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Dishevelled controls cell polarity during *Xenopus* gastrulation

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Although cell movements are vital for establishing the normal architecture of embryos, it is unclear how these movements are regulated during development in vertebrates. Inhibition of *Xenopus* Dishevelled (Xdsh) function disrupts convergent extension movements of cells during gastrulation, but the mechanism of this effect is unclear, as cell fates are not affected[1]. In *Drosophila*, Dishevelled controls both cell fate and cell polarity[2–4], but whether Dishevelled is involved in controlling cell polarity in vertebrate embryos has not been investigated. Here we show, using time-lapse confocal microscopy, that the failure of cells lacking Xdsh function to undergo convergent extension results from defects in cell polarity. Furthermore, Xdsh mutations that inhibit convergent extension correspond to mutations in *Drosophila* Dishevelled that selectively perturb planar cell polarity. Finally, the localization of Xdsh at the membrane of normal dorsal mesodermal cells is consistent with Xdsh controlling cell polarity. Our results show that polarized cell behaviour is essential for convergent extension and is controlled by vertebrate Dishevelled. Thus, a vertebrate equivalent of the *Drosophila* planar cell polarity signalling cascade may be required for normal gastrulation.

The morphogenetic cell movements of gastrulation are critical for establishing the germ layers and embryonic axes in animals, and convergent extension movements are important during gastrulation[5,6]. During *Xenopus* convergent extension, lamellipodia that extend from dorsal mesodermal cells become mediolaterally polarized and make stable contacts with neighbouring cells. Alignment, elongation and intercalation of cells follows, leading to narrowing of the mediolateral axis (convergence) and lengthening of the anteroposterior axis (extension) (Fig. 1b)[7–9]. The molecular mechanisms controlling these polarized cell behaviours are unclear.

Dishevelled (Dsh) and other members of the wingless (Wg)/Wnt pathway have many roles in embryogenesis[10], and dorsal translocation of Xdsh in *Xenopus* is an essential event in establishing dorsal cell fates[11]. Expression of a mutant form of Xdsh, Xdd1, inhibits convergent extension of the dorsal mesoderm, but these defects are specific to morphogenesis and the embryos display no defects in dorsoventral axis specification[1]. These results indicate that Xdsh may have two distinct functions in vertebrate embryogenesis, consistent with the situation in *Drosophila*, where Dsh signalling modulates cell fate through the Wg/Wnt pathway and also mediates cell polarity through the planar cell polarity (PCP) cascade[2–4] (Fig. 1d). However, Dsh has not previously been shown to mediate cell polarity decisions in any vertebrate embryo[4].

We were interested in how polarized cell behaviour in the *Xenopus* dorsal marginal zone (DMZ) was affected by modulation of

