Development 138, 2947-2956 (2011) doi:10.1242/dev.066654
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DeltaC and DeltaD interact as Notch ligands in the zebrafish segmentation clock

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SUMMARY

We describe the production and characterisation of two monoclonal antibodies, zdc2 and zdd2, directed against the zebrafish Notch ligands DeltaC and DeltaD, respectively. We use our antibodies to show that these Delta proteins can bind to one another homo- and heterophilically, and to study the localisation of DeltaC and DeltaD in the zebrafish nervous system and presomitic mesoderm (PSM). Our findings in the nervous system largely confirm expectations from previous studies, but in the PSM we see an unexpected pattern in which the localisation of DeltaD varies according to the level of expression of DeltaC: in the anterior PSM, where DeltaC is plentiful, the two proteins are colocalised in intracellular puncta, but in the posterior PSM, where DeltaC is at a lower level, DeltaD is seen mainly on the cell surface. Forced overexpression of DeltaC reduces the amount of DeltaD on the cell surface in the posterior PSM; conversely, loss-of-function mutation of DeltaC increases the amount of DeltaD on the cell surface in the anterior PSM. These findings suggest an explanation for a long-standing puzzle regarding the functions of the two Delta proteins in the somite segmentation clock – an explanation that is based on the proposition that they associate heterophilically to activate Notch.

KEY WORDS: Notch signalling, DeltaC, DeltaD, Segmentation clock, Zebrafish

INTRODUCTION

Notch signalling mediates communication between adjacent cells through the binding of two related cell-surface proteins: the receptor, Notch, in the membrane of the signal-receiving cell, and a ligand belonging to the DSL (Delta/Serrate/Lag) family in the membrane of the signal-delivering cell. Signals are relayed across the membrane of the receiving cell by proteolytic cleavage of the receptor (Chandu et al., 2006), resulting in release of the intracellular fragment of the receptor, termed Notch^{ICD} or NICD, which translocates to the nucleus and regulates the expression of target genes (Ilagan and Kopan, 2007).

Activation of the Notch pathway depends on specialised biochemical processing not only of Notch itself, but also of its ligands (for reviews, see D'Souza et al., 2008; Le Borgne et al., 2005a). To be able to trigger the cleavage events that release NICD, the ligands have to be ubiquitylated on their intracellular tails, a process that depends on the E3 ubiquitin ligases Neuralized (most important in *Drosophila*) and Mind bomb (most important in vertebrates) (Glittenberg et al., 2006; Haddon et al., 1998a; Itoh et al., 2003; Jiang et al., 1996; Koo et al., 2005a; Koo et al., 2005b; Lai et al., 2001; Lai et al., 2005; Le Borgne et al., 2005b; Pavlopoulos et al., 2001; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). In zebrafish, which have five different Delta proteins, at least four (DeltaA, B, C and D) are substrates of Mind bomb (Mib) and/or its close relative Mib2 (Chen and Casey Corliss, 2004; Itoh et al., 2003; Zhang et al., 2007a; Zhang et al., 2007b).

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Accepted 3 May 2011

When the critical E3 ligase is missing, Notch ligands not only fail to activate Notch, but also accumulate on the cell surface instead of undergoing rapid internalisation. This suggests that Notch activation somehow requires that the Notch ligand should engage with the machinery of endocytosis, and that ligand internalisation is a necessary accompaniment of receptor activation. Indeed, phenotypes resembling those due to loss of Notch function are seen when endocytosis fails [as in the *Drosophila shibire* mutant, which is defective in dynamin (Chen et al., 1991; van der Bliek and Meyerowitz, 1991)]. Notch ligands with a truncated intracellular domain, which presumably lack the sites required for normal ubiquitylation and endocytosis, fail to activate Notch (Chitnis et al., 1995; Glittenberg et al., 2006; Henrique et al., 1997). The picture is further complicated by interactions between Notch and Delta in cis (in the same cell, where the two proteins exert mutual inhibition) as well as in trans (in separate but adjoining cells, mediating cell-cell communication); these interactions can have strong effects on the levels of cell-surface Notch and Delta (del Alamo et al., 2011; Glittenberg et al., 2006; Matsuda and Chitnis, 2009; Sakamoto et al., 2002; Sprinzak et al., 2010). In general, however, a Notch ligand that is seen to accumulate on the cell surface instead of being internalised is unlikely to be activating Notch.

Two members of the zebrafish Delta family, DeltaC and DeltaD, are of special interest to us. We have generated monoclonal antibodies against each of these two proteins. In three previous papers, we have briefly introduced these antibodies and used them for a variety of purposes (Itoh et al., 2003; Crosnier et al., 2005; Giudicelli et al., 2007); using the same antibodies, Matsuda and Chitnis (Matsuda and Chitnis, 2009) have recently shown that, in neural tissue, the internalisation of DeltaA and DeltaD (but not DeltaC) depends not only on their interaction with Mib, but also on their interaction with Notch (specifically Notch1a and Notch3) both in cis and in trans.

Here, we characterise our DeltaC and DeltaD antibodies in greater detail and use them for further investigation of the biochemistry of Delta proteins and of their role in the presomitic mesoderm (PSM). We are especially interested in their functions in the segmentation clock – the gene expression oscillator in the PSM that controls segmentation of the body axis. *deltaC* shows oscillating expression and is thought to thereby provide a cyclic time signal that keeps adjacent cells oscillating in synchrony (Giudicelli et al., 2007; Herrgen et al., 2010; Horikawa et al., 2006; Jiang et al., 2000; Lewis, 2003; Mara et al., 2007; Morelli et al., 2009; Oates et al., 2005; Riedel-Kruse et al., 2007). In *deltaC* mutants, synchrony is lost and segmentation is disrupted. *deltaD* mutants show a broadly similar segmentation phenotype (Jiang et al., 2000; van Eeden et al., 1996; van Eeden et al., 1998); yet *deltaD* appears to be expressed at a steady rate in the critical region of the PSM (Holley et al., 2000; Julich et al., 2005; Mara et al., 2007), arguing against any role for DeltaD as a cyclic time signal.

Using our monoclonal antibodies, we now find that whereas DeltaC is rapidly internalised and degraded, so that it is practically undetectable on the cell surface, cell-surface levels of DeltaD in the posterior PSM are persistently high. By manipulating the expression of DeltaC, we can shift the subcellular localisation of DeltaD in the PSM: overexpression of DeltaC decreases DeltaD on the cell surface, whereas loss-of-function mutation of DeltaC has the opposite effect. We show, moreover, that DeltaC and DeltaD can bind to one another directly. Our observations indicate that interaction between the two proteins is crucial for their function in the PSM, with each contributing to the activation of Notch in a different way. We suggest that DeltaC acts as an oscillating signal, whereas DeltaD serves as its non-oscillatory permissive partner, ineffective as a Notch ligand by itself but facilitating the cyclic action of DeltaC, perhaps by dimerising with it.

MATERIALS AND METHODS

Animals

Embryos were staged according to Kimmel et al. (Kimmel et al., 1995). Homozygous $mib^{ta52b/ta52b}$ mutants were identified by visual inspection of the progeny from genotyped heterozygous carriers. *deltaC* mutant (*bea*) embryos were homozygotes from crosses of homozygous $dlc^{tw212b/tw212b}$ mutant parents, with a cysteine-to-serine missense mutation in the seventh EGF repeat (Julich et al., 2005). *deltaD* mutant (*aei*) embryos were homozygotes from crosses of homozygous $dld^{tr233/tr233}$ mutant parents, with a tyrosine-to-Stop mutation in the fifth EGF repeat (Holley et al., 2000).

A detailed account of the *Tg(UAS:dlc)cj2* transgenic line will be given elsewhere. Briefly, embryos were injected with a linearised plasmid containing five copies of the yeast upstream activating sequence (UAS) followed by a sequence encoding zebrafish DeltaC (with the seventh intron retained but all other introns spliced out), followed by the full-length *deltaC* 3′ UTR and an SV40 polyadenylation signal sequence. ISce-I sites flanked this gene construct, and the plasmid was co-injected with the ISce-I meganuclease to facilitate integration. Transgenic fish were identified by PCR. We crossed these with *Tg(hsp70l:Gal4vp16)vu22* transgenics (Shin et al., 2007) to give progeny in which heat shock induced prolonged DeltaC expression.

Fish welfare was in accordance with a UK Home Office Project License held by J.L.

