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The Endocytic Protein α-Adaptin Is Required for Numb-Mediated Asymmetric Cell Division in *Drosophila*

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Summary

During asymmetric cell division in *Drosophila* sensory organ precursor cells, the Numb protein localizes asymmetrically and segregates into one daughter cell, where it influences cell fate by repressing signal transduction via the Notch receptor. We show here that Numb acts by polarizing the distribution of α-Adaptin, a protein involved in receptor-mediated endocytosis. α-Adaptin binds to Numb and localizes asymmetrically in a Numb-dependent fashion. Mutant forms of α-Adaptin that no longer bind to Numb fail to localize asymmetrically and cause *numb*-like defects in asymmetric cell division. Our results suggest a model in which Numb influences cell fate by downregulating Notch through polarized receptor-mediated endocytosis, since Numb also binds to the intracellular domain of Notch.

Introduction

Cells can use different mechanisms to generate diversity among their progeny. They can divide into two initially identical cells, which then become different by interacting with each other or with their environment. Alternatively, cell divisions can become intrinsically asymmetric when cell fate determinants are segregated into one of the two daughter cells during mitosis and establish a particular cell fate in this cell, but not in its sister cell (Horvitz and Herskowitz, 1992). In the *Drosophila* peripheral nervous system, a combination of these two mechanisms is used to create the four different cell types found in external sensory (ES) organs (Bellaiche and Schweisguth, 2001). ES organs are composed of two outer cells, which differentiate into a hair and a socket, and two inner cells, which form a neuron and a sheath. During development, these four cells arise from a single sensory organ precursor (SOP) cell in a stereotyped lineage (Hartenstein and Posakony, 1989) (Figure 1A). The SOP cell first divides into a posterior pIIa and an anterior pIIb cell. Next, the pIIb cell divides, to generate the pIIIb cell and a glia cell, which migrates away and is not part of the ES organ (Gho et al., 1999). Finally, the pIIa cell gives rise to the hair and the socket while the pIIIb cell generates the neuron and sheath.

The generation of diverse cell fates in the SOP lineage requires the *numb* gene (Rhyu et al., 1994; Uemura et al., 1989). In *numb* mutants, SOP cells divide symmetrically into two pIIa cells. Each of these, in turn, generates two socket cells, so that morphologically abnormal ES organs with four sockets are formed (Figure 1A). Conversely, when the *numb* gene is overexpressed in SOP cells, the opposite cell fate transformation is observed, and, in the most extreme case, all cells are transformed into neurons. *numb* encodes a PTB (phosphotyrosine binding) domain protein (Bork and Margolis, 1995), which is present in all cells and uniformly distributed around the cell cortex in interphase. In mitotic neural precursor cells, however, the protein concentrates in the area of the cell cortex that overlies one of the two centrosomes (Knoblich et al., 1995) and is segregated into one of the two daughter cells upon cytokinesis (Rhyu et al., 1994). Thus, Numb acts as a segregating determinant in the SOP lineage.

Numb influences cell fate by repressing signal transduction through the transmembrane receptor Notch. Notch is part of a conserved signaling pathway that is used many times during development (Artavanis-Tsakonas et al., 1995). Binding of one of the two ligands, Delta or Serrate, to the extracellular domain of Notch leads to proteolytic cleavages, first in the extracellular and then in the intracellular domain. Upon cleavage, the intracellular part of Notch translocates to the nucleus, where it converts the transcription factor Suppressor of Hairless [Su(H)] from a repressor into a transcriptional activator. Analysis of Notch function during asymmetric cell division in the bristle lineage is complicated by the fact that it is also required for lateral inhibition during specification of SOP cells (Hartenstein and Posakony, 1990). In the absence of Notch, too many SOP cells are specified, leading to the generation of supernumerary bristles. However, inactivation of Notch function after SOP cell determination using temperature-sensitive alleles leads to cell fate transformations that are opposite of those observed in *numb*: the SOP cell divides into two pIIb cells, and, in the most extreme cases, four neurons are generated (Hartenstein and Posakony, 1990). Thus, Numb and Notch act antagonistically in specifying correct cell fate in the bristle lineage. Genetically, *numb* acts upstream of *Notch* in this process (Guo et al., 1996). A model has begun to emerge in which Notch is activated in both daughter cells by its ligands Delta and Serrate, which act redundantly in the SOP lineage (Zeng et al., 1998). While Notch signaling induces the pIIa cell fate in one daughter cell, Numb prevents signal transduction in the other daughter cell and allows this cell to assume the pIIb cell fate. In vitro binding and two-hybrid experiments have suggested that Numb