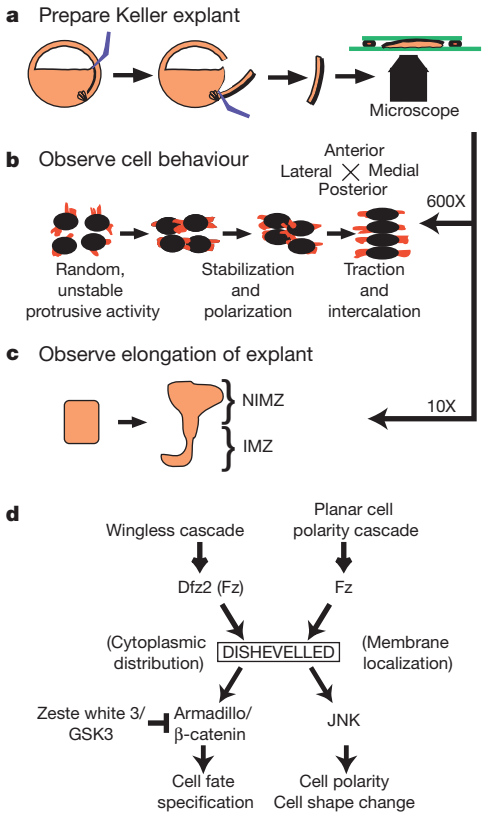

Figure 1 Methods and signalling pathways. **a**, Preparation of Keller explants. DMZs are removed at the onset of gastrulation and immobilized in culture under coverslips. **b**, Time-lapse observation at high magnification allows examination of cell behaviour (black, cell bodies; red, lamellipodia). **c**, Observation at low magnification allows assessment of elongation and morphogenesis; the neural ectoderm (NIMZ) does not elongate; the mesoderm (IMZ) does[7]. **d**, Dishevelled mediates both Wg and PCP signalling cascades[4].

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Dishevelled signalling. Overexpression of Xdd1 inhibits convergent extension of the DMZ[1]. Likewise, overexpression of wild-type Xdsh significantly inhibits convergent extension (see below), consistent with results from *Drosophila*, where overexpression of wild-type Dishevelled elicits dominant disruption of planar cell polarity. We used confocal time-lapse microscopy of Keller explants (Fig. 1a, b) to examine the cell behaviour underlying Xdd1- and Xdsh-mediated disruption of convergent extension.

As expected[9], analysis of control explants expressing a membrane-targeted green fluorescent protein (GFP) confirmed that most lamellipodia in control DMZ explants were stable (Fig. 2a–e, l). In contrast, dorsal mesodermal cells expressing Xdd1 extended and withdrew significantly more protrusions and maintained significantly fewer stable protrusions than did controls (Fig. 2f–l; time-lapse video images available at http://mcb.berkeley.edu/labs/harland/). Thus, cells expressing Xdd1 are not defective in their ability to make membrane protrusions, but are unable to maintain them stably. On the other hand, cells overexpressing Xdsh had no defects in lamellipodial stability (Fig. 2l), indicating that other defects in cell behaviour must underlie the inhibition of convergent extension.

We next examined cell polarity in Keller explants by determining the orientation of membrane protrusions from individual cells. Whereas lamellipodia were predominantly extended from the mediolateral ends of control cells, this polarity was severely disrupted in both Xdd1- and Xdsh-expressing cells (Fig. 2m).

Analysis of cell elongation and orientation of the long axis provide additional indicators of cell polarity in the DMZ[7,8]. As cells begin convergent extension, they align and elongate in the mediolateral axis (Fig. 2n); the mean length-to-width ratio (LWR) of control cells was 1.97 ($\pm$ 0.31), similar to that reported[8]. On the other hand, cells expressing Xdd1 (Fig. 2o; LWR = 1.65 $\pm$ 0.12) or Xdsh (Fig. 2p; LWR = 1.57 $\pm$ 0.08) were not elongated. These LWRs are similar to those of DMZ cells before gastrulation or animal cap cells, which do not converge and extend[8]. Thus, cell elongation is correlated with normal, but not inhibited, convergent extension.

In Fig. 2q, the LWR of each cell is plotted against the angle of its long axis. This plot illustrates the bias of elongate control cells (red diamonds) towards the mediolateral axis and the random angular distribution of cells expressing Xdd1 (black squares) or Xdsh (blue circles). Over 67% of control cells have their long axes in the mediolateral sector (Fig. 2n, q, r). This polarity is severely disrupted in Xdd1-expressing explants (Fig. 2o, q, r) and in Xdsh-expressing explants (Fig. 2p–r). These data show that cell polarity is severely disrupted in Xdd1- and Xdsh-expressing DMZ explants.