Construction, expression and purification of recombinant proteins

DNA sequences corresponding to the entire extracellular regions (including native signal peptide) of zebrafish DeltaA, B, C and D were fused in-frame to sequences corresponding to a C-terminal tag consisting of rat Cd4 domains 3 and 4 (Cd4d3+4) and a 19 amino acid peptide that can be enzymatically monobiotinylated using the *E. coli* BirA protein biotin ligase (Fig. 1A) (Brown et al., 1998; Bushell et al., 2008). The resulting soluble recombinant proteins contained the following sequences: DeltaA IASDVP<u>ST</u>, DeltaB GQTSPS<u>ST</u>, DeltaC NSPALP<u>ST</u> and DeltaD DDGGFP<u>ST</u> (the start of the Cd4 tag is underlined). Proteins were produced by transient transfection of HEK293T cells. For biochemical studies, the proteins were enzymatically biotinylated (Brown et al., 1998). For injection into mice, the soluble recombinant DeltaC and DeltaD proteins were purified from a bulk transfection by immunoaffinity chromatography using their Cd4 tag (Wright et al., 2000).

Generation and purification of monoclonal antibodies to zebrafish DeltaC and DeltaD

Ten-week-old female BALB/c mice were immunised subcutaneously with 20 μg purified DlCCd4d3+4 or DlDCd4d3+4 in complete Freund's adjuvant (once) and incomplete Freund's adjuvant (three times) (Wright et al., 2001). Hybridomas were generated using standard procedures by fusion with the SP2/0-Ag14 myeloma. After selection according to our criteria (see below), hybridomas were cloned once and named.

Assays for antibody specificity and formalin fixation sensitivity

Hybridoma supernatants containing antibody were identified by ELISA using recombinant zebrafish DeltaA, B, C and D proteins as bait. Hybridomas that scored positive were further screened by flow cytometry for the ability of the corresponding antibodies to recognise a formalin fixation-resistant DeltaC or DeltaD epitope at the surface of transfected human cells (DeltaD, see Fig. 1B; DeltaC, data not shown). For this, HEK293T cells that had been transfected with full-length DeltaC or DeltaD were fixed in 4% formalin at room temperature for varying lengths of time. Aliquots of the fixed cells were then incubated with the monoclonal antibodies for 1 hour at 4°C, washed, stained with rabbit anti-mouse FITC conjugate (1:200, DAKO) and analysed by flow cytometry on a FACSCaliber (Beckton Dickinson).

Cross-reactivity with other zebrafish Delta proteins (see Fig. 1C) was tested by ELISA. Hybridomas secreting monoclonal antibodies meeting all these criteria were cloned and named zdc2 (specific for DeltaC) and zdd2 (specific for DeltaD). Two other monoclonal antibodies, zdc1 and zdd1, likewise specific for DeltaC and DeltaD, respectively, were also obtained, but the epitopes they recognise do not survive formalin fixation. Immunoglobulin heavy chains were isotyped as: zdd1 IgG_1, zdd2 IgG_1, zdc1 IgG_{2a}, zdc2 IgG_{2a}.

Assay for homophilic binding

Enzymatically monobiotinylated Delta ectodomains were produced and normalised as described (Bushell et al., 2008), further diluted in Hank's Balanced Saline (HBS) or HBS containing EDTA, heat-treated as appropriate and captured on streptavidin-coated microtitre plates (Nunc). After washing three times in HBS, 50 μl of 1:5000 avidin-alkaline phosphatase (Sigma) was added in HBS containing 0.2% BSA for 1 hour at room temperature. The wells were again washed three times, Sigma 104 colorimetric alkaline phosphatase substrate was added, and absorbance was read at 405 nm.

Immunoprecipitation

HEK293 cells were co-transfected with plasmids encoding full-length DeltaD and DeltaC or an unrelated GFP-tagged control plasma membrane protein (a chimaeric fusion protein containing the extracellular regions of rat Cd200, the transmembrane region of rat Cd200R and a cytoplasmic eGFP) using TransIT-LT1 reagent (Mirus Bio). Forty-eight hours after transfection, cells were lysed in 50 mM Hepes (pH 7.4), 175 mM NaCl, 0.5% Triton X-100, 0.03 μg/μl DNase and RNase. For immunoprecipitation, lysates were incubated with protein G Sepharose beads (Sigma) and either zdd2 or anti-myc 9E10 monoclonal antibody (negative control; in-house CR UK LRI Antibody Service). Western blots were performed under non-reducing conditions using rabbit anti-GFP (Molecular Probes), biotinylated anti-DeltaC (a mixture of zdc1 and zdc2), zdd2, streptavidin-HRP (Perkin Elmer) and the ECL Plus western blotting detection system (GE Healthcare). Antibodies were biotinylated using NHS-LC-LC-biotin (Pierce).

DEVELOPMENT

Immunohistochemistry, in situ hybridisation (ISH) and imaging

For cryosectioning, zebrafish embryos were fixed overnight at 4°C in 4% formalin, washed and then embedded in 1.8% LB agar (Gibco) plus 5% sucrose in PBS before equalisation in a 30% sucrose solution. Sections were cut at 12-15 μm and slides stored at –20°C until use. Purified anti-Delta monoclonal antibodies were diluted in 10% goat serum, 2% BSA, 0.1% (v/v) Triton X-100, 10 mM NaN_3 in PBS to a final concentration of 10 μg/ml and sections were stained for 2 hours at room temperature or overnight at 4°C. Monoclonal antibodies were detected, after washing, using goat anti-mouse Alexa Fluor 594 (1:500, Molecular Probes) with Alexa Fluor 488 phalloidin counterstain (1:50, Molecular Probes) for 2 hours at room temperature. For detecting DeltaC and DeltaD simultaneously, isotype-specific secondary antibodies were used (goat anti-mouse IgG_1 Alexa Fluor 594 and goat anti-mouse IgG_{2a} Alexa Fluor 488; Molecular Probes).

For wholemount immunostaining, embryos were fixed in 4% formalin overnight at 4°C or for 30 minutes at room temperature, rinsed, dechorionated (and in some cases de-yolked) in PBS containing 0.1% Tween 20, incubated with primary antibodies [zdc2, 1:50; zdd2, 1:100; β-catenin antibody (Sigma, C2206), 1:500] for 2-3 hours at room temperature (or overnight at 4°C) in PBS containing 0.5% Triton X-100, 2% BSA, 1% DMSO, 10% goat serum or in PBS containing 0.5% Triton X-100 and 0.2% gelatin, rinsed for 2 hours in PBS containing 0.1% Triton X-100, and incubated for 2 hours at room temperature with secondary antibodies (1:500) diluted similarly. Secondary antibodies for zdc2 and zdd2 detection were as above; for β-catenin we used anti-rabbit IgG Alexa Fluor 633 or 488 (Molecular Probes). For experiments to distinguish extracellular from intracellular staining, detergent was omitted from the initial fixing and staining solutions but included in the second round of staining. Specimens were flat-mounted in SlowFade (Molecular Probes) with TOPRO-3 or DAPI as nuclear counterstain.

ISH followed standard protocols, using NBT/BCIP detection (Roche), with probes for *deltaC* and *deltaD* mRNA as described (Giudicelli et al., 2007).

Fluorescently stained specimens were viewed on an LSM510 or LSM710 confocal microscope (Zeiss). Saved images were linearly adjusted in Photoshop for contrast, brightness and colour balance. We used Mathematica (Wolfram Research) for quantitative analysis of the colocalisation of DeltaD with β-catenin (the DeltaD surface localisation index, see Results).

RESULTS

zdc2 and zdd2 are non-cross-reactive monoclonal antibodies to zebrafish DeltaC and DeltaD that recognise formalin-resistant epitopes

We required antibodies that would not be cross-reactive, would recognise a formalin fixation-resistant epitope and would work on wholemount and sectioned tissue. We therefore expressed the entire extracellular regions of DeltaC and DeltaD in a mammalian expression system as tagged soluble proteins (Fig. 1A) and used these to immunise mice and thereby generate monoclonal antibodies. We screened the hybridomas for production of antibodies that (1) recognised DeltaC or DeltaD both in the unfixed state and after formalin fixation (Fig. 1B), and (2) did not cross-react with other zebrafish Delta proteins (Fig. 1C; see Materials and methods). We named the antibodies that met all these criteria zdc2 (specific for DeltaC) and zdd2 (specific for DeltaD).

The efficacy of the antibodies was checked on western blots (Fig. 1D): both worked well, but only when the gel was run under non-reducing conditions, suggesting that they recognise conformation-sensitive epitopes. Notably, in addition to the main bands at the expected sizes for the monomeric Delta proteins, fainter bands were visible at approximately twice this size, hinting that the proteins might, under some circumstances, occur as dimers.