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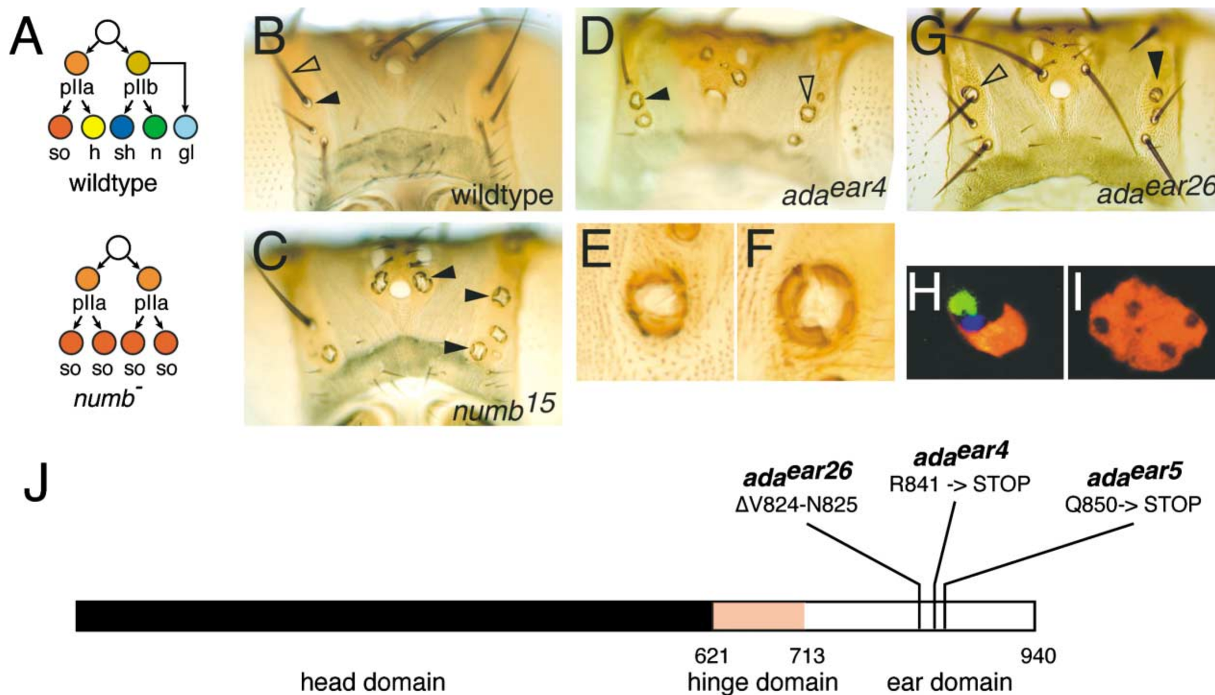


Figure 1. *ada*^{ear} Mutants Cause Cell Fate Transformations in ES Organs

(A) In wild-type ES organs, the SOP cell divides into a pIIa cell, which generates the socket (so) and hair (h) cells, and a pIIb cell, which generates the sheath cell (sh), a neuron (n), and a glia cell (gl), which migrates away. In *numb* mutants the SOP cell divides symmetrically into two pIIa cells, which generate four socket cells.

(B) Wild-type *Drosophila* head. The hair (open arrowhead) and socket (arrowhead) cells form morphologically distinct structures.

(C) When large *numb*^{15} mutant clones are introduced on the head using *eyeless*-Flp, almost all ES organs contain four sockets and no hair (arrowheads).

(D–F) In *ada*^{ear4} mutant clones, most ES organs contain either two (open arrowhead and [E]) or four (arrowhead and [F]) sockets and no hair.

(G) In *ada*^{ear26} mutant clones, many ES organs contain two sockets and no hair (arrowhead) or one hair and three sockets (open arrowhead).

(H) Wild-type ES organs contain one socket (red, Suppressor of Hairless), one sheath (blue, Prospero), and one neuron (green, Elav).

(I) *ada*^{ear4} mutant ES organs contain two (data not shown) or four (I) socket cells.

(J) Domain structure of *Drosophila* α-Adaptin as predicted by the crystal structure. Positions of *ada*^{ear} mutations are indicated. Numbers refer to amino acid positions.

directly binds to the intracellular domain of Notch (Guo et al., 1996), but how Numb binding prevents Notch signaling is unclear.

A mammalian homolog of Numb was shown to localize to endocytic vesicles and to bind to the endocytic protein α-Adaptin (Santolini et al., 2000). α-Adaptin is an essential component of the AP-2 complex, a heterotetramer that functions as an adapter between the intracellular domain of transmembrane receptors destined for endocytosis and the endocytic machinery (Clague, 1998; Robinson, 1994). Recruitment of AP-2 to the plasma membrane promotes the polymerization of clathrin into a cage-like structure, the formation of coated pits, and, ultimately, the internalization of the bound transmembrane receptors. Structural analysis has revealed that AP-2 contains a brick-like core domain, which is connected by flexible linkers to two appendage domains (Heuser and Keen, 1988). These so-called ear domains are formed by the C-terminal-most 25–30 kDa regions of α- and β2-Adaptin. They are dispensable for the formation of clathrin-coated pits (Peeler et al., 1993), suggesting that they might have a regulatory function.

We show here that, like its vertebrate counterpart, *Drosophila* α-Adaptin binds to Numb and that the ear domain is critical for this interaction. Like Numb, α-Adaptin localizes asymmetrically in dividing SOP cells and preferentially segregates into the pIIb cell. α-*adaptin* mutations that affect binding to Numb and abolish asymmetric localization cause cell fate transformations similar to those observed in *numb*. Epistasis experiments place α-*adaptin* downstream of *numb* and upstream of *Notch*, suggesting that it is involved in the suppression of Notch signaling by Numb. Our results suggest that Numb regulates cell fate by polarizing the distribution of the endocytic protein α-Adaptin.

Results

α-Adaptin Is Required for Cell Fate Specification in the ES Organ Lineage

To identify genes required for asymmetric segregation of Numb and for its function in cell fate determination, we carried out a large-scale genetic screen for mutations affecting bristle morphology. Random mutations were induced by EMS treatment and analyzed in large homozygous mutant clones of otherwise heterozygous animals. We used the *eyeless*-Flp/FRT/cell-lethal system

(Newsome et al., 2000), which induces mitotic recombination in all tissues expressing Flp recombinase from a particular *eyeless* promoter fragment. Even though this system was designed to analyze eye development, it can be used to study bristle development, since mutant clones include the whole eye imaginal disc, which gives rise to the head capsule and most of the larger macrochaete and smaller microchaete bristles on the head (Haynie and Bryant, 1986). Details of this genetic screen will be described elsewhere (D.B., T.T., M. Petronczki, S. Bulgheresi, and J.A.K., unpublished data).

