The adaptor protein Dab2 sorts LDL receptors into coated pits independently of AP-2 and ARH

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Summary

Clathrin-mediated endocytosis requires cargo-specific adaptor proteins that recognize specific receptors and recruit them into coated pits. ARH [also called low-density lipoprotein receptor (LDLR) adaptor protein] serves as an adaptor for LDLR endocytosis in liver. However, ARH is dispensable for LDL uptake by some other cell types. Here, we show that the adaptor Dab2 plays a major role in LDLR internalization in HeLa cells and fibroblasts. Dab2 mediates internalization of LDLRs but not transferrin receptors independently of ARH and the classic clathrin adaptor AP-2. If Dab2 is absent, ARH can mediate LDLR endocytosis, but its action requires AP-2. Furthermore, the rate of LDLR endocytosis is decreased when Dab2 is absent and Dab2, but not ARH, catalyzes the efficient clustering of LDLR into coated pits. Dab2 activity requires its binding to clathrin, LDLR and phospholipids. Dab2 is also involved in moving LDLRs off filopodia. We suggest that Dab2 is a cargo-specific endocytic adaptor protein, stably associating with phospholipids and clathrin to sort LDLR to nascent-coated pits, whereas ARH might accelerate later steps in LDLR endocytosis in cooperation with AP-2.

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Key words: AP-2, Clathrin, CLASP, Dab2, ARH, LDL receptor adaptor protein, Myosin VI

Introduction

Clathrin-mediated endocytosis (CME) is responsible for the internalization of a wide range of signaling receptors and essential macromolecules. Receptors are selected for internalization by adaptor proteins, which recruit their cargo into clathrin-coated pits (CCPs) by binding to clathrin and to internalization signals within the cytoplasmic regions of the receptors. The prototypical clathrin adaptor is the AP-2 tetramer, which contains large (α and β2), medium (μ2) and small (σ2) subunits (Pearse, 1988). AP-2 is a major component of cell-surface CCPs and clathrin-coated vesicles (CCVs). AP-2 binds to phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P2] and to some receptor internalization motifs, such as the YxxΦ signal in the transferrin receptor (TfnR), via its μ2 subunit (Kirchhausen, 1999). The β2 subunit binds clathrin (Owen et al., 2000) and the α subunit recruits accessory proteins involved in coated-pit invagination and scission (Traub et al., 1999). Different subunits within AP-2 are thus responsible for binding to cargo and to different components of the endocytosis machinery.

AP-2 was once considered to be the universal adaptor for CME. However, overexpression experiments showed that the low-density lipoprotein receptor (LDLR), TfnR and epidermal growth factor receptor (EGFR) do not compete for entry, implying that there are receptor-specific components to the endocytic machinery (Warren et al., 1998). Indeed, interfering with AP-2 by various methods inhibits TfnR but not LDLR or EGFR internalization (Conner and Schmid, 2003; Hinrichsen et al., 2003; Motley et al., 2003; Nesterov et al., 1999).

Endocytosis of AP-2-independent receptors is believed to depend on alternative adaptor proteins, known as clathrin-associated sorting proteins (CLASPs), including β-arrestin, Numb, ARH and Dab2, but their mechanisms of action are as yet poorly understood (Robinson, 2004; Sorkin, 2004; Traub, 2003).

The LDLR internalization signal, FxNPxY, is essential for LDLR internalization in vivo, and mutations lead to reduced LDL removal by the liver and high levels of cholesterol in the blood (Chen et al., 1990; Davis et al., 1986). Other members of the lipoprotein receptor family (including LRP1, megalin, ApoER2 and VLDLR) contain at least one FxNPxY motif. Both AP-2 and clathrin were reported to bind this signal, but the significance of these interactions is unclear (Kibbey et al., 1998; Pearse, 1988). Recently, mutation of the autosomal recessive hypercholesterolemia genes (arh) gene in humans or mice was found to cause an increase in circulating LDL and to inhibit LDLR endocytosis in liver hepatocytes (Garicia et al., 2001; Jones et al., 2003). The ARH protein (recently renamed LDL-receptor adaptor protein) contains an N-terminal PTB/PID domain with binding sites for the FxNPxY signal and for PtdIns(4,5)P2, and clathrin- and AP-2-binding sites in its C-terminus (He et al., 2002; Mishra et al., 2002b). However, although ARH is required in cultured hepatocytes and lymphocytes for endocytosis of the LDLR, endocytosis in arh−/− fibroblasts is unaffected (Norman et al., 1999; Wilund et al., 2002; Zuliani et al., 1999).

Dab2 is a distant relative of ARH. It contains separate binding sites for FxNPxY and PtdIns(4,5)P2, AP-2 and
clathrin, and colocalizes with LDLR, clathrin and AP-2 in coated pits at the plasma membrane (Mishra et al., 2002a; Morris and Cooper, 2001). Dab2, but not ARH, also contains several NPF motifs, potentially enabling interactions with EH-domain-containing proteins, and a binding site for myosin VI (MyoVI), a minus-end-directed actin motor that has been implicated in endocytosis (Buss et al., 2001; Morris et al., 2002a). Dab2 mediates trafficking of the lipoprotein receptor megalin in vivo. Dab2 is required in the embryonic visceral endoderm and adult kidney proximal tubule for endocytosis of megalin and uptake of megalin ligands (Maurer and Cooper, 2005; Morris et al., 2002b; Nagai et al., 2005). p96, an isoform of Dab2 that binds AP-2 and clathrin, rescues megalin endocytosis in dab2−/− visceral endoderm, whereas p67, an isoform that does not bind clathrin, is only partially functional (Maurer and Cooper, 2005). These in vivo studies implicate Dab2 in megalin uptake, at least in specific cell types, but the molecular details are unclear. In addition, it has been unclear whether Dab2 mediates endocytosis of LDLR. LDLR trafficking is intact in kidney epithelial cells derived from dab2−/− mouse embryos (M.E.M., unpublished results). A dominant-negative form of Dab2 blocks endocytosis of LDLR and ApoER2, but this protein may act non-specifically to block binding of other proteins to the FxNPxY internalization signal (Cuitino et al., 2005; Mishra et al., 2002a). Therefore, the possible role of Dab2 in LDLR internalization remains to be demonstrated.