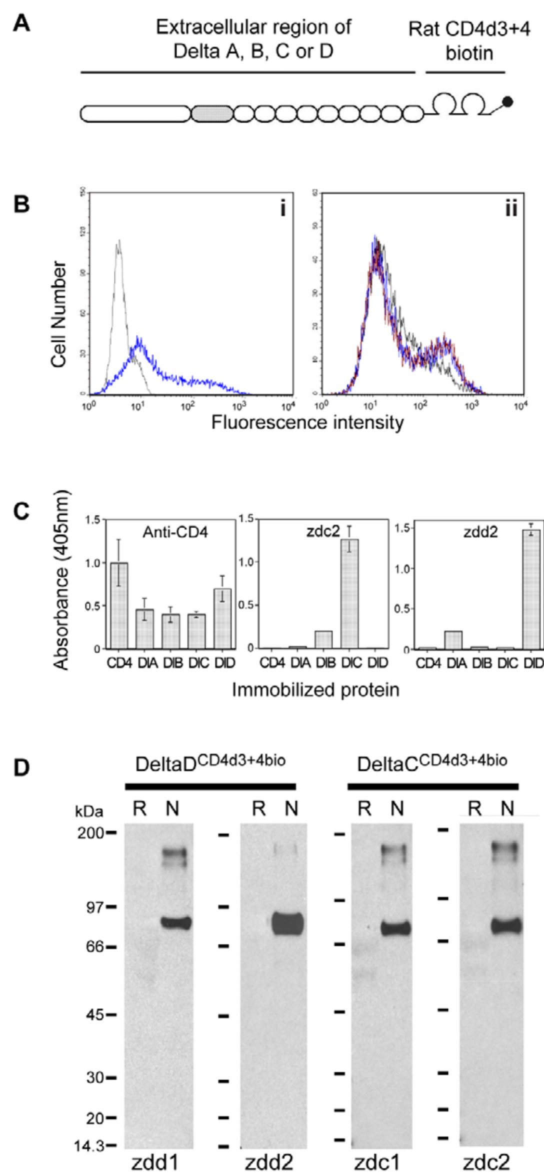

Fig. 1. Selection of non-cross-reactive monoclonal antibodies that recognise formalin-treated zebrafish DeltaC and DeltaD glycoproteins. (**A**) Soluble recombinant Delta proteins used as immunogens, each comprising the extracellular region of zebrafish DeltaA, B, C or D, a C-terminal tag of rat Cd4 domains 3 and 4 (Cd4d3+4) and a short peptide that can be enzymatically monobiotinylated. (**B**) Selection of antibodies that recognise formalin-fixed DeltaD. (**i**) Untransfected (thin black line) or full-length DeltaD-transfected (thick blue line) HEK293T cells were stained with zdd2 monoclonal antibody and analysed by flow cytometry. (**ii**) The sensitivity of the zdd2 epitope to formalin fixation was assessed by flow cytometry of DeltaD-transfected cells fixed for 0 (black line), 40 (blue line) or 90 (red line) minutes before staining with zdd2. (**C**) Anti-zebrafish DeltaC and DeltaD monoclonal antibodies show little cross-reactivity within the zebrafish Delta family. The Cd4 antigenic tag and the four soluble Delta constructs were captured via their biotin tags to a streptavidin-coated ELISA plate before incubation with the indicated monoclonal antibodies. Antibody binding was quantified by a colorimetric assay using a phosphatase-conjugated secondary antibody. Bar chart shows mean ± s.d.; n=3. (**D**) Western blots of recombinant protein secreted from HEK293T cells transfected with tagged Delta constructs. R, reducing conditions; N, non-reducing conditions. All four antibodies reveal bands at the expected sizes for monomeric Delta and, more faintly, at approximately twice this size.

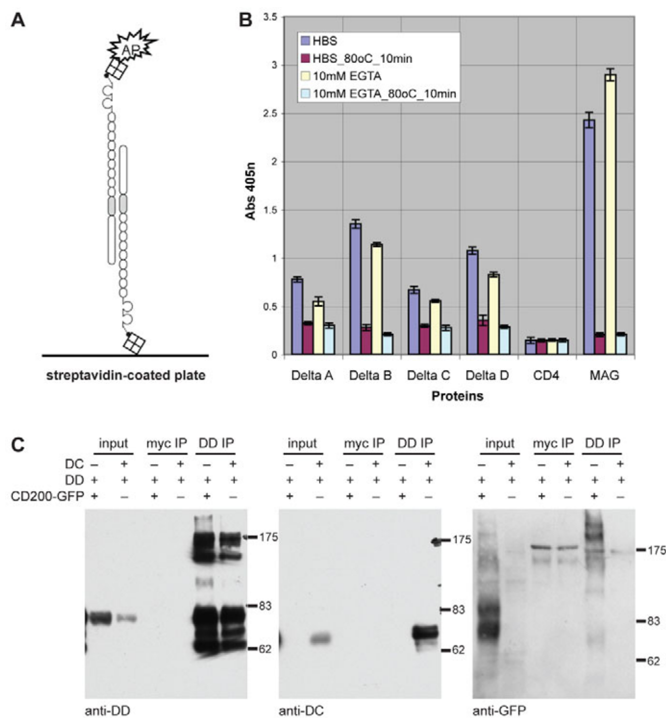

Fig. 2. Binding of Delta to Delta. (**A**) Diagram of the assay used in B. A dimer of monobiotinylated protein molecules binds to a streptavidin-coated plate through one of its biotin moieties, leaving the other exposed and detectable with a streptavidin-coupled alkaline-phosphatase (AP) probe. Binding of this probe indicates presence of dimeric (or multimeric) complexes. (**B**) The ectodomains of DeltaA, B, C and D bind to one another homophilically. Cd4 protein serves as a negative (non-dimerising) control, and Mag protein as a positive (dimerising) control. The dimerisation signal is reduced by heat denaturation and by removal of Ca^{2+} by prior treatment with EGTA. Error bars indicate s.d.; *n*=4. HBS, Hank's Buffered Saline. (**C**) Co-immunoprecipitation experiments demonstrate heterophilic binding of DeltaC to DeltaD. HEK293 cells were co-transfected with plasmids encoding DeltaC (DC), DeltaD (DD) and an unrelated transmembrane protein (CD200-GFP, ~70 kDa) as indicated, followed by immunoprecipitation (IP) with anti-DeltaD and a monoclonal control antibody (myc IP). Cell lysates (input) and immunoprecipitates were analysed by western blot against DeltaC, DeltaD and GFP.

Zebrafish Delta ectodomains associate homophilically

To investigate whether Delta proteins are able to bind to one another, we immobilised enzymatically monobiotinylated ectodomain fragments of the Delta proteins on streptavidin-coated plates and then probed with streptavidin-alkaline phosphatase. As illustrated in Fig. 2A, this would be expected to give a signal only if the biotinylated proteins form dimers (or multimers), allowing attachment to the coated plate via one biotin, while leaving another biotin exposed for interaction with the probe. We detected a strong signal for DeltaA, B, C and D, as compared with a negative control (Cd4 tag alone, Fig. 2B).

To show that this signal was due to a non-covalent association and was dependent on the correct folding of the Delta ectodomains, we heat-denatured the proteins (85°C for 15 minutes) before immobilisation: the signal was largely lost. To show that the binding depended on Ca^{2+} ions, which are known to be structurally important for the Ca^{2+}-dependent EGF domains that form part of the Delta ectodomains, we removed Ca^{2+} ions by prior incubation with EGTA. This treatment again resulted in a reduction of binding for all the Delta proteins, but not for the positive control myelin-associated glycoprotein (Mag), which binds to itself via immunoglobulin superfamily domains, which are not structurally dependent on Ca^{2+} ions. Heat denaturation of the Delta proteins in the presence of EGTA resulted in a further loss of signal. Together, these results show that the ectodomains of DeltaA, B, C and D form homophilic, Ca^{2+}-dependent, non-covalent associations; however, they leave open the question of whether the Delta proteins can also bind to one another heterophilically.

DeltaC and DeltaD associate heterophilically

To determine whether DeltaC and DeltaD can bind to one another, we co-transfected HEK293 cells with plasmids coding for DeltaD and DeltaC or for DeltaD and an unrelated GFP-tagged transmembrane protein (CD200-GFP); we then analysed lysates from these cells by immunoprecipitation and western blotting. Whereas neither DeltaD nor DeltaC was precipitated by a control monoclonal antibody against Myc, DeltaC could be readily detected in the zdd2 (anti-DeltaD) immunoprecipitates (Fig. 2C). In contrast to the highly overexpressed control transmembrane protein (CD200-GFP), the moderately expressed DeltaC protein was found to exist almost entirely in a complex with DeltaD.

DeltaC and DeltaD show punctate distributions in the central nervous system

As a further test of our antibodies, we used them to immunostain cryosections of formaldehyde-fixed zebrafish embryos, focusing on retina (Fig. 3A-F,I-K) and hindbrain (Fig. 3G,H), where *deltaC* and *deltaD* are expressed in well-defined patterns. As expected from previous work (Itoh et al., 2003; Matsuda and Chitnis, 2009), staining with zdd2 revealed a punctate distribution of DeltaD protein, and the same was true for DeltaC as revealed with zdc2. The puncta of staining were generally small and sparse, with only a few per cell in any single optical section of the hindbrain, and even fewer per cell in the retina. As expected, the amount of both DeltaC and DeltaD was greatly increased in *mib* mutants (Fig. 3B,D) (Haddon et al., 1998a; Haddon et al., 1998b; Jiang et al., 1996; Schier et al., 1996). Not only was the number of Delta-expressing cells increased in these mutants (Haddon et al., 1998a; Haddon et al., 1998b; Jiang et al., 1996; Schier et al., 1996), but also the quantity of Delta protein per expressing cell, presumably because of the failure of Mib-dependent endocytosis and degradation. In the wild-type tissue, the puncta of staining were often clearly in the interior of the cell, although they were also seen at or just beneath the cell surface (we could not easily distinguish which). In *mib* mutants, by contrast, the staining usually appeared more strongly concentrated at the cell surface, again as expected. The pattern in most cells was, however, still punctate, suggesting that the Delta proteins cluster together in the plasma membrane even in the absence of Mib.