Among the mutations we identified nine new alleles of *numb*, the strongest of which, $numb^{15}$, caused an almost complete cell fate transformation of all head bristle cells into four sockets (Figure 1C; compare to Figure 1B). Another complementation group consists of the two mutations ada^{ear4} (Figure 1D) and ada^{ear5} (data not shown), which also cause the generation of extra sockets and the loss of shafts (Figures 1E and 1F), and ada^{ear26}, a third allele, which showed these transformations at a lower frequency and also had missing bristles (Figure 1G). To test whether these morphological changes are due to cell fate transformations in the bristle lineage, pupae carrying ada^{ear4} and ada^{ear5} mutant head clones were stained for cell-type-specific markers of the bristle lineage. Wild-type external sensory organs consist of two small inner cells, which express Prospero (sheath cell) or Elav (neuron), and two larger outer cells, which express Su(H) (socket cell) or none of the markers analyzed (hair cell) (Figure 1H). In ada^{ear4} mutant clones, 92% of the macrochaetae on the head show cell fate transformations of inner into outer cells (either pIIb into pIIa or hair to socket). In 66% of the macrochaetae, all cells are transformed into four sockets (Figure 1I). Similar defects, even though at a lower frequency, are observed in microchaetae (44%, outer cell fate transformations; 4%, four sockets). We conclude that ada^{ear4} mutations cause cell fate transformations that are similar to, although somewhat weaker than, those observed in *numb* mutants.

ada^{ear} Mutations Affect the Ear Domain of α-Adaptin

ada^{ear} mutations were mapped to the tip of chromosome arm 2L by recombination with marked P elements of known cytological position (see Experimental Procedures for details). Based on noncomplementation of the deficiency *Df(2L)al*, we further narrowed down the cytological location to 21C1–C7. This deficiency deletes the *Drosophila* homolog of α-*adaptin*, and, indeed, all three alleles of our complementation group fail to complement a previously identified mutation in α-*adaptin* (ada^3) (Gonzalez-Gaitan and Jackle, 1997). We conclude that the ada^{ear} mutations are alleles of *Drosophila* α-*adaptin*.

A genetic analysis of α-Adaptin function in *Drosophila* has been carried out before (Gonzalez-Gaitan and Jackle, 1997). ada^3, an amorphic mutation in α-Adaptin, is late embryonic or early larval lethal and causes defects in synaptic vesicle recycling. Animals carrying the weaker allele ada^1 develop into larvae that move slowly, due to defects in synaptic vesicle recycling, and die as pupae. No obvious cell fate transformations in the bristle lineage were detected in ada^3 or ada^1 mutant clones. ada^3 mosaic animals die as pupae with severe cuticle defects, presumably due to a requirement of α-Adaptin for general endocytosis in all cells. Flies carrying ada^1 mutant clones, in contrast, are viable and show only very mild loss of bristles on the head (data not shown). Furthermore, ada^{ear}/ada^1 transheterozygous flies are viable and have no obvious phenotype, suggesting that the ada^{ear} mutant protein can still function in the endocytosis of synaptic vesicles. Taken together, these results suggest that ada^{ear} mutations preferentially affect functions of α-Adaptin that are required during bristle development.

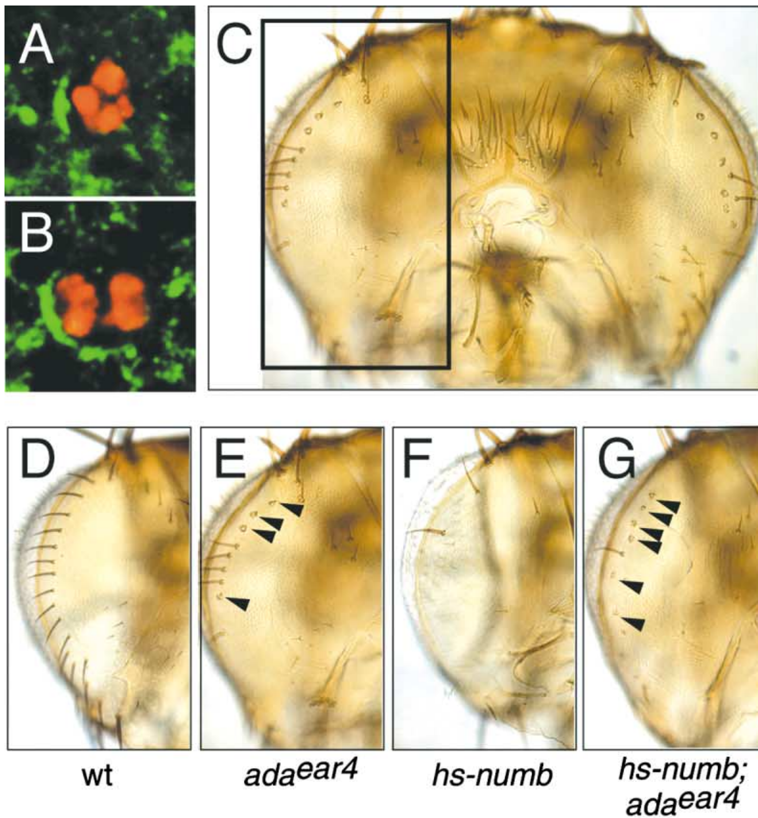
To determine which part of the α-Adaptin protein is affected in these alleles, we analyzed the exact nucleotide exchanges in ada^{ear} mutations (Figure 1J). ada^{ear4} and ada^{ear5} are C to T transitions that change an arginine at position 841 and a glutamine at position 850 into stop codons and are therefore predicted to result in C-terminal truncations of the protein. ada^{ear26} is a six-nucleotide deletion that removes valine 824 and asparagine 825 but leaves the rest of the protein intact. Thus, all of the new α-*adaptin* alleles affect the α-Adaptin ear domain, which extends from amino acids (aa) 713 to 940 of the protein. Based on the available crystal structure for this domain (Owen et al., 1999), we predict the mutations that we have identified to delete or modify a C-terminal subdomain that makes all of the important protein interactions (see Discussion for details).