To identify the molecules regulating LDLR endocytosis, we examined LDLR trafficking in cultured cells depleted of Dab2, ARH and AP-2. We found that endocytosis of endogenous LDLRs in HeLa cells and human dermal fibroblasts (HDFs) requires Dab2 when ARH is absent. This suggests that these adaptors are partially redundant. However, we also found important differences between Dab2- and ARH-mediated endocytosis. Quantitatively, LDLR endocytosis is inhibited more by depletion of Dab2 than by depletion of ARH. Furthermore, ARH requires AP-2 for LDLR internalization whereas Dab2 does not. Dab2 can replace AP-2 completely as an LDLR-specific endocytic adapter protein, using a mechanism similar to AP-2 that relies on binding to PtdIns(4,5)P_{2}, cargo receptors and clathrin. In addition, Dab2 is required for efficient clustering of LDLR into coated pits. This work shows that the monomeric Dab2 protein functions analogously to the tetrameric AP-2 in sorting cargoes into coated pits and mediating their subsequent internalization.

**Results**

**Removal of ARH and Dab2 from HeLa cells inhibits LDLR internalization**

To test the roles of ARH and Dab2 in LDLR internalization, we used HeLa cells, which express ARH and the p96 isoform of Dab2 (Fig. 1A, lane 1). Dab2 and ARH were depleted independently or simultaneously using siRNA. As a control, clathrin heavy chain (CHC) was depleted to block all CME (Motley et al., 2003). As shown by immunoblot analysis, target protein levels were significantly reduced, but total LDLR levels were unchanged, compared to a mitogen-activated protein kinase (MAPK) protein loading control (Fig. 1A).

Steady-state surface levels of LDLR were analyzed using cell membrane-impermeant biotin. Biotinylated proteins were collected using streptavidin beads and the LDLR in surface protein and total protein fractions was detected by immunoblotting. Quantification of several independent experiments (n=4 or 5, some in duplicate) showed that the amount of surface LDLR was unchanged in the absence of ARH, and increased only slightly in the absence of Dab2 (Fig. 1B,C). However, depletion of both ARH and Dab2 caused a significant (P<0.001) increase in cell-surface LDLR (Fig. 1B,C). LDLR also accumulated at the cell surface when CHC was depleted (Fig. 1B,C). These results suggest that Dab2 and ARH function in parallel to mediate LDLR internalization.

To test the effects of Dab2 and ARH depletion on a single cycle of LDLR endocytosis, we labeled surface LDLR at 4°C with antibody against the extracellular domain. Cells were then warmed to 25°C for 10 minutes (to allow internalization) or not warmed (0 minutes). Non-internalized antibody was removed by acid stripping, cells were fixed and permeabilized, and internalized antibody was visualized by indirect immunofluorescence. In control cells, little fluorescence was detectable after 0 minutes at 25°C, demonstrating effective

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**Fig. 1.** Knockdown of both Dab2 and ARH results in accumulation of LDLR at the cell surface. (A) Western blot analysis of HeLa total cell lysates demonstrates that ARH, Dab2 and CHC were efficiently depleted following two rounds of transfection with short RNA duplexes specific for each target. As a control, MAPK levels remained constant in siRNA-treated cells. (B) Surface proteins were biotinylated and precipitated using streptavidin-linked agrose beads followed by western blot analysis for LDLR. Total cell lysates were also analyzed for LDLR on the same gel. (C) Densitometry was used to compare surface (biotinylated) LDLR to total cellular LDLR for each siRNA condition. The steady-state ratio of surface:total LDLR in control cells was set as 1. Depletion of ARH did not significantly change the amount of LDLR at the surface, whereas depletion of Dab2 caused a minor but significant increase (**P<0.01 as determined by t-test analysis). Depletion of both ARH and Dab2, or CHC, caused significant increases in relative surface LDLR (***P<0.001).
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removal of cell-surface antibody by acid stripping (Fig. 2A). After 10 minutes at 25°C, labeled LDLR had been internalized and was present in vesicles within the cell (Fig. 2B). ARH or Dab2 siRNA caused small but statistically insignificant reductions in LDLR internalization (Fig. 2C,D), reducing the number of cells internalizing LDLR by 12.5% and 31%, respectively, compared with control cells (Fig. 2G). Consistent with the biotinylation experiments, LDLR internalization was inhibited in most cells treated with Dab2 and ARH siRNA (Fig. 2E) or CHC siRNA (Fig. 2F). Depletion of both ARH and Dab2 caused a 64% reduction in the number of cells internalizing LDLR (P<0.001), and depletion of CHC reduced internalization by 80% (Fig. 2G). However, when coverslips were stained in parallel for ARH, Dab2 and CHC, we found that 23% of cells treated with siRNA for Dab2 and ARH still expressed at least one target protein, compared with 13% of CHC siRNA cells that still expressed CHC (Fig. 2H). Although antibody limitations precluded staining for Dab2, ARH and internalized LDLR simultaneously, the results suggest that LDLR endocytosis is inhibited in most (>90%) cells that lack both ARH and Dab2 or lack CHC.

To test whether ARH and Dab2 are needed for endocytosis of other receptors, we compared the endocytosis of Tfn and LDLR. Following siRNA depletion of target proteins, HeLa cells were incubated at 4°C with a mixture of Alexa Fluor-488-labeled Tfn and anti-LDLR antibody, and internalization was assayed after 10 minutes at 25°C. In control cells, both Tfn and LDLR antibody were internalized (Fig. 2I-K). As expected, depletion of CHC inhibited endocytosis of both ligands (Fig. 2O-Q). However, double depletion of ARH and Dab2 inhibited endocytosis of the LDLR but not Tfn (Fig. 2L-N). Therefore, in the absence of ARH and Dab2, LDLR but not TfnR endocytosis is specifically inhibited.

LDLR endocytosis in arh−/− primary cells requires Dab2

Human dermal fibroblasts (HDFs) from arh mutant patients exhibit normal LDLR endocytosis (Garcia et al., 2001; Norman et al., 1999). To determine whether Dab2 compensates for the absence of ARH in these cells, we examined LDLR endocytosis in arh−/− HDFs using siRNA to deplete Dab2 or CHC (Fig. 3A). The expression level of LDLR was identical in control and siRNA-depleted cells (Fig. 3A) but single-cycle internalization of LDLR antibody was decreased when Dab2 was removed from arh−/− HDFs. Following 10 minutes at 25°C, internalized LDLR was visible in control cells (Fig. 3B), but not in cells lacking Dab2 (Fig. 3C) or CHC (Fig. 3D). The percent of cells with internalized receptor decreased by 72% in Dab2-depleted cells (**P<0.01) and 64% in CHC-depleted cells (P<0.01). However, when coverslips were stained in parallel for ARH, Dab2 and CHC, we found that 23% of cells treated with siRNA for Dab2 and ARH still expressed at least one target protein, compared with 13% of CHC siRNA cells that still expressed CHC (Fig. 2H). Although antibody limitations precluded staining for Dab2, ARH and internalized LDLR simultaneously, the results suggest that LDLR endocytosis is inhibited in most (>90%) cells that lack both ARH and Dab2 or lack CHC.

