C terminus with EGFP, (2) for full-length DeltaD, and (3) for DeltaD lacking the C-terminal valine residue (DeltaD-TE\*). Both forms of DeltaD are recognized by our zdd2 monoclonal antibody against DeltaD (Itoh et al., 2003). Both full-length DeltaD and DeltaD-TE\* when expressed alone were localized to the plasma membrane. In contrast, MAGI1-EGFP when expressed alone appeared cytosolic with scarcely perceptible membrane localization. However, in cells transfected with the full-length DeltaD and MAGI1-EGFP plasmids together, we observed a dramatic increase in the concentration of MAGI1-EGFP at the plasma membrane, where it was co-localized with the full-length DeltaD (Fig. 3B). This localization pattern was not observed when DeltaD-TE\* was substituted for full-length DeltaD (Fig. 3C). These data support our *in vitro* findings as to the specificity of the interaction and imply that Delta proteins can recruit MAGI proteins to the cell surface.

To see whether DeltaD and MAGI1 proteins interact in the

living embryo, we injected mRNA coding for MAGI1-EGFP into 2- to 4-cell-stage zebrafish embryos and compared the subcellular localization of the tagged MAGI1 to that of endogenous DeltaD at the 10-14 somite stage. DeltaD protein in uninjected embryos has a different intracellular distribution in different cell types: in nascent neurons, cell surface concentrations are below the threshold of detection with our zdd2 antibody, and the protein is seen only in intracellular granules (Itoh et al., 2003); in cells in the anterior parts of somites, DeltaD is detected in a spotty distribution at or near the cell surface (L.A.-M. and François Giudicelli, unpublished). This DeltaD pattern was maintained in embryos injected with the MAGI1-EGFP construct. Although, in these injected embryos, there was no detectable co-localization of MAGI1-EGFP with the intracellular DeltaD granules of the nascent neurons, the MAGI1-EGFP was frequently co-localized with the cell-surface DeltaD of the somite cells (Fig. 3D), suggesting that MAGI1 and DeltaD interact at the cell surface in the intact zebrafish embryo just as they do in cultured HEK293T cells (Fig. 3B). It should be noted that spots of DeltaD without MAGI1 and of MAGI1 without DeltaD were also seen, presumably reflecting the fact that DeltaD is only one of many MAGI1-binding partners (Fig. 1, see Data S1 in supplementary material) and that the levels of MAGI1 protein generated upon mRNA injection may be much higher than endogenous levels.

### A splice-blocking morpholino can be used to deprive DeltaD of its terminal valine in vivo

We have shown that the terminal valine of DeltaC and DeltaD is required for binding to MAGI1. Removal of this valine should therefore provide a way to test the functional importance of the Delta-MAGI interaction while causing minimal disruption of other processes. By good fortune, the terminal valine of the ATEV Delta proteins is encoded on a separate exon. We were thus able to remove it in vivo by blocking the splicing reaction that links this exon to the rest. For this, we used a morpholino, which we shall call MO[dID-V], complementary to the exon/intron junction on the 5' side of the intron preceding the exon that encodes the terminal valine (Fig. 4A). A BLAST search of the zebrafish genome database indicated that this was the only target site for the morpholino. We injected varying doses of MO[dID-V] into fertilized eggs at the one-cell stage, left them to develop as far as 6, 24, 48 or 72 hpf, and used RT-PCR to discover what splice variants of *deltaD* mRNA were present. As shown in Fig. 4B, 0.5 ng of MO[dID-V] was sufficient to give a marked reduction in the amount of the normally spliced mRNA, and with 5 ng or more, this form was undetectable, implying that no DeltaD was being produced with a terminal valine. The block was still fully effective as late as 72 hpf (Fig. 4C). The predominant mRNA instead was a mis-spliced variant with the subterminal intron retained, so as to code for a protein whose terminal valine was replaced by a sequence of 34 amino acids ending in LVLN\*.