α-*adaptin* Acts in the Bristle Lineage Downstream of *numb* and Upstream of *Notch*

α-Adaptin could act at several levels during cell fate specification in the bristle lineage. It could be required for Numb localization, for Notch repression by Numb, or for signal transduction downstream of Notch. To distinguish between these possibilities, we analyzed the asymmetric localization of Numb in ada^{ear4} mutants and performed epistasis experiments between α-*adaptin*, *numb*, and *Notch*.

Numb localization was analyzed by staining SOP cells of the developing eye in ada^{ear4} mutant clones for DNA and Numb. Like in wild-type SOP cells, Numb localizes into a cortical crescent overlying one of the two spindle poles from prophase (Figure 2A) to anaphase (Figure 2B) and segregates into one of the two daughter cells upon cytokinesis. Similar results were obtained for the other ada^{ear} alleles (data not shown). Thus, α-Adaptin is not required for the asymmetric localization of Numb.

To test the epistatic relationship between *numb* and α-*adaptin*, *numb* was overexpressed in α-*adaptin* mutant clones. *numb* overexpression induces transformations of externally visible outer cells (socket and hair) into inner cells (neuron and sheath), presumably because the protein segregates into both daughter cells and represses Notch (Rhyu et al., 1994). Inner cells do not produce any structures that are visible from the outside, and, therefore, these transformations cause an apparent loss of bristles. If *numb* acts downstream of α-*adaptin*, *numb* overexpression in α-*adaptin* mutant clones should revert the outer cell fate transformations observed in these clones. Conversely, if *numb* is upstream, outer cell fate transformations should still be observed. Epistasis experiments were carried out in



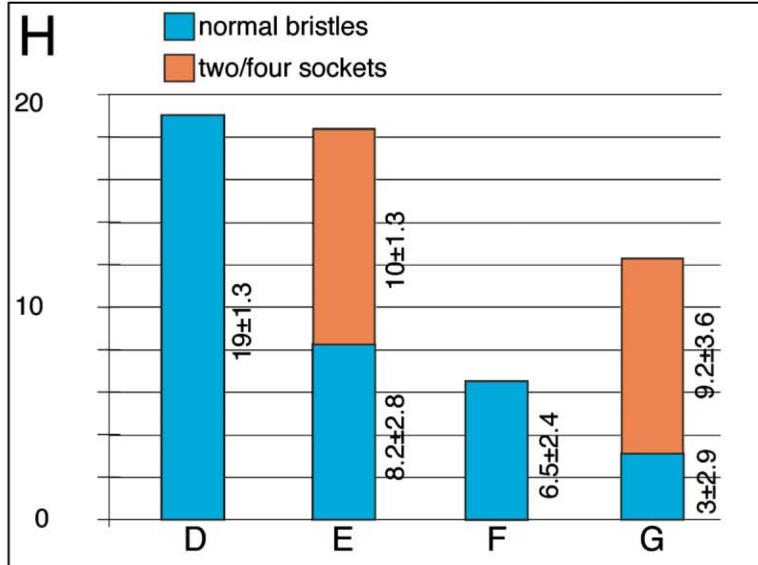


Figure 2. α-*adaptin* Acts Downstream of *numb*

(A and B) Asymmetric localization of Numb is initiated normally during prophase (A) and maintained through anaphase (B) in ada^{ear4} mutant SOP cells. Numb, green; DNA, red.

(C) In fly heads carrying large ada^{ear4} mutant clones, many postorbital ES organs have no hairs and multiple sockets. Frame indicates the region magnified in (E).

(D–G) Postorbital bristles in control (D), ada^{ear4} mutant (E), *numb*-overexpressing (*hs-numb*) (F), and *numb*-overexpressing ada^{ear4} mutant (G) flies. *numb* overexpression results in loss of most postorbital bristles in control (F), but not in ada^{ear4} mutant (G), heads. Arrowheads indicate bristles containing multiple sockets.