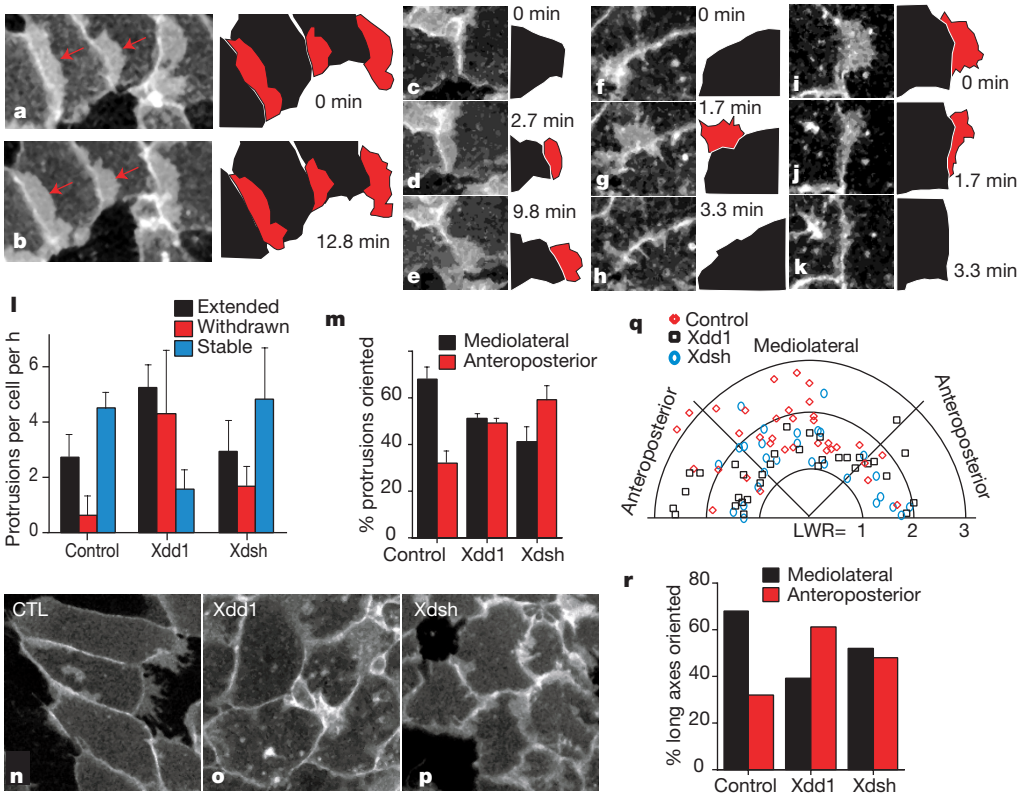

Figure 2 Analysis of cell behaviour and polarity in Keller explants. Overexpression of Xdd-1 or Xdsh severely disrupts cell polarity. **a–j**, Left, single time-lapse frames show representative cell behaviours; right, diagrams of cells with time points. Cell bodies, black; lamellipodia, red; horizontal is mediolateral. **a**,**b**, Arrows, stable mediolaterally biased lamellipodia in control DMZ cells. **c–e**, Lamellipodium extending and stabilizing mediolaterally in a control explant. **f–h**, Xdd1-expressing cell quickly extending and retracting a posteriorly biased lamellipodium. **i–k**, Xdd1-expressing cell quickly retracting a mediolaterally biased lamellipodium. **l**, Protrusive activity: control cells make and withdraw few protrusions but maintain many stable ones. Xdd1-expressing cells make and retract significantly more protrusions than do controls and maintain fewer stable protrusions. Xdsh-expressing cells are stable. **m**, Orientation of protrusions. Lamellipodia are mediolaterally biased in control cells but randomly distributed in cells expressing Xdd1 or Xdsh. Values shown in **l** and **m** are means $\pm$ s.d. between explants. Differences are statistically significant ($P < 0.01$) by the Mann–Whitney *U*-test (control, 33 cells/4 explants; Xdd1, 32 cells/4 explants; Xdsh, 29 cells/3 explants). **n**, Mediolaterally aligned, elongating control cells. Xdd1- (**o**) and Xdsh-expressing cells (**p**) fail to align or elongate. **q**, LWR versus angle of major axis for each of the cells scored in **l**, **m**. Elongate control cells are clustered mediolaterally; Xdd1- and Xdsh-expressing cells are randomly distributed. **r**, Per cent of cells oriented in mediolateral and anteroposterior sectors (derived from **q**). Control cells are mediolaterally biased; Xdsh- and Xdd1-expressing cells are not.