**Fig. 4.** A morpholino, MO[dID-V], targeted to the intron-exon boundary responsible for the addition of the DeltaD terminal valine residue disrupts splicing and selectively removes the PDZ domain binding site for up to 72 hours of development. (A) The genomic structure of the zebrafish DeltaD gene surrounding the PDZ domain binding site, indicating the amino acids at the boundaries of each exon, the MO[dID-V] morpholino annealing site, and primer binding sites used to monitor the effects on splicing by RT-PCR in (B). (B) Effects on *deltaD* mRNA splicing, monitored by RT-PCR, in embryos injected with MO[dID-V] at the one-cell stage and left to develop until the shield stage (6 hpf). Total RNA was extracted from 20 embryos for each dose, reverse transcribed and amplified by PCR. PCR products were cloned and fully sequenced.

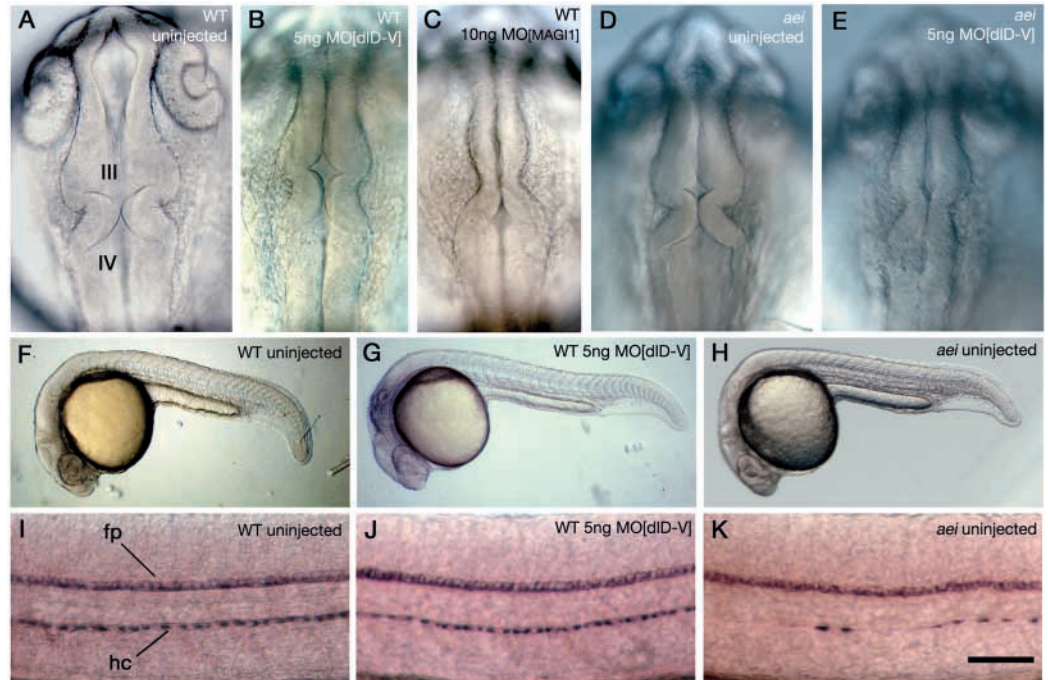
Uninjected embryos produced a 370 bp band that corresponds to the correctly spliced transcript, coding for a protein that ends -ATEV. The amount of correctly spliced product decreases as the amount of injected morpholino increases; at a dose of 5 ng or more per embryo, no correctly spliced product is observable. Products at 469 and 269 bp were mis-spliced as shown and correspond to proteins lacking the terminal ATEV. The band at ~330 bp was not characterized but probably represents the use of a cryptic splice donor site. The 546 bp band may result from small amounts of contaminating genomic DNA in the extracted RNA. The separate small panel below shows RT-PCR analysis of the same cDNA using oligonucleotides targeted to an upstream region of the *deltaD* transcript (nucleotides 389-923) that is unaffected by MO[dID-V]. The total quantity of *deltaD* mRNA is not significantly altered by the MO injections. (C) Time course of the effect. Embryos were injected and allowed to develop until the indicated stages, and RT-PCR was performed as in B.

**Fig. 5.** Effects of morpholino injections that block the interaction of DeltaD with MAGI1. (A-E) Dorsal views of the midbrain-hindbrain region in live embryos at 24 hpf.