(H) Statistical analysis of the results presented in (D)–(G). Normal bristles, blue; bristles containing multiple sockets without hairs, red. The average number of postorbital bristles per head and their standard deviations are indicated for the individual genotypes. At least 20 heads were counted for each genotype.

postorbital bristles, which are located at the posterior edge of the eye and can easily be scored in high numbers (Figures 2C–2H). When ada^{ear4} mutant head clones are generated using *eyeless*-Flp, about 50% of these bristles show the characteristic transformation of hairs into additional sockets (Figures 2E and 2H). The other bristles are unaffected, presumably because they are not included in the mutant clones or due to perdurance of α-Adaptin protein. Overexpression of *numb* in SOP cells, on the other hand, causes a 70% reduction of postorbital bristles (Figures 2F and 2H). When *numb* is overexpressed in ada^{ear4} mutant head clones, the number of bristles bearing outer cell fate transformations is unchanged (Figures 2G and 2H). These data show that the ada^{ear4} mutant phenotype cannot be reverted upon *numb* overexpression and indicate that α-*adaptin* acts genetically downstream of *numb*.

To test whether α-*adaptin* acts upstream or downstream of *Notch*, we analyzed the *Notch*, α-*adaptin* double mutant phenotype. Inactivation of *Notch* during SOP

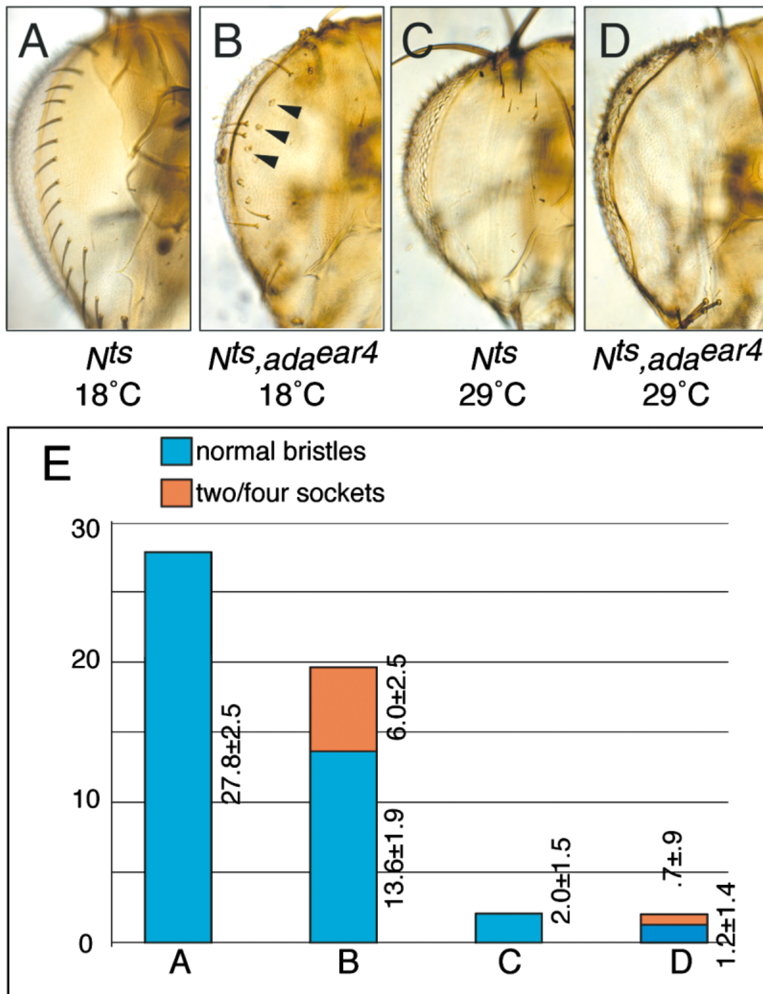


Figure 3. *α-adaptin* Acts Upstream of or in Parallel to *Notch*

(A–D) A temperature-sensitive *Notch* allele (N^{ts}) was used to inactivate *Notch* function during asymmetric cell division. Postorbital bristles are shown for N^{ts} (A and C) and for ada^{ear4} mutant head clones in an N^{ts} background (B and D) at 18°C (A and B) and 29°C (C and D).

(A) At the permissive temperature, normal bristles are formed.

(B) When ada^{ear4} mutant clones are introduced at the permissive temperature, a significant fraction of the bristles are transformed into multisockets without hairs (arrowheads).

(C) At the restrictive temperature, all bristles are absent due to transformation of outer cells into invisible inner cell types (Hartenstein and Posakony, 1990).

(D) At the restrictive temperature ada^{ear4} clones have no effect, indicating that ada^{ear4} does not cause outer cell fate transformations in the absence of functional Notch protein.

(E) Statistical analysis of the results presented in (A)–(D). Colors and labels as in Figure 2H.

division causes transformations of hair and socket cells into inner cells and leads to an apparent loss of bristles (Hartenstein and Posakony, 1989). *Notch*, *α-adaptin* double mutants should have the *Notch* phenotype if *α-adaptin* is upstream, but the *α-adaptin* phenotype if it is downstream, of *Notch*. We used a temperature-sensitive allele of *Notch* ($Notch^{ts}$) (Hartenstein and Posakony, 1989) that has no bristle phenotype at 18°C (Figures 3A and 3E) but causes essentially a complete loss of postorbital bristles when shifted to 29°C during the time of asymmetric cell divisions in the bristle lineage (Figures 3C and 3E). When ada^{ear4} mutant clones are generated in a $Notch^{ts}$ background, outer cell fate transformations are observed at the permissive temperature (Figures 3B and 3E), but not at the restrictive temperature (Figures 3D and 3E). Thus, $Notch^{ts}$, ada^{ear4} double mutant SOP cells have the *Notch* mutant phenotype, indicating that *α-adaptin* acts upstream of, or in parallel to, *Notch*.