Although polarized cell behaviour has been shown to be associated with convergent extension, our results show that experimental disruption of cell polarity is directly associated with failure of both cell elongation and convergent extension. In light of the observed defects, we investigated the possibility that Xdsh may control cell polarity in a manner similar to that of Dsh in *Drosophila*.

Drawing on the separable functions of Dsh[2–4] (Fig. 1d), we tested reagents that differentially modulate signalling in the Wnt and PCP pathways for their ability to affect convergent extension. We assessed convergent extension by observing changes in both length and shape of isolated Keller explants[12] (Fig. 1c). Control explants elongated significantly and underwent typical changes in morphology between the gastrula and neurula stages (Fig. 3a, b), and expression of Xdd1 inhibited both elongation and morphological changes of explants (Fig. 3a, c). This effect was partially prevented by co-expression of wild-type Xdsh (Fig. 3a, d), indicating that the effect was specific.

To investigate whether canonical Wnt signalling is required during convergent extension, we tested whether specific activation of this pathway downstream of Dsh could rescue the effects of Xdd1. Dominant-negative (DN)-GSK3 is a potent downstream activator of the Wnt pathway[13] (Fig. 1d), but co-expression of DN-GSK3 with Xdd1 did not rescue convergent extension (Fig. 3a, e), indicating that downstream activation of the canonical Wnt pathway does not reverse the effects of Xdd1 on convergent extension. Similar results were obtained in intact embryos (J.B.W. and R.M.H., unpublished data). Overexpression of DN-GSK3 alone in the DMZ did not significantly affect convergent extension (Fig. 3a), but overexpression of wild-type Xdsh alone strongly inhibited convergent extension (Fig. 3a, f). In *Drosophila*, overexpression of wild-type Dsh produces a planar polarity defect, but does not affect Wg/Wnt signalling[2]. Thus the contrasting effects of DN-GSK3 and Xdsh (both of which activate the Wnt pathway) indicate that the control of cell polarity in the DMZ by Xdsh is largely independent of canonical Wg/Wnt signalling.

To investigate whether Xdsh signals through a PCP-like cascade in the *Xenopus* DMZ, we used deletions of Xdsh similar to those that uncouple Wingless and planar polarity phenotypes in *Drosophila*[2–4]. Xdsh-ΔPDZ (a PDZ domain deletion similar, but not identical, to Xdd1) and Xdsh-ΔDEP (a deletion of the DEP domain) are both functional for Wnt signalling in *Xenopus* and *Drosophila*, but have dominant, inhibitory effects on planar polarity in *Drosophila*[2,14] (Fig. 4). Expression of either Xdsh-ΔPDZ or Xdsh-ΔDEP strongly inhibited convergent extension in Keller explants (Fig. 3a, g, h). These phenotypes were partially rescued by co-expression of wild-type Xdsh (Fig. 3a). As both Xdsh-ΔPDZ and Xdsh-ΔDEP can activate the Wnt pathway (Fig. 4), it is important to note that the induced failure of convergent extension was not the result of excessive Wnt signalling; co-expression of additional wild-type Xdsh (which is fully active for both Wnt and PCP signalling) did not exacerbate the convergent extension phenotypes of the mutant Xdsh constructs, but instead ameliorated these effects (Fig. 3a).