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**Fig. 2.** Endocytosis of LDLR, but not the TfnR, is inhibited in the absence of both ARH and Dab2. (A-F) LDLR antibody internalization. siRNA-treated cells on coverslips were incubated at 4°C with anti-LDLR for 1 hour, followed by internalization for 0 or 10 minutes at 25°C. Non-internalized antibody was removed by acid stripping and cells were fixed and permeabilized. Internalized antibody was detected by immunofluorescence using a DeltaVision microscope. A 4 μm flattened Z-projection of a representative cell is shown for each condition. Bar, 15 μm. (G) The internalization experiment was repeated three times and for each experiment approximately 100 cells were assessed for internalization for each condition. Percent of cells internalizing antibody was averaged for each experiment. Depletion of both ARH and Dab2 or CHC caused a significant reduction in LDLR endocytosis (**P<0.001, as determined by t-test). (H) On separate coverslips, knockdown efficiency was evaluated for each target by immunofluorescence. Approximately 100 cells were analyzed for each siRNA. (I-Q) LDLR antibody and Alexa Fluor-488–Tfn internalization. LDLR internalization was analyzed as for A-F with the addition of labeled-Tfn to assess TfnR endocytosis. A 4 μm Z-stack projection is shown for a representative field for control (I-K), Dab2/ARH-depleted (L-N), and CHC-depleted cells (O-Q). LDLR endocytosis was intact in control cells (I), but was inhibited by depletion of ARH/Dab2 (L) and CHC (O). Tfn was internalized in control (J) and ARH/Dab2-depleted cells (M), but not in CHC-depleted cells (P). Bar, 15 μm.
cells compared with controls (Fig. 3E). These results confirm that Dab2 is required for LDLR endocytosis when ARH is absent.

LDLR endocytosis rate is decreased in Dab2-depleted cells

To determine whether the rate of endocytosis is affected by depletion of adaptor proteins, we used FACS to measure import of antibodies bound to cell surface receptors. For these studies, we utilized a chimeric mini-receptor (miniLDLR), which contains truncated extracellular and transmembrane domains from LRP1 and the cytoplasmic tail of LDLR (Li et al., 2001). The extracellular domain contains an HA-epitope tag, allowing tracking of the receptor in live or non-permeabilized cells. The miniLDLR receptor behaved similarly to endogenous LDLR in biotinylation and antibody internalization experiments (supplementary material Fig. S1), confirming that Dab2 and ARH act on the cytoplasmic domain of the LDLR.

HeLa cells were detached from tissue culture plates using 10 mM EDTA, and the cell suspension was incubated with anti-HA antibody at 4°C. Steady-state levels of surface miniLDLR were assessed by FACS. Cells depleted of either ARH or Dab2 showed only a slight increase in steady-state surface receptor levels, whereas depletion of both ARH and Dab2 caused a significant increase in surface miniLDLR levels (Fig. 4A).

Receptor endocytosis was calculated from the decrease in surface-bound anti-HA antibody following incubation for various times at 25°C (Fig. 4B, supplementary material Fig. S2). The rate of endocytosis was not significantly decreased in ARH-depleted cells compared with control cells. However, depletion of Dab2 alone significantly slowed endocytosis by approximately 60%. Likewise, CHC-depletion, or double depletion of ARH and Dab2, significantly reduced miniLDLR endocytosis, inhibiting uptake almost completely. Therefore, Dab2 supports rapid endocytosis of the LDLR in ARH-depleted HeLa cells, whereas ARH supports inefficient endocytosis of LDLR in Dab2-depleted HeLa cells.

Dab2 functions independently of AP-2

Both Dab2 and ARH bind to, and colocalize with, AP-2, and may cooperate with AP-2 to mediate endocytosis. To learn more about the mechanism of adaptor-mediated endocytosis, we examined Dab2 and ARH function when AP-2 was depleted. MiniLDLR trafficking was monitored in cells depleted, in various combinations, of Dab2, ARH and AP-2 μ2, a subunit shown previously to be required for AP-2-complex formation and TfnR endocytosis (Motley et al., 2003). Immunofluorescence showed that μ2 siRNA resulted in loss of AP-2 α from coated pits (Fig. 5A,B), and increased the steady-
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state levels of TfnR on the cell surface (Fig. 5C,E). This effect was observed when μ2 was depleted singly or in combination with Dab2 and ARH. Also as expected (Motley et al., 2003), μ2 depletion caused only a slight increase in surface miniLDLR (Fig. 5D,F). A similar effect was observed when both AP-2 and ARH were depleted, showing that Dab2 can compensate for the loss of both adaptor proteins. Surprisingly, removal of AP-2 and Dab2 caused maximal accumulation of miniLDLR at the cell surface, to the same level as when all three adaptor proteins were depleted (Fig. 5D,F). This suggests that Dab2 internalizes LDLRs independently of AP-2 but, in the absence of Dab2, ARH-mediated LDLR internalization requires AP-2.

Single-cycle antibody endocytosis experiments confirmed that Dab2 is sufficient for LDLR endocytosis in the absence of AP-2 μ2. siRNA-treated cells were incubated with anti-HA antibody at 4°C, washed, and incubated at 25°C for 10 minutes prior to acid stripping and fixation. Acid stripping efficiently removed surface antibody from control cells prior to internalization (Fig. 5G). Control cells internalized their receptor-antibody complexes after warming for 10 minutes (Fig. 5H). Simultaneous depletion of Dab2 and ARH inhibited miniLDLR endocytosis (Fig. 5I). As expected, AP-2 μ2 depletion had no effect on miniLDLR endocytosis (Fig. 5J).