Numb Binds to α-Adaptin In Vitro and In Vivo

Numb has been shown to bind to the intracellular domain of Notch in vitro (Guo et al., 1996). Our epistasis experiments place *α-adaptin* between *numb* and *Notch* in the pathway for cell fate specification in the bristle lineage. To test whether Numb functions as a linker between α-Adaptin and Notch, we tested a physical association between α-Adaptin and Numb. In vitro-translated full-length α-Adaptin protein binds to a GST-Numb fusion protein, but it does not bind to GST alone (Figure 4A). When the C-terminally-truncated Ada^{ear4} protein or the mutant form Ada^{ear26} is used in the binding assay, however, the interaction with Numb is strongly reduced (Figure 4A), suggesting that the ear domain is crucial for binding. Conversely, binding of α-Adaptin is strongly reduced when the C-terminal amino acids 426–546 are deleted (Figure 4B). Interestingly, the C-terminus of Numb contains a consensus α-Adaptin binding DPF motif and was shown before to be essential for Notch repression by Numb in cell culture (Frise et al., 1996).

To test whether Numb and α-Adaptin also interact in vivo, Numb was immunoprecipitated from embryo extracts using a C-terminal peptide antibody (Figure 4C). α-Adaptin can be detected in the immunoprecipitate, but the protein is not found in a control experiment where the Numb antibody is preincubated with the peptide that had been used as an antigen (Figure 4C). Thus, a fraction of α-Adaptin is bound to Numb in vivo, presumably due to a direct physical association between the two proteins that requires the α-Adaptin ear domain.

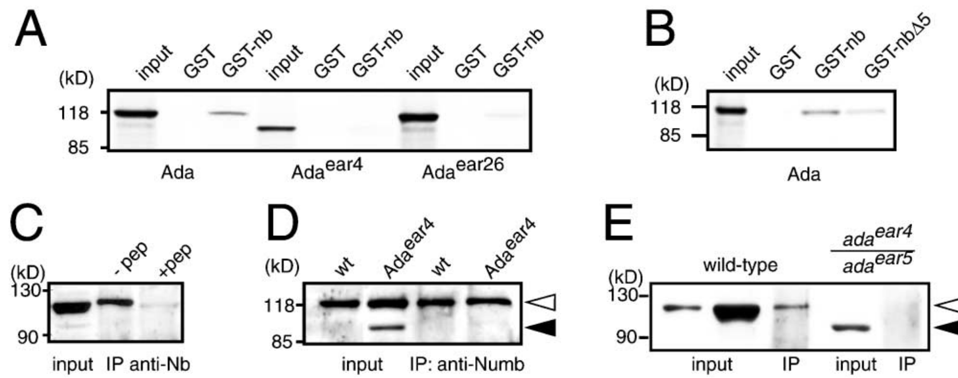


Figure 4. Numb Binds Wild-Type, but Not Mutant, α-Adaptin

(A) In vitro-translated α-Adaptin (Ada) protein binds GST-Numb, but not GST. The mutant forms Ada^{ear4} and Ada^{ear26} do not bind. One-tenth of the amount used in the binding assays is shown in the lanes marked "input."

(B) In vitro-translated α-Adaptin protein binds GST-Numb, but binding to GST-NumbΔ5 (Frise et al., 1996) lacking amino acids (aa) 426–546 is strongly reduced. Input is 1/10th of the amount used in the binding assay. Note that equal amounts of GST-Numb and GST-NumbΔ5 fusion proteins (determined by Coomassie staining) were used in the reactions.

(C) Numb was immunoprecipitated from embryo extracts using a peptide antibody. α-Adaptin can be detected in the immunoprecipitate (−pep), but not when the antibody is blocked by the immunogenic peptide (+pep). A total of 1/100th of the protein amount used in the immunoprecipitation is shown in the lane marked "input."

(D) Numb was immunoprecipitated from wild-type embryos (wt) and from embryos expressing truncated Ada^{ear4} protein from a transgene (Ada^{ear4}). The full-length (open arrowhead), but not the truncated (arrowhead), form can be coimmunoprecipitated (input is 1/100th of the protein amount used in the immunoprecipitation).

(E) Anti-Numb coimmunoprecipitates full-length α-Adaptin (open arrowhead) from wild-type, but not C-terminally truncated α-Adaptin (arrowhead) from *ada^{ear4}/ada^{ear5}* transheterozygous mutant larvae (input is 1/150th and 1/50th [wild-type] or 1/50th [mutant] of the protein amount used in the immunoprecipitation).

To test whether the ear domain is also required for Numb binding in vivo, we generated a transgene that expresses the C-terminally truncated, *ada^{ear4}* mutant form of α-Adaptin. Overexpression of this form—like overexpression of full-length α-Adaptin—has no effect on asymmetric cell division (data not shown). In embryos expressing Ada^{ear4}, both the truncated form and the endogenous, full-length version can be detected in immunoblots. When Numb is immunoprecipitated from these embryos, however, only the endogenous full-length form of α-Adaptin can be detected in the immunoprecipitate (Figure 4D). Furthermore, Numb and α-Adaptin can be coimmunoprecipitated from wild-type, but not from *ada^{ear4}/ada^{ear5}* transheterozygous mutant larvae (Figure 4E). Thus, the in vivo interaction between Numb and α-Adaptin is disrupted in *ada^{ear}* mutants. The *numb*-like cell fate transformations observed in these mutants indicate that binding to α-Adaptin is crucial for Numb to carry out its function during asymmetric cell division.