(A) Uninjected wild-type control. (B) Wild-type embryo injected with 5 ng of MO[dID-V]; note narrowed third (III) and fourth (IV) ventricles and irregular texture due to dying cells in the walls of the hindbrain.

(C) Similar phenotype produced by injection of 10 ng of MO[MAGI1]. (D) *aei* uninjected embryo; note normal morphology. (E) *aei* embryo injected with 5 ng of MO[dID-V]; note phenotype similar to that seen in (B) and (C). (F-H) Lateral views of live embryos at 24 hpf showing somite segmentation. Somite boundaries are disorganized below the eighth somite in *aei* (H), but are unaffected by MO[dID-V] treatment (G).

(I-K) Lateral views, anterior towards the left, of embryos at 26 hpf analysed by *in situ* hybridization for *col2a1* expression to mark floor-plate (fp) and hypochord (hc). *aei* embryos exhibit a reduction in hypochord cell number. This effect is not observed upon MO[dID-V] treatment (J). Scale bar: 50  $\mu$ m for I,J,K.



We examined the effect of MO[dID-V] on the DeltaD-MAGI1 interaction by artificially expressing MAGI1-EGFP alone or together with the morpholino in sibling embryos. In the absence of MO[dID-V], we observed co-localization of DeltaD and MAGI1-EGFP in somite cells, as described above (Fig. 3D). Upon addition of MO[dID-V] to the mRNA injection mixture, however, this co-localization was abolished while the distribution of DeltaD appeared to be unaffected (compare Fig. 3D,E). Although it is difficult to put these observations on a firm quantitative footing, the effect seemed clear. We conclude (1) that treatment with MO[dID-V] is effective in generating a mis-spliced variant of DeltaD which, although expressed at normal levels, is incapable of binding MAGI proteins; and (2) that the co-localization of MAGI1 with DeltaD seen *in vivo* is indeed dependent on direct interaction between the two proteins.

### Specific and non-specific effects of the DeltaD splice-blocking morpholino can be distinguished by analysis of *aei/deltaD* mutants

To test the functional significance of the DeltaD-MAGI1 interaction, we examined the phenotype of the embryos that developed from eggs injected with 5 ng of MO[dID-V], so that the only forms of DeltaD produced would be lacking their terminal valine.

At a gross anatomical level, the injected embryos showed clear and reproducible abnormalities in the hindbrain and midbrain, such that at 24 hpf, the width of the roofplate and the lumen of the third and fourth ventricles of the neural tube were markedly reduced (Fig. 5A,B, Table 2). A similar narrowed-ventricle effect was seen when we injected a morpholino (MO[MAGI1]) targeted against the translational start of *MAGI1* (Fig. 5C), whereas injection of other

**Table 2.** Effect of morpholino injections on midbrain and hindbrain structure, scored at 24 hpf

Embryo genotype	Injected reagent:	Phenotype (%):			Embryos scored <i>n</i>
		Severe	Mild	WT	
Wild type	uninjected	1	3	96	99
Wild type	5 ng MO[dID-V]	52	30	18	98
Wild type	5 ng MO[MAGI1]	17	38	45	53
Wild type	10 ng MO[MAGI1]	53	39	8	49
Wild type	5 ng mis-match MO	3	6	91	35
<i>aei</i> <sup>AR33</sup>	uninjected	6	14	81	101
<i>aei</i> <sup>AR33</sup>	5 ng MO[dID-V]	33	51	16	67

WT, brain ventricles very similar to uninjected.

Mild, brain ventricles have formed but are misshapen and reduced in size.

Severe, ventricles absent or severely reduced in size and severely misshapen.

morpholinos, including a five base-pair mismatch control for MO[dID-V], did not produce this phenotype (data not shown, Table 2). These findings strongly suggested that the anatomical abnormality was a specific effect of disrupting the DeltaD-MAGI1 interaction. Similar structural abnormalities have, however, been seen as an effect of morpholino mistargeting (Ekker and Larson, 2001), and they are not easily explainable in terms of known functions of DeltaD or MAGI1. Spurred on by the doubts of a referee, we therefore performed a further, and more decisive, control experiment.