Simultaneous depletion of AP-2 and ARH also had no effect, showing that Dab2 alone is sufficient for LDLR endocytosis (Fig. 5K). However, depleting AP-2 and Dab2 together completely blocked miniLDLR endocytosis (Fig. 5L), to a similar extent as depleting AP-2, Dab2 and ARH together (Fig. 5M). Therefore, Dab2-mediated endocytosis of lipoprotein receptors does not require AP-2 or ARH, whereas ARH-mediated endocytosis requires either Dab2 or AP-2. This suggests that there are two independent mechanisms for LDLR internalization, a Dab2-mediated pathway and an AP-2–ARH-mediated pathway, that operate in parallel.

Dab2 mediates clustering of LDLRs into clathrin-coated pits

To determine which step of LDLR endocytosis is blocked in the absence of Dab2 and ARH, we examined the steady-state distribution of miniLDLR on the surface of siRNA-treated cells. In control cells, miniLDLR was localized in coated pits at the cell surface (Fig. 6A). This suggests that the rate-limiting step for LDLR endocytosis occurs after LDLRs have entered coated pits but before they have pinched off from the plasma membrane, at which time the LDLR would become inaccessible to antibody. Depletion of ARH alone did not significantly change the localization of miniLDLR (Fig. 6B).

Fig. 5. Dab2-mediated endocytosis does not require AP-2. (A,B) Treatment with siRNA to μ2 adaptin causes loss of α-adaptin from coated pits. Single 0.2-μm sections at the bottom surfaces of representative cells are shown. (C-F) Steady-state surface levels of TfnR and miniLDLR were measured in control and siRNA-treated cells. Cell surface proteins were biotinylated. For TfnR analysis, lysates were immunoprecipitated using a mouse anti-TfnR antibody, and precipitated proteins were analyzed by western blot analysis first using streptavidin-HRP to detect biotinylated receptor (C, top), and then with rabbit anti-TfnR to detect total cellular TfnR (C, bottom). (E) Ratios of surface to total TfnR were determined for all conditions and compared with control cells. Depletion of AP-2 μ2 in combination with any other protein caused an increase in TfnR at the surface. For the miniLDLR, biotinylated proteins were precipitated with streptavidin-agarose beads and analyzed by western blotting using anti-HA antibody to detect the receptor (D, top). Total cell lysates were also analyzed to determine the total amount of miniLDLR (D, bottom). (F) Ratios of surface to total miniLDLR were determined for each condition and compared with controls. Depletion of AP-2 μ2 caused a large increase in miniLDLR at the surface only if Dab2 was also depleted. Error bars in E and F represent range of duplicate experiments. (G-M) Endocytosis of miniLDLR was measured in siRNA-treated cells. 4 μm Z-stack projections are shown for a representative field from each condition. No internal receptor was detected at 0 minutes (G). Following a 10-minute incubation, miniLDLR was internalized in control cells (H) but not in ARH/Dab2-depleted cells (I). Depletion of AP-2 μ2 alone (J) or in combination with ARH (K) did not affect miniLDLR endocytosis, but in combination with Dab2 (L) or Dab2/ARH (M), endocytosis was completely blocked. Bars, 15 μm.
However, knockdown of Dab2 caused a shift in receptor distribution (Fig. 6C). Even though the amount of miniLDLR at the surface was only slightly increased in the absence of Dab2 (Figs 1 and 4, supplementary material Fig. S1), the receptor became diffuse, and less was present in CCPs. As a result, although the number of miniLDLR-positive CCPs was unchanged, the size of these punctae was reduced by over 50% in Dab2-depleted cells (Table 1). Depletion of both Dab2 and ARH resulted in a similar, but more extreme, shift in receptor localization (Fig. 6D). Receptor distribution was similarly changed in cells depleted of CHC (Fig. 6E).

We quantified the changes in receptor localization by making a histogram of the profile of fluorescent pixels in areas from the ventral surfaces of ten to 14 cells from each coverslip (Fig. 6F). Bright fluorescence, from receptors in clusters (CCPs), was significantly reduced by Dab2 depletion (Fig. 6F, inset, open arrow) relative to control (closed arrow). By contrast, dim fluorescence, from receptors outside CCPs, was increased by Dab2 depletion (Fig. 6F, open arrowhead) relative to control (closed arrowhead). We set a threshold that included

![Figure 6](https://example.com/figure6.jpg)

**Fig. 6.** Surface distribution of miniLDLR is altered in the absence of Dab2. (A-E) Surface miniLDLR distribution in non-permeabilized siRNA-treated cells. The miniLDLR was detected with anti-HA antibody and visualized using a DeltaVision microscope. Single 0.2-μm sections at the bottom surfaces of representative cells are shown. Dab2 and ARH immunofluorescence (not shown) was used to confirm depletion. (F) To quantify receptor distribution, a 100×100 pixel area was analyzed for 10-14 cells of each condition. The average histogram values for these images were plotted. Punctate staining (as in control and ARH-depleted cells) results in a high number of black pixels (arrowhead) and bright white pixels spread out over a broad range of intensities (arrow, magnified region). Diffuse staining (as in Dab2, Dab2/ARH, and CHC siRNA cells) results in fewer black pixels, more grey pixels (open arrowhead) and fewer white pixels. Based on the histogram for control cells, a threshold (TH) was set. Pixels above this TH indicate clustered receptor and below this threshold indicate diffuse receptor. Similar results were obtained in independent experiments. (G) A plot of the mean pixel intensity above and below the TH shows that in Dab2, ARH/Dab2, and CHC siRNA-treated cells, there is less clustered receptor and more diffuse receptor compared with control cells and ARH siRNA treated cells (**P<0.001).** (H-L) Surface TfnR distribution was compared in siRNA-treated cells. Only in CHC-depleted cells is there a change in receptor localization, where there is more diffuse receptor accumulating on the surface. Bars, 15 μm.

### Table 1. Effect of siRNA on number and size of punctae containing miniLDLR

|                    | Number of punctae (per 100×100 pixel area) | Mean area (pixels) |
|--------------------|--------------------------------------------|--------------------|
| Control            | 56±7                                       | 18.8±2.8           |
| ARH siRNA          | 56±9                                       | 19.1±3.7           |
| Dab2 siRNA         | 61±17                                      | 8.8±1.7            |

ImageJ was used to count particles in the thresholded 100×100 pixel images analyzed in Fig. 5. The number of particles is unaffected by depletion of either ARH or Dab2, but the size is decreased in the absence of Dab2.
Therefore, Dab2 association with LDLR is independent of ARH and was reduced to only 1.3% of the cell surface. However, upon depletion of Dab2, miniLDLR or Dab2 (11.7%), or in the extent of colocalization between them (7.4%).