Another deletion of Dsh, Xdsh-ΔDIX, has only very weak effects on PCP signalling in *Drosophila* assays (Fig. 4), and likewise only very weakly affects convergent extension (Fig. 3a); most explants expressing Xdsh-ΔDIX do change shape and elongate (Fig. 3i). This result is particularly telling, as Xdsh-ΔDIX and Xdd1 have very different effects on convergent extension but both have equivalent weak ability to activate the Wnt signalling pathway in a *Xenopus*

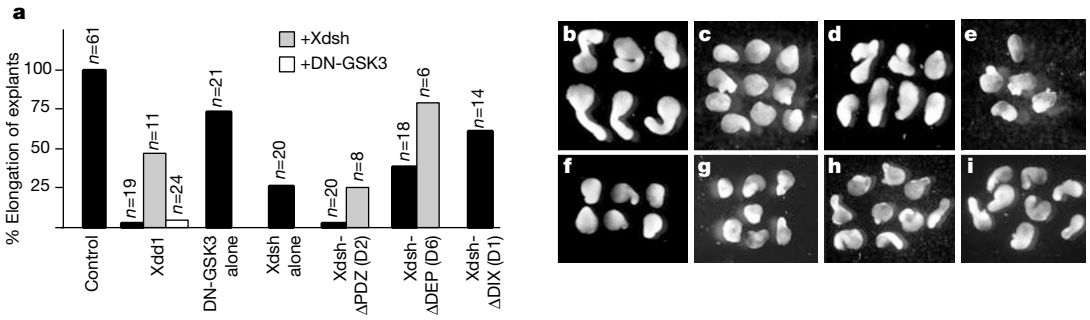

Figure 3 Analysis of convergent extension in Keller explants. **a**, Relative elongation of DMZ explants compared with controls. Constructs expressed are indicated below graph; co-injected constructs are indicated in legend above graph. All differences shown are statistically significant ($P < 0.01$) by the Mann–Whitney *U*-test. **c–i**, Keller explants from representative experiments. **b**, DMZ explants from uninjected embryos elongate substantially between stages 10+ and 19, reorganizing into an IMZ 'tail' and a NIMZ 'head' (Fig. 1c). **c**, Explants expressing Xdd1 fail to elongate. Co-expression of wild-type Xdsh (**d**) partially rescues the phenotype; co-expression of DN-GSK3 does not (**e**); ventral expression of DN-GSK3 induces ectopic axes (not shown), indicating that the dose used activated Wnt signalling. Explants overexpressing wild-type Xdsh (**f**), Xdsh-ΔPDZ (**g**) or Xdsh-ΔDEP (**h**) fail to elongate. Effects of mutant Xdsh constructs were rescued by co-expression with wild-type Xdsh (**a**). Most explants expressing Xdsh-ΔDIX (**i**) change shape normally and extend the IMZ: overall elongation is only slightly reduced.

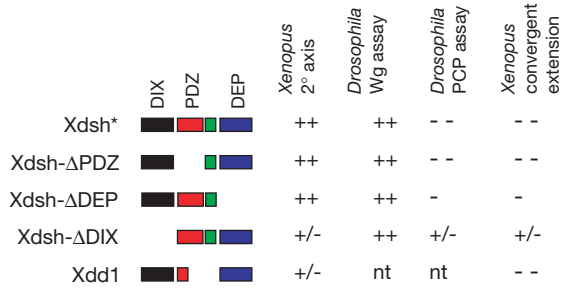

Figure 4 Xdsh constructs and their effects in Wnt, PCP and convergent extension assays. ++, strongly active; +, weakly active; –, weakly inhibitory; —, strongly inhibitory; nt, not tested. *Xenopus* secondary axis assay from ref. 14; *Drosophila* assays from refs 2, 3; convergent extension assay from Fig. 3a. Asterisk, Dsh hyperactivation phenotype.

secondary axis assay (Figs 3a, 4), indicating again that inhibition of convergent extension is not a result of activating Wnt signalling.