α-Adaptin Localizes Asymmetrically in Dividing SOP Cells

Numb localizes asymmetrically and segregates into one of the two daughter cells during SOP cell division. To test whether α-Adaptin shows a similar subcellular localization, we stained wild-type pupae for Numb, α-Adaptin, and the SOP marker Asense. During interphase Numb is uniformly cortical, and the α-Adaptin protein is detected in dots that are distributed in the cytoplasm and accumulate at the cell cortex. During metaphase, however, Numb localizes asymmetrically, and the α-Adaptin protein is concentrated in the same area of the cell cortex as Numb (Figure 5A; asymmetric localization of α-Adaptin was seen in 18 of 18 metaphase SOP cells). The asymmetric localization of both proteins is maintained during anaphase (Figure 5B) and telophase (Figure 5C), and, after division, α-Adaptin is preferentially found in the pIIb cell that also inherits Numb (Figure 5D). Unequal distribution of α-Adaptin is only found in a subset of two-cell pairs that show Numb in only one daughter cell, probably indicating a higher turnover rate of α-Adaptin protein.

α-Adaptin localization requires Numb. When large mitotic clones homozygous for the strong allele *numb^{15}* are induced by the *eyeless*-Flp/FRT system, no Numb protein can be detected in mutant SOP cells by antibody staining. α-Adaptin no longer localizes asymmetrically in these cells (0 of 33 *numb^{15}* mutant metaphase SOP cells scored) but instead is uniformly distributed around the cell cortex throughout mitosis (Figure 5E). To test whether the α-Adaptin ear domain is required for asymmetric localization of the protein, the distribution of α-Adaptin was analyzed in *ada^{ear}* mutant clones. In *ada^{ear4}* and *ada^{ear5}* mutant cells, the cortical localization of α-Adaptin is very weak, and it is difficult to assess whether the residual cortical protein is asymmetrically localized (data not shown). In *ada^{ear26}* mutant SOP cells, in contrast, the protein is concentrated at the cortex but fails to localize asymmetrically during mitosis, even though Numb localization is unaffected (Figure 5F). Thus, binding to Numb seems to be required for the asymmetric localization of α-Adaptin. Furthermore, the cell fate transformations observed in α-Adaptin mutants that disrupt Numb binding suggest that Numb functions