Clustered receptors on control images (Fig. 6F) and calculated the mean pixel intensity of pixels above the threshold (Fig. 6G, open bars) and mean pixel intensity of pixels below the threshold (Fig. 6G, shaded bars). Depletion of Dab2 caused a significant reduction in fluorescent intensity from clustered receptors and significant increases in fluorescent intensity from non-clustered receptors (P<0.001; Fig. 6G).

The shift in receptor localization in the absence of Dab2 but not ARH is striking because it indicates that Dab2 and ARH have different roles in LDLR endocytosis. The implication is that Dab2 facilitates the movement of receptors to, or their retention in, CCPs. When Dab2 is depleted, coated-pit entry or retention becomes rate limiting, and this slows the rate of receptor internalization (Fig. 4B). This is consistent with recent findings that Dab2-containing CCPs are more likely to contain LDLR (Lakadamyali et al., 2006). Given that the number of punctae did not change in the absence of either Dab2 or ARH, we conclude that these adaptors are not essential for formation of coated pits but rather for cargo flow into and out of the pits.

To confirm that these effects are specific, we monitored the surface distribution of TfnR. We found no difference in TfnR distribution in control cells compared with cells depleted of ARH and/or Dab2. Receptor was clustered similarly in these cases (Fig. 6H-K). Only upon depletion of CHC did receptor accumulate at the surface and become more diffusely distributed (Fig. 6L). This supports previous results that ARH and Dab2 are specific adaptors for lipoprotein receptors.

### Dab2 brings LDLRs to ARH

Our results suggest that Dab2 and ARH have somewhat different roles in LDLR endocytosis. Further evidence for this came from comparing the localization of the miniLDLR, Dab2 and ARH. In control cells, the miniLDLR, Dab2 and ARH were punctate and covered 10.5%, 9.9% and 8.5%, respectively, of the areas examined (Table 2). In these cells, colocalization of miniLDLR and Dab2 covered 7.3% of the area, and colocalization of miniLDLR and ARH covered 3.5% of the area. (In all cases, approximately 1% colocalization is expected by chance). Upon depletion of ARH, there was no significant change in distribution of either miniLDLR (9.4%) or Dab2 (11.7%), or in the extent of colocalization between them (7.4%). However, upon depletion of Dab2, miniLDLR became more diffuse, with clustered receptor only covering 5.4% of the area examined. The area covered by ARH remained constant (10.9%) but colocalization of miniLDLR and ARH was reduced to only 1.3% of the cell surface. Therefore, Dab2 association with LDLR is independent of ARH, whereas Dab2 – or perhaps clustered LDLR – is required for efficient association between LDLR and ARH. These results imply that Dab2 recruits LDLR to an ARH-positive compartment, which may account for the decreased rate of LDLR endocytosis in Dab2-depleted cells.

Interestingly, in the absence of CHC, the cell surface occupied by Dab2 decreases but the area covered by either miniLDLR or ARH does not (Table 2). MiniLDLR becomes more abundant at the surface but less clustered, explaining the steady coverage. The decrease in Dab2 coverage suggests that in the absence of CCPs, Dab2 localization becomes more diffuse, revealing a requirement for clathrin for Dab2 localization. However, consistent with published reports that only a fraction of ARH localizes to CCPs, ARH distribution is unaffected by CHC depletion (Mishra et al., 2002b; Nagai et al., 2003).

### Dab2 activity requires binding to clathrin, lipoprotein receptors and phospholipids

Dab2 has multiple protein-protein interaction sites that allow it to interface with the endocytic machinery (Fig. 7A). To learn more about the mechanism of Dab2 activity we examined the importance of these binding sites during LDLR endocytosis. Rescue experiments were performed by re-expressing T7-tagged wild-type or mutant forms of mouse Dab2 in HeLa cells depleted of endogenous Dab2 and ARH. In control cells expressing empty vector (pCGT), miniLDLR was clustered in punctate spots as shown previously (Fig. 7B’). In cells depleted of both ARH and Dab2 and expressing pCGT, receptors accumulated at the surface and were diffuse (Fig. 7C’). These results were quantified as described for Fig. 6, and mean receptor fluorescence in clusters (coated pits) and diffusely over the surface are shown (Fig. 7H,I). Wild-type T7-Dab2p96, detected by immunofluorescence with anti-T7 antibodies, was present in CCPs (Fig. 7D) (Morris and Cooper, 2001), and it rescued miniLDLR distribution in the absence of endogenous ARH and Dab2 (Fig. 7D’,H,I). The p67 isoform of Dab2 lacks clathrin-binding and some AP-2-binding sites, and as a result, T7-p67 was diffusely cytoplasmic and not associated with CCPs (Fig. 7E) (Morris and Cooper, 2001). T7-p67 did not significantly rescue miniLDLR movement into CCPs (Fig. 7E’,H,I). Because Dab2 and AP-2 function independently in LDLR endocytosis, we infer that the clathrin-binding sites absent from p67 are important for Dab2 endocytic activity.

The PTB domain of Dab2 has two distinct ligand-binding pockets, one that recognizes the FxNPxY internalization peptide and one that recognizes phosphoinositides at the plasma membrane (Fig. 7A) (Mishra et al., 2002a; Yun et al.,...
Mutation of a single residue in the PTB domain of Dab2 (K53Q) or Dab1 (K45Q) ablates binding to phospholipids. The point mutation S114T in Dab1 blocked binding to the FxNPxY peptide, and it was predicted that mutation of a conserved residue (S122) in Dab2 would similarly inhibit peptide binding (Huang et al., 2005; Stolt et al., 2004; Xu et al., 2005; Yun et al., 2003). These mutations were generated in the context of T7-p96 to test the requirement for these interactions in LDLR endocytosis. The subcellular localization of both mutant forms of p96 remained punctate, consistent with data that the PTB domain is not required for Dab2 localization to CCPs (Morris and Cooper, 2001) (Fig. 7F,G). However, neither T7-p96 S122T nor T7-p96 K53Q rescued miniLDLR clustering (Fig. 7F’,G’,H,I). Furthermore, mislocalization of miniLDLR at the membrane translated into endocytic defects when we used an antibody to follow a single round of receptor internalization for 10 minutes at 25°C (data not shown). These experiments demonstrate that Dab2-mediated trafficking of lipoprotein receptors requires binding to clathrin, the LDLR endocytosis signal and phosphoinositides.