As Xdsh regulates cell polarity in the DMZ (Fig. 2) and its effects are independent of Wnt signalling (Fig. 3), there should be evidence of Xdsh signalling through the PCP pathway in normal DMZ cells. To identify such activity, we made use of the fact that activation of the PCP pathway localizes Dsh to the inner surface of the cell membrane, whereas activation of the canonical Wnt pathway leaves Dsh in the cytoplasm[2]. In posterior dorsal mesoderm cells that have not yet begun to converge and extend[8], an Xdsh–GFP construct was localized to the cytoplasm (Fig. 5a). A similar distribution was observed in ventral marginal zone cells (not shown), which do not converge and extend. On the other hand, in anterior chordamesoderm cells that were actively engaged in convergent extension, the Xdsh–GFP signal was localized to the cell membrane (Fig. 5b). Furthermore, Xdsh–GFP was found in the cytoplasm in naive animal cap cells, which do not converge and extend (Fig. 5c). But in animal cap cells treated with activin to induce convergent extension[15], Xdsh–GFP was present at cell membranes (Fig. 5d). This localization of Xdsh to the cell membrane during convergent extension is consistent with Xdsh regulating cell polarity through a PCP-like cascade.

Although the role of Dsh in mediating Wnt signalling and cell fate has been extensively examined, its ability to mediate cell polarity during vertebrate development has remained largely unexplored. Vertebrate Dsh signals through a cascade similar to the *Drosophila* PCP pathway[16], but no *in vivo* function for this activity has been identified in any vertebrate. Here we show that Dishevelled controls essential cell polarity decisions during convergent extension is *Xenopus*. Furthermore, analysis of Xdsh deletion mutants and Xdsh subcellular localization indicate that Xdsh signals through a vertebrate PCP cascade, indicating a common mechanism for vertebrate and *Drosophila* Dishevelled in regulating cell polarity. Coupled with its important role in early specification of the dorsoventral axis[11], these results place vertebrate Dishevelled at the nexus of the two events critical to establishing the normal architecture of the embryo, specification of cell fate and coordination of cell behaviour during morphogenesis. □

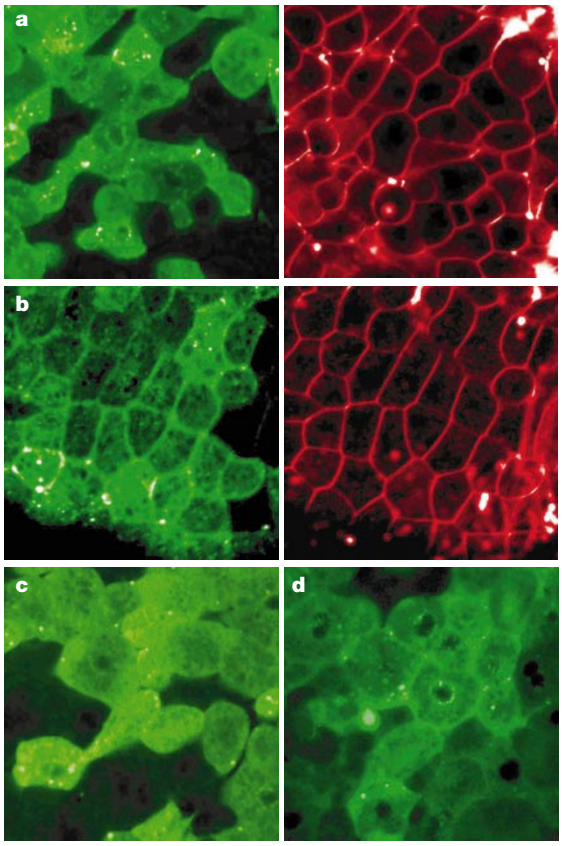

Figure 5 Xdsh is localized to the membrane in cells undergoing convergent extension. **a**, Left, Xdsh–GFP signal is found in a punctate, cytoplasmic distribution in posterior dorsal mesoderm cells that are not undergoing convergent extension at stage 10.75. **b**, Left, in anterior dorsal mesoderm cells engaged in convergent extension, Xdsh–GFP is localized to the cell membrane, consistent with signalling through the planar cell polarity cascade[2]. **a**, **b**, Right, FM 4-64 membrane staining of the cells shown on the left. **c**, Xdsh–GFP is cytoplasmic in naive animal cap cells. **d**, Xdsh–GFP is present at the membrane in activin-treated animal cap cells undergoing convergent extension.