In the posterior PSM, DeltaC is internalised but DeltaD remains at the cell surface

The situation in the presomitic and somitic mesoderm was strikingly different from that seen in the CNS. Many ISH studies (Giudicelli et al., 2007; Haddon et al., 1998b; Holley et al., 2000; Jiang et al., 2000; Julich et al., 2005; Mara et al., 2007; Smithers et al., 2000) have shown that both *deltaC* and *deltaD* are expressed in the PSM and in the recently formed somites (see Fig. 4A,B),

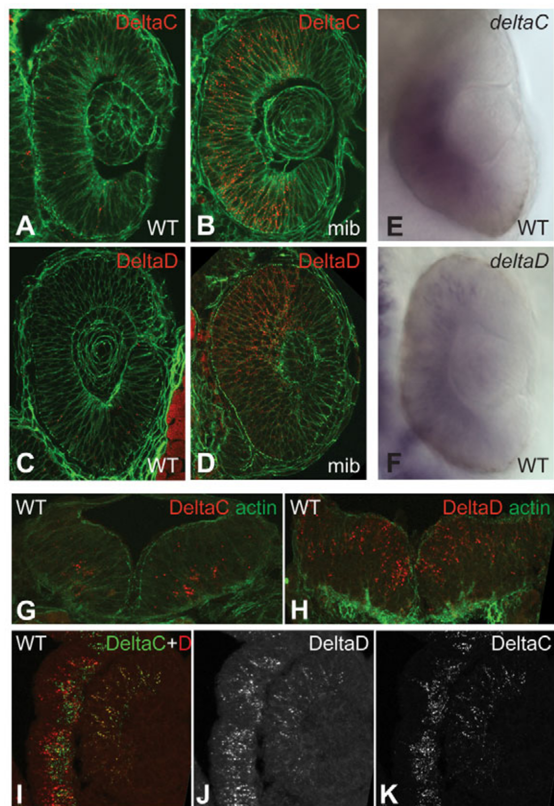

Fig. 3. Expression of DeltaC and DeltaD in retina and brain. (**A-D**) Sections of wild-type (WT) and *mib* mutant zebrafish retina at 24 hpf, immunostained with zdc2 and zdd2. (**E**,**F**) In situ hybridisation (ISH) of wild-type specimens (wholemounts) with probes detecting *deltaC* and *deltaD* mRNA. (**G**,**H**) Sections of hindbrain at 24 hpf, immunostained for DeltaC and DeltaD. (**I-K**) Section of retina and adjacent brain tissue in a 48 hpf embryo, doubly immunostained for DeltaC and DeltaD. In the retina the two proteins largely colocalise, but in the brain they are in distinct sets of cells. A-D and I-K are single confocal optical sections of 15 μm cryosections; G,H are projections of a small *z*-stack. A-D and G,H are counterstained for actin with fluorescent phalloidin (green).

with levels of mRNA that vary with position and according to the phase of the segmentation clock cycle. The posterior half of the PSM is the region in which the clock exerts its control over the future segmentation behaviour of the cells (Giudicelli et al., 2007); here, levels of *deltaC* mRNA oscillate, but those of *deltaD* mRNA are steady (Giudicelli et al., 2007; Holley et al., 2000; Jiang et al., 2000; Julich et al., 2005; Mara et al., 2007; Oates and Ho, 2002; Oates et al., 2005). In the anterior half of the PSM, levels of both mRNAs are increased, and, from its stripy pattern, it seems that *deltaD* also becomes subject to regulation by the clock or by the clock read-out machinery, along with *deltaC*. In the mature somites, both genes are expressed at low levels – *deltaC* in the posterior part of each somite and *deltaD* in the anterior.

As expected (Giudicelli et al., 2007), wholemounts immunostained with our DeltaC and DeltaD antibodies revealed both proteins in patterns that correlated well with their corresponding mRNA patterns (Fig. 4C,D), although the immunostaining for DeltaC was only seen clearly at sites with strong ISH signal, indicating that in the PSM, as in the retina, immunostaining is less sensitive than ISH in detecting expression of the genes.

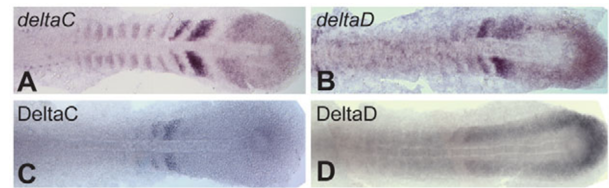

Fig. 4. ISH and antibody staining patterns compared in flat-mounted ~10-somite stage zebrafish embryos. (**A**,**B**) ISH for *deltaC* and *deltaD*. (**C**,**D**) Immunohistochemical staining of DeltaC with zdc2 and of DeltaD with zdd2.

Immunostained optical sections at higher magnification revealed a more complex picture. In the anterior PSM, where expression was strongest, both proteins occurred mainly as intracellular puncta (Figs 5-7), as in the central nervous system. In the posterior PSM, however, there was a striking difference. Levels of immunostaining for DeltaC were so low as to be barely detectable in an optical section, but again took the form of intracellular puncta (Fig. 5B). DeltaD was much more easily detected, and was concentrated at the surface of each cell (Fig. 6C-C″). A similar distinction was seen in the mature somites, where DeltaC was detected in sparse intracellular puncta, whereas DeltaD was localised (in the anterior part of each somite) at the cell surface (Fig. 5 and Fig. 6A).

To confirm that the surface staining in the paraxial mesoderm corresponded to DeltaD at the cell surface, we devised the following protocol, taking advantage of the fact that the zdd2

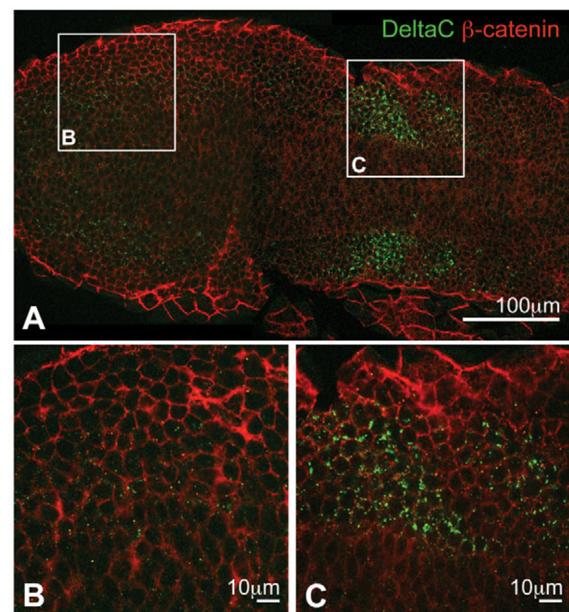

Fig. 5. Optical sections of PSM immunostained for DeltaC and β-catenin in a flat-mounted, 10-somite stage wild-type zebrafish embryo. (**A**) Low-magnification overview. DeltaC, green; β-catenin, red. (**B**,**C**) Higher magnifications of the boxed regions in A showing posterior (B) and anterior (C) presomitic mesoderm (PSM). DeltaC staining is always observed as intracellular puncta, with very faint expression in the posterior but strong upregulation in the anterior PSM.