Discussion

In this paper, we have described Dab2-dependent AP-2-independent and ARH-dependent AP-2-dependent mechanisms for LDLR endocytosis in HeLa cells. Depletion of Dab2 partially but significantly inhibited LDLR endocytosis, whereas depletion of ARH had little effect. However, when both Dab2 and ARH were depleted, LDLR endocytosis was strongly inhibited to the same degree as when clathrin was absent. TfnR endocytosis depends on clathrin but not Dab2 and ARH (Figs 1-4). LDLR endocytosis by arh/−/−human fibroblasts required Dab2 (Fig. 3). This suggests that Dab2 and ARH are partly redundant for LDLR but not TfnR endocytosis. Similar observations have been made by Keyel et al. (Keyel et al., 2006). By depleting AP-2 µ2, we showed that Dab2 activity is independent of AP-2, whereas ARH-mediated endocytosis requires AP-2 in the absence of Dab2 (Fig. 5). Although these studies suggested that Dab2 and ARH/AP-2 are on parallel pathways, further analysis using tagged, chimeric receptors revealed that Dab2 is more efficient than ARH at clustering of LDLR into CCPs (Figs 6 and Table 1), and that colocalization of LDLR with ARH is reduced in the absence of Dab2 whereas LDLR colocalization with Dab2 is ARH-independent (Table 2). Similar to AP-2, Dab2 activity requires simultaneous binding to clathrin, phosphoinositides and LDLR (Fig. 7).

These results suggest a model in which Dab2 and ARH/AP-2 have different modes of mediating LDLR endocytosis (Fig. 8). Dab2 and ARH bind
many of the same partners, including LDLR, PtdIns(4,5)P₂, clathrin and AP-2, although Dab2 binds the AP-2 α subunit whereas ARH binds the β2 subunit (Mishra et al., 2002b; Morris and Cooper, 2001). ARH also binds AP-2 more tightly than Dab2, with a \( K_d \) of 1-2 μM compared with ~120 μM (Mishra et al., 2005; Owen et al., 2000). Another difference is that Dab2 has two clathrin-binding sites, allowing it to nucleate clathrin-lattice formation, whereas the single clathrin-binding site on ARH is not sufficient for this activity (Mishra et al., 2002a; Mishra et al., 2002b). Finally, only Dab2 binds to MyoVI (Morris et al., 2002a). We suggest that these differences between Dab2 and ARH result in different endocytosis mechanisms (Fig. 8). We propose that Dab2 binds to PtdIns(4,5)P₂ in the membrane and to the endocytosis signal on LDLRs, and actively conveys LDLRs to nascent CCPs (Fig. 8, step 1), perhaps making use of MyoVI and the cortical actin network. Alternatively, Dab2 might initiate clathrin-lattice formation once bound to the receptor (Fig. 8, step 2). Together, these activities provide a stable mechanism for trapping LDLR that may explain why Dab2 is more effective than ARH in clustering LDLR. AP-2 is not essential for this process, although Dab2 and AP-2 colocalize in vitro and probably work together in normal cells. Presumably, in the absence of AP-2, Dab2 binds the accessory proteins required for CCP budding from the membrane (Fig. 8, step 3). When AP-2 is present, it might facilitate clathrin lattice formation, although Dab2 binding to the α appendage of AP-2 might interfere with accessory protein binding, and Dab2 might still be important for accessory protein recruitment.

A second mechanism for LDLR internalization entails ARH and AP-2 functioning together (Fig. 8, step 4). ARH links LDLR receptors to the clathrin machinery, although it might passively trap receptors in CCPs rather than recruit them from distant locations. ARH is not required for receptor clustering in lymphocytes (Michaely et al., 2004), and LDLR is diffusely localized on the basolateral surface of hepatocytes (Pathak et al., 1990), even though ARH is required for LDLR endocytosis in both of these cell types. These results are consistent with ARH being inefficient at recruitment of LDLR into CCPs, although it can promote LDLR clustering when overexpressed (Garuti et al., 2005). By binding to AP-2, ARH has a mechanism for recruiting accessory proteins involved in budding and scission (Fig. 8, step 6). However, unlike Dab2 and AP-2, ARH is not enriched in CCVs (Mishra et al., 2002b). Since ARH and clathrin compete for binding to the AP-2 β2 subunit, clathrin-lattice formation may displace ARH before budding off occurs and LDLRs may remain in the pit by binding to AP-2 or clathrin (Fig. 8, step 5). The fact that ARH and AP-2 can perform all the functions of Dab2 but are inefficient at bringing LDLR into coated pits explains why LDLR internalization is slowed when Dab2 is missing.

An intriguing difference between the Dab2-mediated vs ARH/AP-2-mediated pathways might be their ability to deliver LDLR to distinct populations of early endosomes. Recent evidence indicates that AP-2-dependent cargos, such as Tfn, are trafficked into a static population of early endosomes, whereas AP-2-independent cargos, such as LDL, are preferentially sorted into more dynamic early endosomes (Lakadamyali et al., 2006). Further studies will determine whether the LDL receptor is differentially sorted into dynamic vs static early endosomes via Dab2-mediated and ARH–AP-2-mediated endocytosis, respectively.

Given our findings that Dab2 and ARH have somewhat overlapping roles in LDLR endocytosis, it is surprising that Dab2 and ARH mutant animals have strikingly different phenotypes. Although the primary phenotype of arh<sup>−/−</sup> mice is hypercholesterolemia, dab2<sup>−/−</sup> mice show no overt phenotype related to defects in LDLR endocytosis. However, given that mutation of the LDL receptor itself only results in phenotypes associated with LDL uptake in the liver, and that ARH mediates LDLR-mediated uptake in the absence of Dab2, this is not surprising. Further work with dab2<sup>−/−</sup> primary cells is needed to determine whether there are other cell types that require Dab2 for LDLR endocytosis.