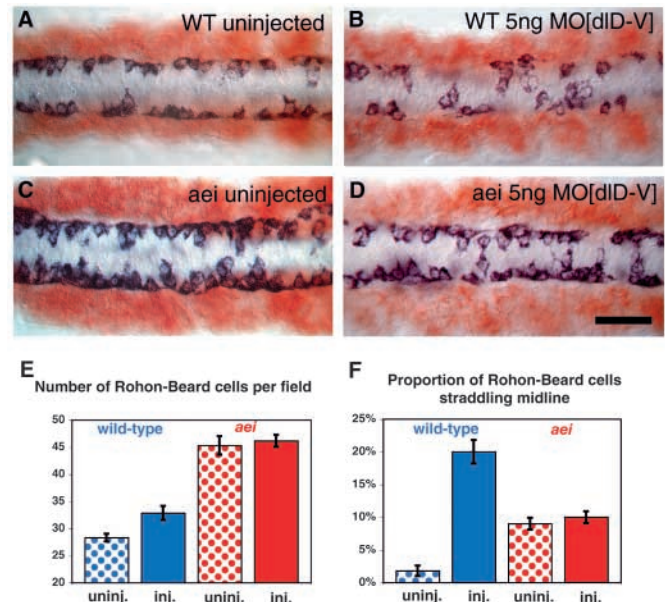
For this, we took advantage of the *aei*<sup>AR33</sup> mutant, which has a stop codon in the fifth EGF repeat of the extracellular domain of *deltaD*, leading to a DeltaD loss-of-function phenotype (Holley et al., 2000). Since the C terminus of DeltaD is already missing in *aei*<sup>AR33</sup> homozygotes, we might perhaps expect that they would show the narrowed-ventricle phenotype even without any morpholino injection; and in any case their phenotype should certainly show no further change when MO[dID-V] is injected, if the only effect of this morpholino is to block the DeltaD-MAGI1 interaction. As it turned out, our expectations were confounded on both scores. The uninjected *aei*<sup>AR33</sup> homozygotes did not show a narrowed-ventricle phenotype, but when they were injected with MO[dID-V] they did show it (Fig. 5D,E, Table 2). We can only conclude that the narrowed-ventricle phenotype resulting from MO[dID-V] is due to a non-specific toxic effect of the morpholino.

### Disruption of the DeltaD-MAGI interaction does not significantly affect the known functions of DeltaD as a Notch ligand

The *aei*<sup>AR33</sup> mutant is useful not only as a negative but also as a positive control, displaying defects that result from loss of DeltaD: somite segmentation is disrupted (Holley et al., 2002; Jiang et al., 2000; van Eeden et al., 1996); primary neurons are produced in excessive numbers in the embryonic CNS (Holley et al., 2000); and the numbers of hypochord (ventral midline) cells are reduced (Latimer et al., 2002). These abnormalities have been well documented and reflect the functions of DeltaD as a Notch ligand. To see whether the DeltaD-MAGI1 interaction is important for these functions, we compared wild-type and *aei*<sup>AR33</sup> mutant embryos with genetically normal embryos that were injected with MO[dID-V].

First of all, somite patterning appeared normal in the MO[dID-V] injected embryos, with no sign of the disruption of somite segmentation that is seen in *aei* and in other Notch pathway loss-of-function mutants (Fig. 5F-H) (Holley et al., 2002; Jiang et al., 2000; van Eeden et al., 1996).