DEVELOPMENT

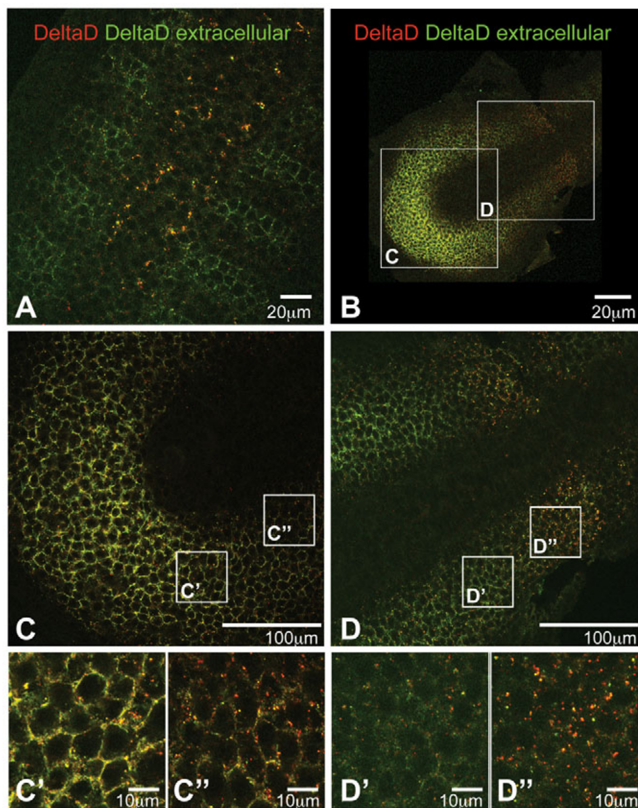

Fig. 6. Optical sections of flat-mounted embryos stained to determine the ratio of extracellular to intracellular DeltaD. zdd2 staining before permeabilisation is in green and that after permeabilisation is in red. A low red:green ratio indicates extracellular staining, whereas a high red:green ratio indicates intracellular. (**A**) Trunk region of a 16 hpf wild-type zebrafish embryo: DeltaD is mainly intracellular and granular in presumptive neuroblasts of the neural tube, but is mainly at the cell surface in the anterior part of each somite. (**B-D″**) PSM of a 10-somite stage wild-type embryo. In the posterior PSM, DeltaD is mainly located at the cell membrane (yellow cell outlines in C-C″); in the anterior PSM DeltaD is predominantly intracellular (red dots in D-D″).

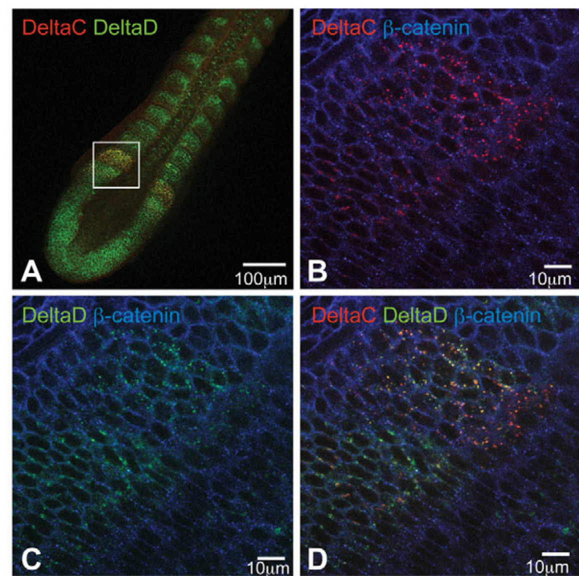

Fig. 7. DeltaC and DeltaD are colocalised in puncta in the anterior PSM. (**A-D**) Optical sections of a flat-mounted 14-somite stage wild-type zebrafish embryo immunostained for DeltaC (red), DeltaD (green) and β-catenin (blue). DeltaC and DeltaD are upregulated in the same region within the anterior PSM and colocalise within the same intracellular granules.

antibody was raised against the extracellular region of DeltaD and performing two immunostainings in succession. First, we detected zdd2 in the non-permeabilised embryos using a green fluorescent secondary antibody. We then permeabilised the embryos with detergent and followed this with a further zdd2 incubation, but this time using a red fluorescent secondary antibody to label the whole DeltaD protein content of the cells. The variations in the green:red ratio can then be taken to indicate variations in the extracellular/intracellular localisation of DeltaD. The images clearly show the highest green:red ratio in the posterior PSM and mature somites (Fig. 6A,C-C″), with a decreasing gradient from posterior to anterior within the PSM (Fig. 6C-D″). This suggests that DeltaD is predominantly in the plasma membrane in the posterior PSM and becomes progressively more internalised as cells mature towards the anterior PSM. After segmentation, expression is reinitiated in the anterior half of each somite and the protein remains at the plasma membrane. By contrast, DeltaD-expressing cells in the neural tube showed a very low green:red ratio, indicating that most of the protein is located intracellularly (Fig. 6A).

Within cells that express both *deltaC* and *deltaD*, simultaneous detection of the two proteins revealed that they mostly colocalise in the intracellular puncta. This was evident in the anterior PSM (Fig. 7), as well as in the retina (Fig. 3I-K). This suggests that the intracellular puncta represent vesicles where the two types of protein are internalised together.

Given that DeltaD is strongly internalised in the anterior but not the posterior PSM, an obvious possibility is that the two regions differ in the susceptibility of DeltaD to the action of Mib. To test this, we examined the zdd2 immunostaining pattern in parallel in the PSM of wild-type embryos and embryos homozygous for the loss-of-function alleles mib^{ta52b} and mib^{tfi91}. In the mutants, we saw DeltaD protein localised at the cell surface throughout the PSM (Fig. 8): use of the same staining protocol as before resulted in a uniform green:red ratio throughout the paraxial mesoderm, showing complete loss of the gradient of internalisation of DeltaD observed in wild-type embryos. The presence of a normal functional *mib* gene is therefore necessary and sufficient to internalise DeltaD in the anterior PSM, but is not sufficient to internalise it in the posterior PSM. This could perhaps reflect a regional difference in, for example, the phosphorylation state of DeltaD, which has several potential phosphorylation sites in its intracellular domain.

In any case, given that loss of *mib* function elsewhere in the body leads to a failure of Notch activation, our findings suggest that in the normal posterior PSM, where DeltaD fails to be internalised, DeltaD by itself is unable to activate Notch.

In the PSM, DeltaC negatively regulates the level of DeltaD on the cell surface

In the somitic mesoderm and the PSM, there is a correlation between the subcellular localisation of DeltaD and the presence of DeltaC: DeltaD is punctate and intracellular where DeltaC is

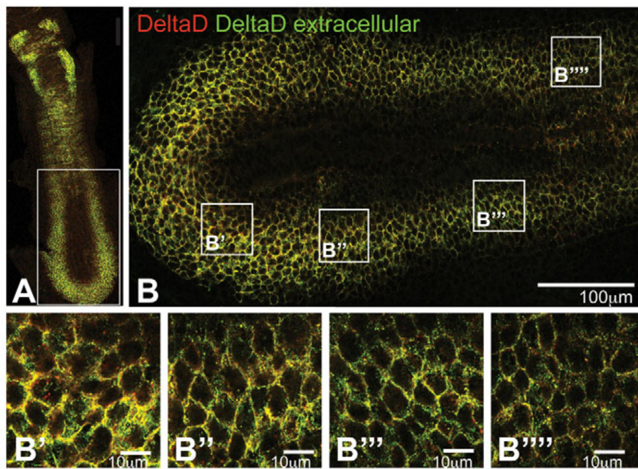

Fig. 8. The subcellular localisation of DeltaD in the PSM of a *mib* mutant zebrafish embryo. (**A-B''''**) Optical sections of a flat-mount immunostained with zdd2 before (green) and after (red) permeabilisation, as in Fig. 6, to distinguish between intracellular and extracellular DeltaD. The caudorostral gradients in the quantity of DeltaD per cell and in its degree of internalisation, as evident in wild type (see Fig. 6), are lost in the *mib* mutant, producing a uniform distribution of DeltaD that is at the cell surface throughout the PSM (see B-B'''').

plentiful, but is on the cell surface where DeltaC is scarce or absent. To examine whether this relationship was causal, we manipulated the expression of DeltaC and looked for effects on the localisation of DeltaD.

To obtain an objective quantitative measure of the extent to which DeltaD was localised on the cell surface, we used immunofluorescent co-staining with two antibodies: zdd2 to detect DeltaD and an antibody against β-catenin to mark the cell surface (Fig. 9A). For each region of interest, we generated a two-dimensional scatter plot in which each pixel was represented by a point with x and y coordinates corresponding to the intensities of the fluorescence signals for the two antibodies. We then computed the correlation coefficient for this scatter plot; we call this the surface localisation index. The maximum possible value for the surface localisation index is 1, which would indicate perfect colocalisation of the two markers, with the DeltaD signal strictly proportional to the β-catenin signal throughout, implying that DeltaD was concentrated on the cell surface. Lower values of the surface localisation index signify lower degrees of cell-surface localisation of DeltaD.

We compared three regions of the PSM – posterior, middle and anterior – in each of three genotypes: (1) wild type; (2) *dlc*$^{tw212b/tw212b}$ (*bea*) mutants, with a loss-of-function mutation in *deltaC*; and (3) transgenics in which DeltaC was artificially overexpressed (Fig. 9B). For the latter, we generated a new line of zebrafish, *Tg(UAS:dlc)cj2*, containing a transgene in which *deltaC* is placed under UAS control (see Materials and methods). We crossed this with the *Tg(hsp70l:Gal4vp16)vu22* transgenic line, in which heat shock induces expression of Gal4-VP16. When heat-shocked at 39°C for 45 minutes at 10 hpf (bud stage), progeny containing both transgenes showed DeltaC overexpression beginning within 3 hours of heat shock and continuing for at least a further 6 hours, as indicated by zdc2 immunostaining. We fixed at 14 hpf (10 somites) and the results are shown in Fig. 9.