Another explanation for the tissue-specific phenotypes of dab2- and arh-mutant mice might be that Dab2 and ARH function at opposing surfaces of polarized cells. Dab2 is required for apical lipoprotein receptor trafficking in VE and kidney epithelial cells (Maurer and Cooper, 2005; Morris et al., 2002b; Nagai et al., 2005), whereas ARH is required for LDLR endocytosis at the basolateral surface of hepatocytes (Garcia et al., 2001; Jones et al., 2003). Since kidney epithelial cells and hepatocytes do express low levels of ARH or Dab2, respectively, we suspect that these adaptor proteins are specialized to mediate endocytosis from the apical versus basolateral surface. Apical endocytosis is sensitive to perturbations of the actin cytoskeleton (Apodaca, 2001). A connection between Dab2 and actin is suggested by our findings that tagged LDLR accumulates in actin-rich filopodia in HeLa cells depleted of Dab2 but not ARH (supplementary material Fig. S3). Dab2 interacts with the minus-
end-directed motor protein MyoVI (Inoue et al., 2002; Morris et al., 2002a), which has been implicated in endocytosis, and both MyoVI and Dab2 localize to filopodia in HeLa cells (supplementary material Fig. S4A,B). Furthermore, depletion of MyoVI caused a similar, but less severe, phenotype as Dab2 depletion: receptor accumulated in filopodia and became more diffuse on the plasma membrane (supplementary material Fig. S4C-H). This suggests that these proteins collaborate to move receptors off filopodia and cluster them into CCPs. Analogously, in polarized cells, apical receptors need to move to the base of the microvilli before entering CCPs. The polarity of F-actin in microvilli is such that MyoVI and Dab2 may move lipoprotein receptors towards the cell body (Biemesderfer et al., 2002). Indeed, megalin seems to use adaptors other than Dab2 (and ARH) for endocytosis in non-polarized cells: our analysis of chimeric LRPI and megalin mini-receptors shows that neither needs Dab2 or ARH for endocytosis in HeLa cells (supplementary material Fig. S5). Both these receptors contain FxNPxY and AP-2-binding traffic signals, and the latter may mediate endocytosis in the absence of Dab2 and ARH (Li et al., 2000). Thus, the special requirement for Dab2 for apical endocytosis of megalin in polarized epithelial cells may reflect a requirement for moving megalin down microvilli rather than an essential role in endocytosis per se (Biemesderfer et al., 2002).

Our structure-function studies identified multiple regions of Dab2 that are required for endocytic activity. These include the clathrin-binding sites absent from the p67 isoform, as well as the PTB-domain-binding sites for the FxNPxY endocytosis signal and PtdIns(4,5)P2. Binding to the FxNPxY motif and either clathrin or AP-2 was recently shown to be required for ARH to support LDLR endocytosis by hepatocytes (Garuti et al., 2005). AP-2 also binds to clathrin, endocytosis signals and PtdIns(4,5)P2, suggesting an analogous mechanism for all of these adaptor proteins. Given that Dab2, ARH and AP-2 each bind the same components, why is ARH not sufficient for LDLR endocytosis in the absence of AP-2 and Dab2? The answer may be that Dab2 has additional uncharacterized binding partners. In addition to clathrin, PtdIns(4,5)P2 and cargo, AP-2 recruits accessory proteins that promote membrane curvature and fission of the clathrin-coated pit through its α-subunit appendage. Dab2 might recruit similar proteins. Dab2 contains five NPF motifs, which typically interact with Eps15-homology (EH) domains, found in the endocytic proteins Eps15/Eps15R and intersectin (Santolini et al., 1999; Wong et al., 1995; Yamabhai et al., 1998), and a proline-rich region that binds SH3 domains (Xu et al., 1998). These motifs could greatly expand the network of proteins recruited by Dab2. Future studies will focus on identifying other binding partners, both intracellular adaptor proteins and other receptors that rely on Dab2 for trafficking. Identifying other binding partners might help explain the mechanism behind other activities attributed to Dab2, such as acting as a signaling protein and potential tumor suppressor, and also expand the range of activities regulated by clathrin-associated sorting proteins.

Materials and Methods

Cell culture, DNA constructs and antibodies

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. arh-7 fibroblasts (GM00697) were obtained from the Human Genetic Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). Cells were cultured in Eagle’s minimal essential medium (MEM) supplemented with 15% FCS, 2 mM L-glutamine, and 2% non-essential amino acids. The miniLDLR, miniLRP1, and mini-megalin constructs were obtained from Guojun Bu (Li et al., 2001) Constructs were modified by moving them from the pcDNA3 backbone to the pBabe-puro retroviral vector. Retroviral particles were generated by co-transfection of 293T cells with pBabe-mini receptor constructs and ecotropic 6 packaging vector. HeLa cells were infected with retrovirus, and 48 hours later selected with 2 μg/ml puromycin. Stable clones were selected, and protein expression examined by immunoblotting. For rescue experiments, vector alone (pCGT) or vector encoding T7-tagged mouse Dab2 (p67, p96, p96-K35Q and p96-S122T) were transfected into HeLa cells using polyethylenimine (PEI). K35Q and S122T mutants were generated by site-directed PCR mutagenesis using the following primers and their respective reverse complements: K35Q forward 5’-GTCTAACTAAGCCCAATGTCGTTTTTATTGATGTATG3’; S122T forward 5’-CCAGTAAATAAGATTACATTCATTGCTCG-TGAT3’. Antibodies used in these experiments included: mouse anti-LDLR (clone C7, Amersham); rabbit anti-Dab2 (Santa Cruz); mouse anti-Dab2 (BD Transduction Laboratories); rabbit anti-ARH (kind gift from L. Traub, University of Pittsburgh, Pittsburgh, PA); rabbit anti-Myc-MyoVI (against the whole globular tail, kind gift from F. Buss, University of Cambridge, Cambridge Institute for Medical Research, Cambridge, UK); mouse anti-Chitinin (BD Transduction Laboratories); mouse anti-α-adaptin (clone AP6, Calbiochem); mouse anti-T7 (Novagen); mouse anti-TfnR (Ab-1, Oncogene Research Products); mouse anti-TfnR (clone HA-11, Covance).

siRNA knockdown

Knockdown experiments in HeLa cells were performed as described by Motley et al. (Motley et al., 2003) with the following changes. 3×10⁶ HeLa cells were plated per well in 12-well plates. Two hours after plating, cells were transfected with 50 nmol of a pool of four siRNA oligonucleotides specific for ARH, Dab2, CHC, AP-2 p2, or MyoVI (Dharmacon) using Oligofectamine (Invitrogen). Oligonucleotide transfection was repeated twice, on day 1 and day 3. Twenty-four hours following each transfection, cells were split. On day 2, cells were split 1:2.5 into 6-well plates, and on day 4 cells were split onto either fibronectin-coated coverslips or 12-well plates. Assays were performed on day 5. A similar protocol was also used to knockdown Dab2 and CHC in arh-7 primary human fibroblasts. Similar results were obtained using different oligos targeting the same protein, implying knockdown effects were specific. For Dab2 only, individual RNA oligonucleotides were tested to find one specific for human Dab2. This oligonucleotide (sense sequence 5’-GAAACGAGCCUCACCCUUUUU-3’) was used for rescue experiments in which mouse Dab2 cDNAs were re-expressed in Dab2-depleted cells. For these experiments, cells were transfected with DNA vectors on day 4, to be assayed 24 hours later.