Our findings with regard to hypochord formation were similar. Defects in hypochord formation are readily observed in *aei/deltaD* mutants as well as *deltaA* loss-of-function mutants and morpholino-induced DeltaC knock-down embryos (Appel et al., 1999; Latimer et al., 2002); the phenotype can be seen by *in situ* hybridization with a probe for *alpha-1 collagen type II (col2a1)* which labels the floor plate and hypochord (Yan et al., 1995). Using this method, we saw a reduction in hypochord cell number in *aei*<sup>AR33</sup> embryos, as expected. However, we detected only a slight increase in hypochord defects in wild-type embryos treated with MO[dID-V] (Fig. 5I-K). Moreover, we observed no effect of MO[dID-V] treatment on expression of *her4* or *ntl* (data not shown); these genes are both regulated – *her4* positively and *ntl*



**Fig. 6.** Disruption of the DeltaD-MAGI1 interaction causes mislocalization of Rohon-Beard neurons. (A-D) Dorsal views of embryos at 16 hpf stained by *in situ* hybridization for *islet1* (black) to mark Rohon-Beard neurons (as well as other primary neurons below the plane of focus). *myoD* (brown) expression serves as a reference for somite number and position. *aei* embryos (C,D) show a 1.6-fold increase in the number of Rohon-Beard cells compared to wild-type embryos (A), whereas the number of these cells is only slightly increased in wild-type embryos injected with MO[dID-V] (B). The MO[dID-V] embryos are abnormal, however, in that many of the Rohon-Beard cells stray into the midline region. The proportion of such mislocalized cells is not affected by the morpholino in *aei* embryos, where DeltaD is missing (C,D). (E,F) Cell counts. The distribution of the neurons was quantified for a region corresponding approximately to somites 5 to 10; 10-13 embryos were analysed for each condition. Error bars represent s.e.m. Scale bar: 50  $\mu$ m for A-D.

negatively – by Delta-Notch signalling during normal hypochord development (Latimer et al., 2002). These findings indicate that the DeltaD-MAGI1 interaction is not important for the function of DeltaD as a Notch ligand in specification of the hypochord.

Lastly, Delta-Notch signalling, mediating lateral inhibition, is well known to regulate the proportion of cells committed to differentiate as neurons (Appel and Eisen, 1998; Chitnis et al., 1995; Haddon et al., 1998), and this is reflected in the phenotype of *aei/deltaD* mutants, which produce neurons in excess (Holley et al., 2000). If interaction with MAGI1 is important for DeltaD's function in lateral inhibition, we would expect to see alterations in neurogenesis when the interaction is blocked. We therefore counted neurons, at the 12-16 somite stage, in genetically wild-type embryos injected with MO[dID-V] and in uninjected controls, using *in situ* hybridization for *islet1* as a neuronal marker (Haddon et al., 1998; Korzh et al., 1993). We found that the MO[dID-V] injection produced little or no change in the number of *islet1*-positive cells. In contrast, *aei*<sup>AR33</sup> mutants showed a 1.6-fold increase (Fig. 6A-E). Specifically, in the dorsal neural tube, in the region corresponding to the middle five somites, we counted  $28.3 \pm 0.6$  (mean  $\pm$  s.e.m.,  $n=10$ ) *islet1*-positive neurons per wild-type



embryo,  $32.8 \pm 1.3$  ( $n=13$ ) per MO[dID-V]-injected wild-type embryo,  $45.3 \pm 1.7$  ( $n=10$ ) per *aei*<sup>AR33</sup> embryo, and  $46.2 \pm 1.1$  ( $n=13$ ) per *aei*<sup>AR33</sup> embryo injected with MO[dID-V]. We conclude that the DeltaD-MAGI interaction has very little influence on the function of DeltaD in the regulation of neuronal commitment.