Methods

Time-lapse confocal analysis of cell behaviour

We injected embryos dorsally with 1 ng of Xdd1, Xdsh or control β-galactosidase messenger RNA and 100 pg of memEGFP mRNA (the ras membrane-localization (CAAX) sequence fused to the carboxy terminus of GFP)[17]. Open-face Keller explants were made at stage 10.5 (Fig. 1a); care was taken to remove head mesoderm and involuted axial mesoderm. We observed deep DMZ cells in the vegetal alignment zone[8] by time-lapse confocal microscopy. Protrusive activity was quantified by counting new protrusions extended, existing protrusions withdrawn, or stable protrusions (present in both the first frame and the last frame of the movie). Orientation of lamellipodia was based on 90° sectors from the middle of the cell body (Fig. 1b). LWRs and angle of long axes were measured using NIH image.

Convergent extension in Keller explants

Embryos were injected dorsally with 1–1.5 ng of the mRNAs indicated and 75 pg of GFP. Explants were cut to an initial length of 1 mm. At stage 19, we measured and photographed GFP-positive explants. In Fig. 3a, the mean difference in length of uninjected control explants between stages 10.5 and 19 defines 100% elongation. Values for elongation of experimental explants are expressed as percentage of the control value; data shown are means of two or more experiments.

Xdsh localization

Embryos were injected with 60 pg of Xdsh—GFP[14] mRNA and explants were prepared as above but cultured with 0.01 mg ml^{-1} FM 4-64 to label cell membranes. Animal caps were removed at stage 9 and cultured in 1 × MMR[12] ± activin. Samples were scanned simultaneously with FITC (Fig. 5a and b, left, and c, d) and Texas Red (Fig. 5a and b, right) filter sets. The low dose of Xdsh–GFP injected had no effect on development.

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A receptor for phosphatidylserine-specific clearance of apoptotic cells

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The culmination of apoptosis *in vivo* is phagocytosis of cellular corpses. During apoptosis, the asymmetry of plasma membrane phospholipids is lost, which exposes phosphatidylserine externally[1–4]. The phagocytosis of apoptotic cells can be inhibited stereospecifically by phosphatidylserine and its structural analogues, but not by other anionic phospholipids, suggesting that phosphatidylserine is specifically recognized[1,5–10]. Using phage display, we have cloned a gene that appears to recognize phosphatidylserine on apoptotic cells. Here we show that this gene, when transfected into B and T lymphocytes, enables them to recognize and engulf apoptotic cells in a phosphatidylserine-specific manner. Flow cytometric analysis using a monoclonal antibody suggested that the protein is expressed on the surface of macrophages, fibroblasts and epithelial cells; this antibody, like phosphatidylserine liposomes, inhibited the phagocytosis of apoptotic cells and, in macrophages, induced an anti-inflammatory state. This candidate phosphatidylserine receptor is highly homologous to genes of unknown function in *Caenorhabditis elegans* and *Drosophila melanogaster*, suggesting that phosphatidylserine recognition on apoptotic cells during their removal by phagocytes is highly conserved throughout phylogeny.

Several potential candidates for phosphatidylserine (PS) recognition on apoptotic cells have been put forth, including CD36, CD68, CD14 and LOX-1 (refs 11–14). In addition, β2GP1 may enhance uptake by bridging PS on the apoptotic cell to receptors on macrophages[15]. Several observations suggest, however, that receptors other than these scavenger/pattern-recognition molecules must exist. These molecules do not appear to discriminate between PS and other anionic phospholipids including phosphatidylinositol (PI)[14,16–19], whereas uptake of apoptotic cells by PS-recognizing macrophages is not blocked by phosphatidylinositol or other anionic phospholipids[1,7].