On visual inspection (Fig. 9A,B), it seemed that in the anterior PSM of the *dlc*$^{tw212b/tw212b}$ (*deltaC* loss-of-function) mutants, DeltaD was no longer internalised as in wild type but instead was concentrated on the cell surface; conversely, it seemed that in the heat-shocked transgenics overexpressing DeltaC, cell-surface levels of DeltaD in the posterior PSM were reduced by comparison with wild type. Quantitative image analysis (Fig. 9C) confirmed these impressions. As expected, in the wild type the surface localisation index was highest in the posterior PSM and lowest in the anterior PSM. In the *dlc*$^{tw212b/tw212b}$ mutants, the index was raised relative to wild type in both the middle and the anterior PSM. Conversely, in the heat-shocked transgenics overexpressing DeltaC, the index was reduced relative to wild type in the posterior PSM. All these differences were statistically significant ($P \leq 0.01$). Thus, a decrease in the levels of functional DeltaC causes an increase in cell-surface DeltaD; conversely, increased expression of DeltaC drives a decrease in cell-surface DeltaD.

We also examined the distribution of DeltaC protein in *deltaD* (*aei*) mutant embryos. We saw no tendency for DeltaC to accumulate on the cell surface instead of being internalised: DeltaC (in the anterior PSM) was concentrated in intracellular puncta, just as in wild-type controls (data not shown).

Together, these results indicate that in the PSM, DeltaC is competent for internalisation independently of DeltaD, but that internalisation of DeltaD depends on DeltaC.

DISCUSSION

The Delta and Serrate/Jagged families of Notch ligands are type I membrane proteins and must be present at the cell surface to encounter their receptor Notch on an adjacent cell. Previous work has shown, however, that these proteins are largely localised in intracellular vesicles and that engagement with the endocytosis machinery is necessary for them to be able to activate Notch (for reviews, see D'Souza et al., 2008; Le Borgne et al., 2005a). Endocytosis, which depends on the ubiquitylation of the ligands by Mib and, at least in some cases, on their interaction with Notch (Matsuda and Chitnis, 2009), keeps their levels at the cell surface very low (Itoh et al., 2003). These insights have depended on the use of antibodies against the Notch ligands and, in particular, on our monoclonal antibodies against zebrafish DeltaC and DeltaD. We report here the characteristics of these antibodies in detail and use them for further exploration of the biochemistry and function of DeltaC and DeltaD. We provide evidence that the two proteins form homophilic and heterophilic associations, we compare their patterns of localisation in the central nervous system and the PSM, we show that DeltaC regulates the subcellular localisation of DeltaD and, on the basis of these observations, we suggest a solution to a puzzle concerning the functions of DeltaC and DeltaD in the segmentation clock.

The distribution of DeltaC and DeltaD that we see in the central nervous system matches expectations based on earlier work (Matsuda and Chitnis, 2009), but in the somitic mesoderm our findings come as a surprise. DeltaC is internalised as expected throughout this tissue, but DeltaD is not, and instead resides at the cell surface both in the posterior PSM and in the mature somites. Given the known relationship between internalisation and the ability to activate Notch, this protein distribution suggests that in the posterior PSM DeltaC is functional as a Notch ligand, whereas DeltaD is not. Yet the *deltaC* (*bea*) and *deltaD* (*aei*) mutant phenotypes indicate that both proteins are required to keep the segmentation clocks of adjacent cells synchronised in this region (Giudicelli et al., 2007; Horikawa et al., 2006; Jiang et al., 2000;

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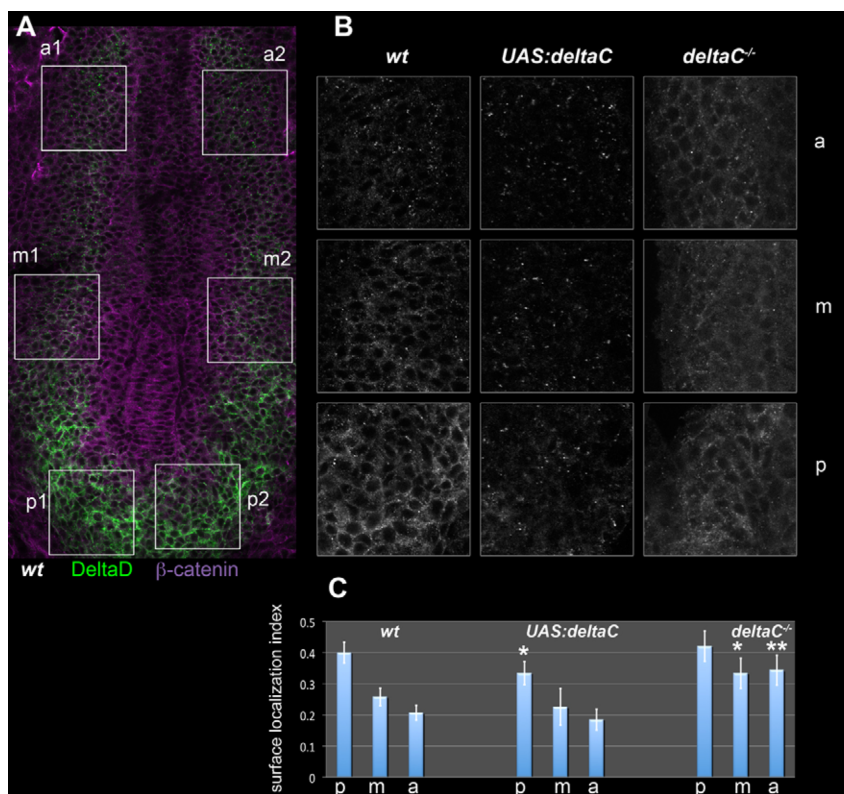

Fig. 9. The subcellular localisation of DeltaD is controlled by DeltaC. (**A**) PSM of a flat-mounted wild-type (wt) zebrafish embryo immunostained for DeltaD (green) and β-catenin (magenta). Boxes show the left and right anterior (a), middle (m) and posterior (p) PSM regions that were sampled for quantitative analysis. (**B**) Enlargements of the corresponding boxed a, m and p regions from wild-type, DeltaC-overexpressing [heat-shocked *Tg(UAS:dlc)*^{cj2}*;Tg(hsp70l:Gal4vp16)*^{vu22}, '*UAS:deltaC*'] and DeltaC-defective (*dlc*$^{tw212b/tw212b}$, '*deltaC*$^{–/–}$') embryos; the DeltaD staining is shown in each case. (**C**) DeltaD surface localisation index computed for each region in each genotype. Values are means of *n* samples (left and right sides of *n*/2 embryos), where *n*=28 for wild type, *n*=10 for DeltaC overexpressing, and *n*=20 for DeltaC defective. Error bars show 95% confidence intervals. *, $P<0.01$; **, $P<0.0001$; versus the corresponding region of the wild type (one-tailed *t*-test).

Lewis, 2003; Mara et al., 2007; Oates et al., 2005; Ozbudak and Lewis, 2008; Riedel-Kruse et al., 2007; van Eeden et al., 1996; van Eeden et al., 1998). If DeltaD is ineffective as a Notch ligand, how does it contribute to this function?

The background to this puzzle has been reviewed elsewhere (Giudicelli and Lewis, 2004; Gossler and Hrabé de Angelis, 1998; Lewis and Ozbudak, 2007; Pourquie, 2007; Saga and Takeda, 2001) and will be briefly summarised here. The PSM is a region of undifferentiated tissue at the tail end of the embryo; through growth, cells continually overflow from the anterior end of the PSM and adjust their contacts so as to form a succession of new somites. The regular spacing of the boundaries between successive somites is controlled by a temporal oscillation of gene expression in the cells of the PSM. The oscillating genes in the zebrafish include *deltaC*, *her1* and *her7*; the phase of the *her1/her7/deltaC* oscillation in each cohort of cells as these cells emerge across a certain 'frontier of determination' in the PSM dictates how the cells will behave subsequently when they undergo overt physical segmentation. The frontier of determination lies about half way along the PSM: it is the *her1/her7/deltaC* oscillation in the posterior half of the PSM that is critical (Giudicelli et al., 2007). Mutations in any of a variety of components of the Notch signalling pathway disrupt segmentation. This is apparently because when Notch signalling fails, the oscillators in the individual PSM cells lose coordination and fall out of synchrony, creating a pepper-and-salt mixture of cells in different phases of the oscillation cycle (Horikawa et al., 2006; Jiang et al., 2000; Mara et al., 2007; Morelli et al., 2009; Ozbudak and Lewis, 2008; Riedel-Kruse et al., 2007). Similar patterns of disruption are seen when Notch signalling is blocked chemically with the gamma-secretase inhibitor DAPT, and in Notch pathway mutants, including *deltaC*$^{–/–}$ (*bea*) mutants and *deltaD*$^{–/–}$ (*aei*) mutants. In all these cases, the first few somites are spared and segment correctly, and this appears to be because the oscillations are initiated synchronously throughout the population of PSM cells at the beginning of somitogenesis and then take several cycles to drift out of synchrony when the synchronisation mechanism is defective.