### When the DeltaD-MAGI interaction is disrupted, Rohon-Beard neurons are mislocalized

In the course of the above experiment, we noticed that Rohon-Beard neurons (primary sensory neurons in the dorsal part of the fish neural tube), although normal in numbers, were consistently mislocalized in the MO[dID-V]-injected embryos. In wild-type and *aei*<sup>AR33</sup> mutant embryos at the 12-16 somite stage, the cell bodies of these *islet1*-positive neurons are located in two paraxial strips lying 3-4 cell diameters apart. In MO[dID-V]-treated wild-type embryos, by contrast, the corresponding cells were often located more medially, between these strips (Fig. 6A-C,F). This effect was not observed when *aei*<sup>AR33</sup> mutants were injected with the morpholino, demonstrating that it is indeed a DeltaD-dependent effect and not a nonspecific artefact (Fig. 6D,F). For a quantitative estimate of the abnormality, we counted the proportion of the dorsal *islet1*-positive cells that spanned the midline. These amounted to  $1.8 \pm 0.9\%$  (mean  $\pm$  s.e.m.,  $n=10$ ) of the population in wild-type embryos,  $20.0 \pm 1.8\%$  ( $n=13$ ) in MO[dID-V]-injected wild-type embryos,  $9.0 \pm 0.9\%$  ( $n=10$ ) in *aei*<sup>AR33</sup> mutants, and  $10.0 \pm 0.9\%$  ( $n=13$ ) in MO[dID-V]-injected *aei*<sup>AR33</sup> mutants (Fig. 6A-D,F). This suggests that the DeltaD-MAGI interaction could play some part in regulating migration or dispersal of the Rohon-Beard cells – and conceivably of other neurons – within the neural tube. Since relatively little mislocalization is seen in *aei*<sup>AR33</sup> mutants, which lack functional DeltaD, an implication would be that the suggested aberrant migratory behaviour depends on DeltaD and is normally restrained by the interaction of DeltaD with MAGI proteins (see Discussion). The slight degree of mislocalization seen in *aei*<sup>AR33</sup> mutants may reflect production of slightly ectopic neurons as a result of loss of lateral inhibition.

## Discussion

All vertebrates, apparently, and some invertebrates such as echinoderms, possess at least one Delta family member with a conserved ATEV motif at its intracellular terminus. We have found by in vitro studies that this terminus of the ATEV Deltas binds selectively to members of the MAGI family of PDZ-containing scaffold proteins. We have shown, moreover, that the binding is direct, is to all three MAGIs, and is specifically to their PDZ4 domain. The interaction is demonstrable in cultured cells, where it enables Delta protein to recruit MAGI protein to the plasma membrane. Using the zebrafish as a model system, we have observed that *magil* is widely expressed and is present in particular in many, if not all, of the cells that express the ATEV Deltas. Additionally, in at least some cell types in the intact embryo, we have seen co-localization of DeltaD and MAGI1 at the plasma membrane and given evidence that the co-localization depends on the ATEV motif. All this amounts to a strong argument that the Delta-MAGI interaction is a real physiological phenomenon and is in some way functionally important.

### Morpholino experiments require stringent controls

To discover what the function of the interaction might be, we exploited a morpholino, MO[dID-V], which, when injected into the early zebrafish embryo, specifically blocks the interaction by interfering with the splicing of the *deltaD* message so as to alter the C terminus of the DeltaD protein. In any morpholino experiment, however, there is a possibility that non-specific side-effects may also be produced. In our system, we were indeed initially misled. We saw a marked narrowing of the third and fourth ventricles of the neural tube in embryos injected with MO[dID-V], performed several controls of the sort that are conventionally done to check specificity of morpholino action, and concluded that the ventricle abnormality was a specific effect of disruption of the DeltaD-MAGI interaction. Fortunately, a *deltaD* loss-of-function mutant, *aei*, was available and allowed us to perform a more stringent control experiment. This showed convincingly that the ventricle abnormality was after all a non-specific side-effect of the morpholino. We offer this cautionary tale as a footnote to our other findings: it may serve as a warning of the risks of misinterpreting morpholino experiments, where often there is no mutant available to provide a stringent test for non-specific side-effects.

### Delta-Notch signalling appears to be independent of the Delta-MAGI interaction

DeltaD has several well-characterized roles in Notch signaling, clearly revealed in the *deltaD* loss-of-function mutant *aei*, where disorders are seen in somitogenesis, hypochord formation, and neurogenesis (Holley et al., 2000; Jiang et al., 2000; Latimer et al., 2002; van Eeden et al., 1996). Upon disruption of the DeltaD-MAGI interaction, however, these processes are largely unaffected. The fact that this treatment does not phenocopy *aei* indicates that the interaction of DeltaD with MAGI proteins is not important for the function of DeltaD as an activating ligand for Notch, at least in these processes. This finding is perhaps not surprising given that, as explained in the Introduction, several forms of Delta protein lacking the MAGI-binding motif are already known to be effective Notch ligands.