Surface biotinylation

Following knockdown, cells were grown overnight in 12-well plates. Cells were rinsed with ice-cold PBS prior to incubation with 0.5 mg/ml sulfo-NHS-biotin (Pierce) in PBS for 1 hour on ice. Following surface labeling, cells were washed once with 1% BSA-PBS to deplete any remaining biotinylation reagent. After three PBS rinses, cells were lysed in LDLR-immunoprecipitation (IP) buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1% Triton X-100, 1 mM PMSF, 1 μg/ml Aprotinin). Lysates were spun for 10 minutes at 16,000 g, and the pellet was discarded. 150 μg of total protein was incubated with 25 μl of 50-50 slurry of streptavidin-agarose beads for 2 hours at 4°C. Following three rinses with IP buffer, beads were boiled with sample buffer. Proteins were analyzed by SDS-PAGE, side by side with 15 μg of total cell lysates, followed by western blotting for specific cellular receptors.

Antibody internalization assays

For immunofluorescence-based assays, siRNA-treated cells were plated on fibronectin-coated glass coverslips. Cells were transferred to ice and rinsed with cold PBS. Mouse anti-LDLR, mouse anti-HA, and/or Alexa Fluor-488-labeled Tfn (Pierce) in PBS for 1 hour on ice. Following surface labeling, cells were washed once with 1% BSA-PBS to deplete any remaining biotinylation reagent. After three PBS rinses, cells were lysed in LDLR-immunoprecipitation (IP) buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1% Triton X-100, 1 mM PMSF, 1 μg/ml Aprotinin). Lysates were spun for 10 minutes at 16,000 g, and the pellet was discarded. 150 μg of total protein was incubated with 25 μl of 50-50 slurry of streptavidin-agarose beads for 2 hours at 4°C. Following three rinses with IP buffer, beads were boiled with sample buffer. Proteins were analyzed by SDS-PAGE, side by side with 15 μg of total cell lysates, followed by western blotting for specific cellular receptors.

Immuno-fluorescence

For immunofluorescence of intracellular proteins, fixed cells were permeabilized in 0.1% Triton X-100 for 5 minutes at 25°C. For cell-surface receptors, non-permeabilized cells were used with extracellular epitope antibodies. Prior to primary antibody addition, cells were blocked in a solution of 2% BSA, 5% normal goat serum, PBS for 1 hour at 25°C. Primary antibodies were diluted into blocking solution and added either for 3-4 hours at 25°C or overnight at 4°C. Coverslips were
rinned three times in PBS before addition of Alexa Fluor-488, Alexa Fluor-568 or Alexa Fluor-647-labeled secondary antibodies, diluted 1:1000, or Alexa Fluor-647-labeled phalloidin, diluted 1:50 (all from Molecular Probes, Invitrogen) for 1 hour at 25°C. Alexa Fluor-labeled secondary antibodies included goat anti-mouse, goat anti-mouse IgG1-isotype-specific and IgG2b-isotype-specific antibodies, and goat anti-rabbit. The isotype-specific secondary antibodies gave less robust signal intensities compared with the non-isotype-specific goat anti-mouse secondary antibodies. Following several PBS rinses, coverslips were mounted using ProLong Gold antifade and mounting medium (Molecular Probes). Cells were visualized using a 63× oil objective on a Zeiss DeltaVision microscope. Images were recorded using fixed camera settings, and deconvolved using Softworx software. For antibody internalization, Z-series projections were prepared, and approximately 100 cells from each experiment were scored as positive or negative for endocytosed antibody. To quantify cell-surface-receptor distribution, deconvolved images from single planes corresponding to the ventral surfaces of the cells were analyzed using Image J, as described in Fig. 6 legend. Figures were assembled using Adobe Photoshop and Deneba Canvas. Levels were adjusted equally for all images in a set. Contrast (gamma) was not adjusted.

FACS internalization experiments
siRNA-treated cells were detached from 6-cm dishes using 10 mM EDTA-PBS for 10 minutes at 37°C. Cells were rinsed in ice-cold PBS, pelleted by centrifugation and resuspended into cold assay medium. Mouse anti-HA antibody was diluted into cold assay medium (1:4000) and cells were incubated with antibody on ice for 30 minutes, after which they were rinsed two times in cold PBS. Cells were transferred to 25°C assay medium for either 0, 5 or 10 minutes before fixing in cold 4% paraformaldehyde-PBS for 20 minutes. Surface antibody was quantified by FACS analysis after staining with an Alexa Fluor-488 goat anti-mouse secondary antibody. Profiles were gated on intact cells, based on morphology, and the arithmetic mean intensity compared with the non-isotype-specific goat anti-mouse secondary antibody. Alexa Fluor-labeled secondary antibodies included goat anti-mouse, goat anti-rabbit. The isotype-specific secondary antibodies gave less robust signal intensities compared with the non-isotype-specific goat anti-mouse secondary antibody. Alexa Fluor-488 (gamma) was not adjusted.

To quantify cell-surface-receptor distribution, deconvolved images from single 25°C assay medium for either 0, 5 or 10 minutes before fixing in cold 4% paraformaldehyde-PBS for 20 minutes. Surface antibody was quantified by FACS analysis after staining with an Alexa Fluor-488 goat anti-mouse secondary antibody. Profiles were gated on intact cells, based on morphology, and the arithmetic mean intensity compared with the non-isotype-specific goat anti-mouse secondary antibody. Alexa Fluor-labeled secondary antibodies included goat anti-mouse, goat anti-rabbit. The isotype-specific secondary antibodies gave less robust signal intensities compared with the non-isotype-specific goat anti-mouse secondary antibody. Alexa Fluor-488 (gamma) was not adjusted.

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