Although the phenotypes of the *deltaC* and *deltaD* mutants are similar, they are not identical: in the *deltaC* mutants, for example, only the first three to five somites segment normally, whereas in *deltaD* mutants the first seven to nine do so (Jiang et al., 2000; Mara et al., 2007; van Eeden et al., 1996). There are also profound differences in the way the two genes are expressed in the posterior PSM: at the RNA level, *deltaC* oscillates, driven up and down by the oscillating levels of the Her1 and Her7 gene regulatory proteins (Giudicelli et al., 2007; Jiang et al., 2000), whereas levels of *deltaD* mRNA appear more or less constant. Mara et al. have tested the roles of *deltaC* and *deltaD* by genetic manipulations and through detailed analysis of mRNA expression patterns; they find, for example, that injection of *deltaC* mRNA, giving steady overexpression, hinders the re-establishment of synchrony after a transient DAPT block, whereas injection of *deltaD* mRNA helps this recovery (Mara et al., 2007).

The oscillatory behaviour of *deltaC* and its direct regulation by Her1 and Her7 suggest a straightforward explanation of its role in synchronisation (Jiang et al., 2000). If we assume that the DeltaC protein level oscillates like that of its mRNA, the level of DeltaC protein at the surface of each cell will serve as an indicator of oscillation phase, providing information to neighbours that can drive them to adjust their own clocks so as to maintain synchrony (Lewis, 2003; Morelli et al., 2009; Giudicelli et al., 2007; Herrgen et al., 2010; Horikawa et al., 2006; Ozbudak and Lewis, 2008).

The role of *deltaD* is more puzzling. If expression of DeltaD in the posterior PSM does not oscillate, it cannot be serving as a synchronising signal in this way. So why should its loss lead to loss of synchrony? Also, if it is normally constantly present to activate

Notch, maintaining a steady background of Notch activity, how can levels of Notch activation oscillate as proposed in response to DeltaC?

We propose that DeltaD at the cell surface, while not itself oscillating, and unable by itself to activate Notch, potentiates the ability of DeltaC to activate Notch. We suggest that it does so by forming a dimeric or multimeric complex with DeltaC, and that Notch activation depends on this heterophilic association. DeltaD–DeltaD dimers would be ineffective as Notch ligands because they fail in internalisation, whereas DeltaC–DeltaD dimers would be effective because the DeltaC would carry the internalisation signal; DeltaC–DeltaC dimers would also be subject to internalisation and be effective, but would be very scarce when DeltaC is scarce. This hypothesis fits with our observation that (in the paraxial mesoderm) DeltaD is predominantly on the cell surface where DeltaC protein is absent or scarce – that is, in the anterior parts of somites and the posterior part of the PSM – whereas DeltaD is in intracellular puncta where DeltaC protein is plentiful – that is, in the anterior part of the PSM. The correlation reflects a causal relationship: when DeltaC is defective, we find that cell-surface levels of DeltaD are increased, at least in the regions where DeltaC would normally be plentiful; and when DeltaC is artificially overexpressed, we find cell-surface levels of DeltaD reduced, at least in the regions where DeltaC would normally be scarce.

These are the results that would be expected if DeltaC, by forming a complex with DeltaD, either hinders its delivery to the cell surface or facilitates its removal from the cell surface. We saw (Fig. 8) that DeltaD accumulates on the cell surface in the anterior PSM of the *mib* mutant, where there is a defect in internalisation of Delta by endocytosis from the plasma membrane. It therefore seems most likely that, in the normal embryo, DeltaC exerts its effect on DeltaD localisation not by blocking its delivery to the cell surface, but by facilitating its removal from the surface by Mib-dependent endocytosis, an activity that is associated with activation of Notch.

Rapid turnover of DeltaC, which is necessary for its rapid oscillation in the posterior PSM, means that DeltaC concentrations must be low there compared with DeltaD (as we observe), so that DeltaC–DeltaC complexes will be rare compared with DeltaC–DeltaD complexes (provided that the Delta:Delta binding constants are similar). Loss of DeltaD will thus impair, but not totally abolish, signalling by DeltaC, explaining why *deltaD*$^{-/-}$ (*aei*) mutants show a loss-of-synchronisation phenotype, but one that is less severe than in *deltaC*$^{-/-}$ (*bea*) mutants. This hypothesis also explains why the phenotype of double (*bea; aei*) mutants is the same as that of *bea* mutants (Jiang et al., 2000; Julich et al., 2005; van Eeden et al., 1998), and it accounts for the observations of Mara et al. (Mara et al., 2007) on the effects of overexpressing DeltaC, DeltaD and chimaeric DeltaD-DeltaC proteins.

Our biochemical experiments (see Fig. 2) show that DeltaC and DeltaD do indeed form homo- and heterodimers, a finding that is consistent with immunoprecipitation experiments showing that chicken Delta proteins associate with one another homophilically (Sakamoto et al., 2002). A tendency of the Delta protein molecules to associate with one another is also consistent with the observation that DeltaC and DeltaD at the cell surface tend to cluster in a punctate distribution (see Fig. 3), and with the observation that DeltaC and DeltaD colocalise in intracellular vesicles (see Fig. 3I-K and Fig. 7).

Further experiments will be needed to test directly whether Delta proteins must combine as dimers or oligomers to activate Notch, to determine why DeltaC and DeltaD show such different trafficking behaviour in the PSM, and to clarify precisely how DeltaC regulates levels of DeltaD on the cell surface. The monoclonal antibodies that we have characterised in detail in this paper will be important tools in the study of these and other aspects of the biochemistry of Delta-Notch signalling.

Acknowledgements
We thank Phil Taylor, Darren Martin and Chris Sergeant for fish care. Our work was funded by Cancer Research UK and by EMBO Fellowships for F.G. and A.H.

Competing interests statement
The authors declare no competing financial interests.

References
Brown, M. H., Boles, K., van der Merwe, P. A., Kumar, V., Mathew, P. A. and Barclay, A. N. (1998). 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J. Exp. Med.* **188**, 2083-2090.
Bushell, K. M., Sollner, C., Schuster-Boeckler, B., Bateman, A. and Wright, G. J. (2008). Large-scale screening for novel low-affinity extracellular protein interactions. *Genome Res.* **18**, 622-630.
Chandu, D., Huppert, S. S. and Kopan, R. (2006). Analysis of transmembrane domain mutants is consistent with sequential cleavage of Notch by gamma-secretase. *J. Neurochem.* **96**, 228-235.
Chen, M. S., Obar, R. A., Schroeder, C. C., Austin, T. W., Poodry, C. A., Wadsworth, S. C. and Vallee, R. B. (1991). Multiple forms of dynamin are encoded by shibire, a Drosophila gene involved in endocytosis. *Nature* **351**, 583-586.
Chen, W. and Casey Corliss, D. (2004). Three modules of zebrafish Mind bomb work cooperatively to promote Delta ubiquitination and endocytosis. *Dev. Biol.* **267**, 361-373.
Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* **375**, 761-766.
Crosnier, C., Vargesson, N., Gschmeissner, S., Ariza-McNaughton, L., Morrison, A. and Lewis, J. (2005). Delta-Notch signalling controls commitment to a secretory fate in the zebrafish intestine. *Development* **132**, 1093-1104.
D'Souza, B., Miyamoto, A. and Weinmaster, G. (2008). The many facets of Notch ligands. *Oncogene* **27**, 5148-5167.
del Alamo, D., Rouault, H. and Schweisguth, F. (2011). Mechanism and significance of cis-inhibition in Notch signalling. *Curr. Biol.* **21**, R40-R47.
Giudicelli, F. and Lewis, J. (2004). The vertebrate segmentation clock. *Curr. Opin. Genet. Dev.* **14**, 407-414.
Giudicelli, F., Ozbudak, E. M., Wright, G. J. and Lewis, J. (2007). Setting the tempo in development: an investigation of the zebrafish somite clock mechanism. *PLoS Biol.* **5**, e150.
Glittenberg, M., Pitsouli, C., Garvey, C., Delidakis, C. and Bray, S. (2006). Role of conserved intracellular motifs in Serrate signalling, cis-inhibition and endocytosis. *EMBO J.* **25**, 4697-4706.
Gossler, A. and Hrabé de Angelis, M. (1998). Somitogenesis. *Curr. Top. Dev. Biol.* **38**, 225-287.
Haddon, C., Jiang, Y.-J., Smithers, L. and Lewis, J. (1998a). Delta-Notch signalling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the mind bomb mutant. *Development* **125**, 4637-4644.
Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D. and Lewis, J. (1998b). Multiple delta genes and lateral inhibition in zebrafish primary neurogenesis. *Development* **125**, 359-370.
Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquié, O., Ish-Horowicz, D. and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signaling in the embryonic chick retina. *Curr. Biol.* **7**, 661-670.
Herrgen, L., Ares, S., Morelli, L. G., Schroter, C., Julicher, F. and Oates, A. C. (2010). Intercellular coupling regulates the period of the segmentation clock. *Curr. Biol.* **20**, 1244-1253.
Holley, S. A., Geisler, R. and Nusslein-Volhard, C. (2000). Control of her1 expression during zebrafish somitogenesis by a delta-dependent oscillator and an independent wave-front activity. *Genes Dev.* **14**, 1678-1690.
Horikawa, K., Ishimatsu, K., Yoshimoto, E., Kondo, S. and Takeda, H. (2006). Noise-resistant and synchronized oscillation of the segmentation clock. *Nature* **441**, 719-723.
Ilagan, M. X. and Kopan, R. (2007). SnapShot: notch signaling pathway. *Cell* **128**, 1246.
Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L. et al. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4**, 67-82.
Jiang, Y.-J., Brand, M., Heisenberg, C. P., Beuchle, D., Furutani-Seiki, M., Kelsh, R. N., Warga, R. M., Granato, M., Haffter, P., Hammerschmidt, M. et