### The Delta-MAGI interaction may be important in the control of neuron migration

In assaying neurogenesis in embryos injected with MO[dID-V] to block DeltaD-MAGI interaction, we observed a mislocalization of Rohon-Beard sensory neurons, which frequently strayed into the dorsal midline of the neural tube – a phenomenon rarely seen in wild-type or *aei* embryos. This effect needs further investigation, but it suggests that the DeltaD-MAGI interaction is important in some way either in determining the site of production of the neurons, or in governing their migratory behaviour as they move away from their birthplace.

We are attracted by the latter possibility, since other studies have reported effects of Delta protein on cell motility: in particular, De Jossineau et al. (De Jossineau et al., 2003) found that Delta in *Drosophila* sense-organ precursor cells promoted extension of filopodia, while Lowell and Watt (Lowell and Watt, 2001) found that mammalian keratinocytes showed enhanced motility when they expressed a truncated form of Delta1 lacking most of the intracellular domain

(including the ATEV motif), but showed reduced motility when they expressed full-length Delta1. This latter pair of observations is consistent with what we saw in the zebrafish embryo: the mis-localization of the Rohon-Beard neurons suggests that they became abnormally motile when their DeltaD protein was deprived of the C-terminal motif that mediates binding to MAGI proteins. If this interpretation is correct, the implication would be that free Delta protein favours motility, and that this action of Delta is inhibited by the binding of Delta to MAGI.

The above account supposes that Delta and MAGI influence motility directly (cell-autonomously) in the cells that express them. An alternative possibility is that these proteins influence the ability of cells to serve as a substratum for the movement of other cells: it could be that cells expressing DeltaD that is not bound to MAGI encourage the Rohon-Beard neurons to move over them, while cells expressing DeltaD that binds to MAGI protein do not. This suggestion has an echo in the *magie oko* (*nok*) zebrafish, where there is a mutation in a MAGUK scaffolding protein – that is, a protein related to the MAGI family. In this mutant, the polarity of the retinal epithelium is disrupted and the migrations of neurons within it are disordered, apparently in consequence of the neuroepithelial polarity defect (Wei and Malicki, 2002). Gray et al. (Gray et al., 2001) have also reported similar phenomena: they find that mutation of *des/notch1a* alters the migration of neural crest cells and has a non-cell-autonomous effect on axon outgrowth.

Whichever of these interpretations is correct, there are many possibilities for the detailed molecular mechanism. Delta and Delta-MAGI complexes could have direct effects on the adhesive or locomotor properties of the cell surface or actin cortex (Lowell and Watt, 2001); they could influence motility by binding (or failing to bind) to Notch on neighbouring cells (Franklin et al., 1999); or they could regulate gene expression cell-autonomously to exert their effects. This last possibility is suggested by recent studies (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six et al., 2003) showing that, like Notch, Delta itself can be cleaved to release an intracellular fragment that can enter the nucleus and act as a gene-regulatory protein. MAGI proteins could influence such a reverse signalling activity of ATEV Deltas by regulating their cleavage or their translocation to the nucleus.

## Conclusion

As Notch ligands, the Delta proteins play a central part in the development and maintenance of a great variety of tissues in the vertebrate body. We have established that a conserved subset of the Delta family – the ATEV Deltas – interact through their intracellular tails with the MAGI family of scaffolding proteins, not only in vitro but also in the living embryo. The interaction does not appear to be critical for Delta-Notch signalling, and yet its evolutionary conservation implies that it must have an important physiological function. The challenge is to discover what that function is. The MAGI proteins, with their multiple PDZ and other protein-recognition domains, have an extraordinary variety of binding partners, as listed in Data S1 (see supplementary material), and could mediate cross-talk between the Delta-Notch pathway and many other pieces of intracellular or extracellular machinery. MAGI may exert important effects on cell behaviour by influencing the

location of Delta, or Delta may do so by influencing the location of MAGI. In this paper we have excluded some possible roles of the Delta-MAGI interaction and found preliminary evidence for an effect on cell positioning; further experiments will be needed to explore its functional significance fully.

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## Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/131/22/5659/DC1>

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