We generated monoclonal antibodies against human macrophages (HMDM) treated with tranforming growth factor-beta (TGF-β) and β-glucan to induce PS recognition[7]. Two IgM antibodies bound more strongly to stimulated HMDM than to unstimulated HMDM (Fig. 1a), but binding was inhibited by preincubating the macrophages with PS liposomes at 4 °C before staining (Fig. 1b; $n = 4$; $P = 0.0008$). Liposomes containing PI or pure phosphatidylcholine (PC) (data not shown) had no effect. Binding was also reduced by downregulating the antigen with PS liposomes for 30 min at 37 °C (data not shown). Both antibodies inhibited the uptake of apoptotic cells by stimulated HMDM, but not that by unstimulated HMDM (Fig. 1c).

We then used monoclonal antibody 217 to examine adherent and suspension cells by flow cytometry. The binding of macrophages to mAb 217 was not species specific: mouse bone-marrow-derived macrophages only bound if they were stimulated to recognize PS[6], whereas thioglycollate-elicited macrophages that constitutively

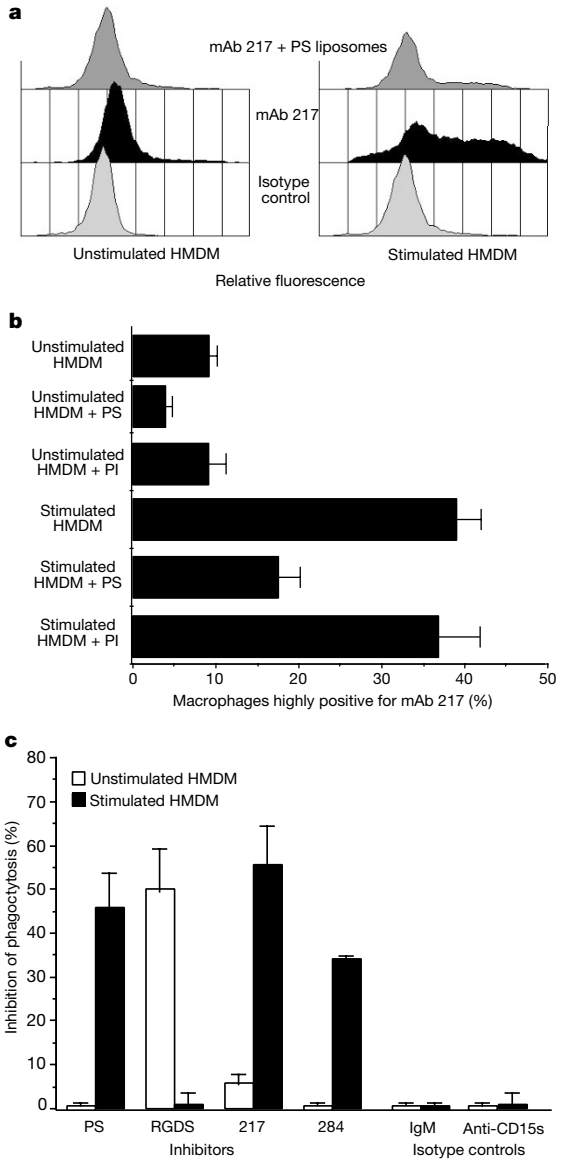

Figure 1 Monoclonal antibody 217 identifies a surface receptor expressed at high levels on human macrophages that recognize phosphatidylserine on apoptotic cells. **a**, Binding of mAb 217 to unstimulated and stimulated (TGF-β/β-glucan) human macrophages. Results represent one of four separate experiments, using macrophages from four different donors. **b**, Liposomes containing PS but not PI inhibit the binding of mAb 217 to stimulated macrophages ($n = 4$; $P = 0.0008$). **c**, Monoclonal antibodies 217 and 284 inhibit uptake of apoptotic cells by stimulated but not unstimulated HMDM. For these experiments, 48.9 ± 8.6% unstimulated macrophages contained apoptotic bodies and 55.2 ± 6.1% stimulated macrophages contained apoptotic bodies ($n = 5$; bars show mean ± s.e.m). Liposomes containing PI and the tetrapeptide control RGES had no effect on uptake (not shown).