DEVELOPMENT

al. (1996). Mutations affecting neurogenesis and brain morphology in the zebrafish, *Danio rerio*. *Development* **123**, 205-216.

Jiang, Y. J., Aerne, B. L., Smithers, L., Haddon, C., Ish-Horowicz, D. and Lewis, J. (2000). Notch signalling and the synchronization of the somite segmentation clock. *Nature* **408**, 475-479.

Julich, D., Hwee Lim, C., Round, J., Nicolaije, C., Schroeder, J., Davies, A., Geisler, R., Lewis, J., Jiang, Y. J. and Holley, S. A. (2005). beamter/deltaC and the role of Notch ligands in the zebrafish somite segmentation, hindbrain neurogenesis and hypochord differentiation. *Dev. Biol.* **286**, 391-404.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Koo, B. K., Lim, H. S., Song, R., Yoon, M. J., Yoon, K. J., Moon, J. S., Kim, Y. W., Kwon, M. C., Yoo, K. W., Kong, M. P. et al. (2005a). Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. *Development* **132**, 3459-3470.

Koo, B. K., Yoon, K. J., Yoo, K. W., Lim, H. S., Song, R., So, J. H., Kim, C. H. and Kong, Y. Y. (2005b). Mind bomb-2 is an E3 ligase for Notch ligand. *J. Biol. Chem.* **280**, 22335-22342.

Lai, E. C., Deblandre, G. A., Kintner, C. and Rubin, G. M. (2001). Drosophila neuralized is a ubiquitin ligase that promotes the internalization and degradation of delta. *Dev. Cell* **1**, 783-794.

Lai, E. C., Roegiers, F., Qin, X., Jan, Y. N. and Rubin, G. M. (2005). The ubiquitin ligase Drosophila Mind bomb promotes Notch signaling by regulating the localization and activity of Serrate and Delta. *Development* **132**, 2319-2332.

Le Borgne, R., Bardin, A. and Schweisguth, F. (2005a). The roles of receptor and ligand endocytosis in regulating Notch signaling. *Development* **132**, 1751-1762.

Le Borgne, R., Remaud, S., Hamel, S. and Schweisguth, F. (2005b). Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in Drosophila. *PLoS Biol.* **3**, e96.

Lewis, J. (2003). Autoinhibition with transcriptional delay: a simple mechanism for the zebrafish somitogenesis oscillator. *Curr. Biol.* **13**, 1398-1408.

Lewis, J. and Ozbudak, E. M. (2007). Deciphering the somite segmentation clock: beyond mutants and morphants. *Dev. Dyn.* **236**, 1410-1415.

Mara, A., Schroeder, J., Chalouni, C. and Holley, S. A. (2007). Priming, initiation and synchronization of the segmentation clock by deltaD and deltaC. *Nat. Cell Biol.* **9**, 523-530.

Matsuda, M. and Chitnis, A. B. (2009). Interaction with Notch determines endocytosis of specific Delta ligands in zebrafish neural tissue. *Development* **136**, 197-206.

Morelli, L. G., Ares, S., Herrgen, L., Schroter, C., Julicher, F. and Oates, A. C. (2009). Delayed coupling theory of vertebrate segmentation. *HFSP J.* **3**, 55-66.

Oates, A. C. and Ho, R. K. (2002). Hairy/E(spl)-related (Her) genes are central components of the segmentation oscillator and display redundancy with the Delta/Notch signaling pathway in the formation of anterior segmental boundaries in the zebrafish. *Development* **129**, 2929-2946.

Oates, A. C., Mueller, C. and Ho, R. K. (2005). Cooperative function of deltaC and her7 in anterior segment formation. *Dev. Biol.* **280**, 133-149.

Ozbudak, E. M. and Lewis, J. (2008). Notch signalling synchronizes the zebrafish segmentation clock but is not needed to create somite boundaries. *PLoS Genet.* **4**, e15.

Pavlopoulos, E., Pitsouli, C., Klueg, K. M., Muskavitch, M. A., Moschonas, N. K. and Delidakis, C. (2001). neuralized Encodes a peripheral membrane protein involved in delta signaling and endocytosis. *Dev. Cell* **1**, 807-816.

Pitsouli, C. and Delidakis, C. (2005). The interplay between DSL proteins and ubiquitin ligases in Notch signaling. *Development* **132**, 4041-4050.

Pourquie, O. (2007). Building the spine: the vertebrate segmentation clock. *Cold Spring Harb. Symp. Quant. Biol.* **72**, 445-449.

Riedel-Kruse, I. H., Muller, C. and Oates, A. C. (2007). Synchrony dynamics during initiation, failure, and rescue of the segmentation clock. *Science* **317**, 1911-1915.

Saga, Y. and Takeda, H. (2001). The making of the somite: molecular events in vertebrate segmentation. *Nat. Rev. Genet.* **2**, 835-845.

Sakamoto, K., Ohara, O., Takagi, M., Takeda, S. and Katsube, K. (2002). Intracellular cell-autonomous association of Notch and its ligands: a novel mechanism of Notch signal modification. *Dev. Biol.* **241**, 313-326.

Schier, A. F., Neuhauss, S. C., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z. et al. (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* **123**, 165-178.

Shin, J., Poling, J., Park, H. C. and Appel, B. (2007). Notch signaling regulates neural precursor allocation and binary neuronal fate decisions in zebrafish. *Development* **134**, 1911-1920.

Smithers, L., Haddon, C., Jiang, Y.-J. and Lewis, J. (2000). Sequence and embryonic expression of *deltaC* in the zebrafish. *Mech. Dev.* **90**, 119-123.

Sprinzak, D., Lakhanpal, A., Lebon, L., Santat, L. A., Fontes, M. E., Anderson, G. A., Garcia-Ojalvo, J. and Elowitz, M. B. (2010). Cis-interactions between Notch and Delta generate mutually exclusive signalling states. *Nature* **465**, 86-90.

van der Bliek, A. M. and Meyerowitz, E. M. (1991). Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. *Nature* **351**, 411-414.

van Eeden, F. J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y.-J., Kane, D. A. et al. (1996). Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. *Development* **123**, 153-164.

van Eeden, F. J., Holley, S. A., Haffter, P. and Nüsslein-Volhard, C. (1998). Zebrafish segmentation and pair-rule patterning. *Dev. Genet.* **23**, 65-76.

Wang, W. and Struhl, G. (2005). Distinct roles for Mind bomb, Neuralized and Epsin in mediating DSL endocytosis and signaling in Drosophila. *Development* **132**, 2883-2894.

Wright, G. J., Puklavec, M. J., Willis, A. C., Hoek, R. M., Sedgwick, J. D., Brown, M. H. and Barclay, A. N. (2000). Lymphoid/neuronal cell surface OX2 glycoprotein recognizes a novel receptor on macrophages implicated in the control of their function. *Immunity* **13**, 233-242.

Wright, G. J., Jones, M., Puklavec, M. J., Brown, M. H. and Barclay, A. N. (2001). The unusual distribution of the neuronal/lymphoid cell surface CD200 (OX2) glycoprotein is conserved in humans. *Immunology* **102**, 173-179.

Zhang, C., Li, Q. and Jiang, Y. J. (2007a). Zebrafish Mib and Mib2 are mutual E3 ubiquitin ligases with common and specific delta substrates. *J. Mol. Biol.* **366**, 1115-1128.

Zhang, C., Li, Q., Lim, C. H., Qiu, X. and Jiang, Y. J. (2007b). The characterization of zebrafish antimorphic mib alleles reveals that Mib and Mind bomb-2 (Mib2) function redundantly. *Dev. Biol.* **305**, 14-27.

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