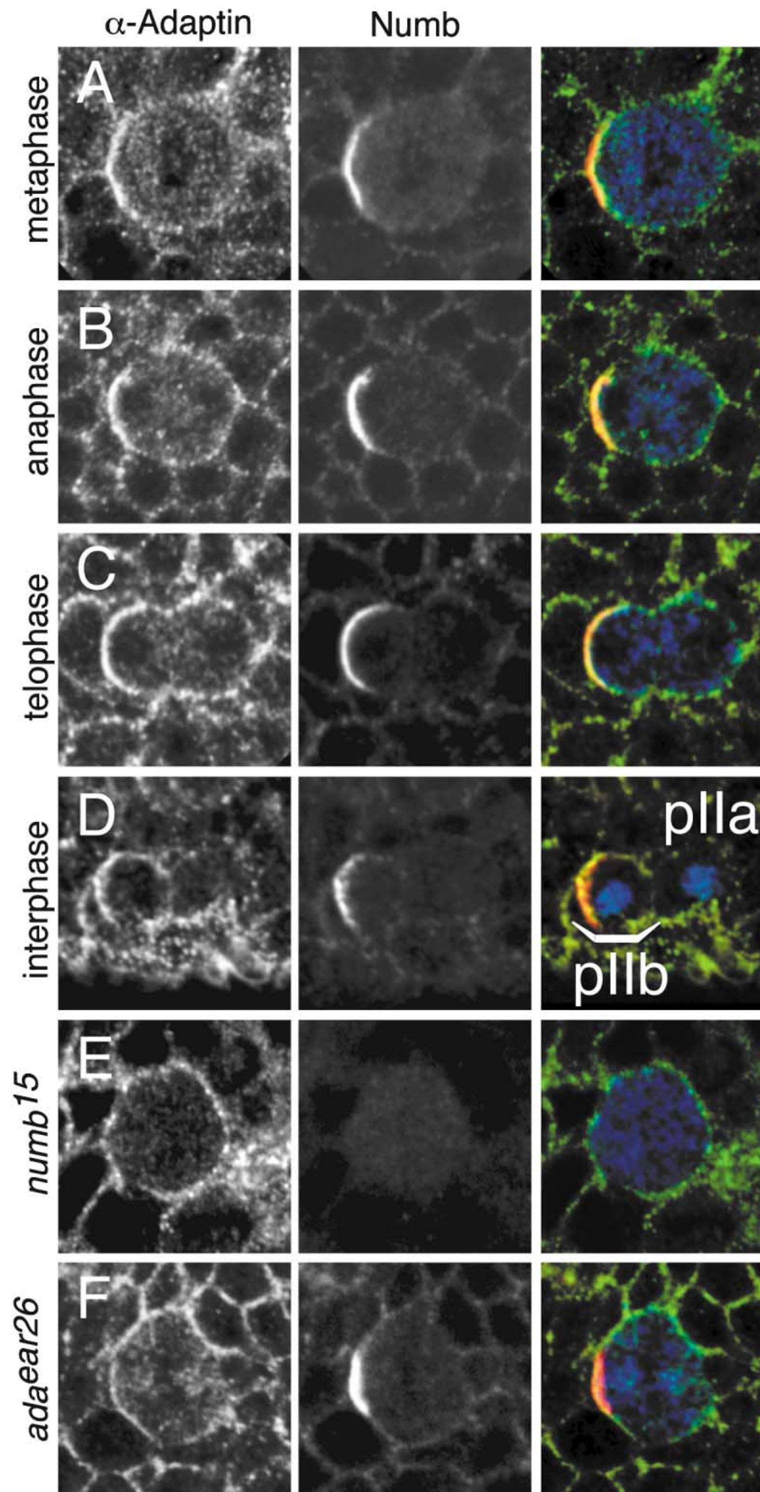


Figure 5. Numb-Dependent Asymmetric Localization of α-Adaptin

(A–F) Mitotic pupal SOP cells costained for α-Adaptin (left), Numb (middle), and Asense, a marker for SOP cells. Right panels show the merged images (α-Adaptin, green; Numb, red; Asense, blue). Mitotic cells were identified based on cytoplasmic localization of Asense and on condensed DNA, which is visible as an area of lower cytoplasmic background staining.

(A–D) In wild-type pupae, α-Adaptin is concentrated in the area of the cell cortex, where Numb is localized during metaphase (A), anaphase (B), and telophase (C). After division (D), higher levels of α-Adaptin are often found in the pIIb cell that also inherits Numb. Note however, that α-Adaptin levels in the two daughter cells are different in only a subset of interphase cells that also have unequal levels of Numb.

(E) In *numb*^{15} mutant clones, Numb is not detectable, and α-Adaptin is not asymmetric.

(F) In *ada*^{ear26} mutant clones, α-Adaptin is cortical but does not accumulate in the cortical area where Numb is localized.

by recruiting α-Adaptin to one side of the cell cortex during asymmetric cell division.

Discussion

During asymmetric cell division, the Numb protein segregates into one of the two daughter cells, where it inhibits signaling via the transmembrane receptor Notch. The results we present here suggest that the main function of Numb in SOP cells is to polarize the distribution of α-Adaptin, a component of the endocytic machinery. We show that Numb binds to the endocytic protein α-Adaptin and is responsible for its asymmetric localization during mitosis. Mutations in α-Adaptin that

affect asymmetric localization of the protein (*ada^ear^* mutations) cause phenotypes similar to those observed in *numb* mutants. Genetically, α-*adaptin* acts upstream of *Notch* and downstream of *numb*. Together with the previous observation that Numb binds to the intracellular domain of Notch, our results suggest that Numb influences cell fate by linking the Notch receptor to α-Adaptin on one side of an asymmetrically dividing cell.

ada^{ear} Mutations Affect a Subset of α-Adaptin Functions

α-Adaptin serves a dual function. During receptor-mediated endocytosis, ligand binding induces recruitment of the protein to the intracellular domain of transmembrane receptors. This regulates signal transduction by targeting the receptor for endocytosis. Genetic analysis of this function during signal transduction is hampered by a second role in constitutive, ligand-independent endocytosis of certain transmembrane receptors, for example, the transferrin receptor. Constitutive endocytosis is essential for cell viability, and, therefore, α-Adaptin null mutant cells do not survive (Gonzalez-Gaitan and Jackle, 1997). The new *ada^{ear}* alleles we have identified do not affect cell viability. Several observations indicate that they are not simply hypomorphs but that they specifically affect particular functions of α-Adaptin during signal transduction. First, *ada^{ear}* mutants are viable over known α-*adaptin* hypomorphs. These hypomorphs affect larval motility due to impaired synaptic transmission at the neuromuscular junction (Gonzalez-Gaitan and Jackle, 1997). No such defects are observed in *ada^{ear}* mutants, suggesting that α-Adaptin function during synaptic transmission is not, or is only weakly, impaired. Second, *ada^{ear}* mutants have defects in asymmetric cell division, but no such defects have been described for any of the known alleles.

All *ada^{ear}* mutants affect the C-terminal so-called ear (or appendage) domain of the protein. This domain was shown by electron microscopy to stick out from a brick-shaped central core in the AP-2 complex (Heuser and Keen, 1988). The crystal structure of this domain (Owen et al., 1999) reveals an N-terminal subdomain (lysine 713–Lysine 826 in the *Drosophila* protein) and a C-terminal subdomain (Phenylalanine 827–Phenylalanine 940) connected by a short linker. While the C-terminal subdomain makes all the known protein interactions, the N terminus forms a scaffold that displays the C-terminal domain in the correct orientation. All *ada^{ear}* mutants affect the C-terminal subdomain: *ada^{ear4}* and *ada^{ear5}* introduce stops at the beginning of this subdomain. *ada^{ear26}* deletes two amino acids in the linker between the two subdomains and might disrupt the correct spatial arrangement of the C-terminal subdomain. Thus, *ada^{ear}* mutations are predicted to generate forms of α-Adaptin that lack functional ear domains.

Our experiments suggest that the ear domain is not required for α-Adaptin function in general endocytosis. While recent experiments have suggested a role of Adaptin ear-domains in clathrin assembly (Clairmont et al., 1997), our results are more consistent with previous experiments in which removal of this domain by proteolytic cleavage did not affect the ability of AP-2 to assemble clathrin-coated pits and form endocytic vesicles in vitro (Keen and Beck, 1989; Peeler et al., 1993). The α-Adaptin ear domain was shown to bind other important endocytic proteins, like Eps-15 and Epsin (Owen et al., 1999), and these important interactions should be disrupted in *ada^{ear}* mutants. However, both proteins also bind to the β2-Adaptin ear domain (Owen et al., 2000), and redundancy between the binding sites could explain why the mutant protein is still partially functional.

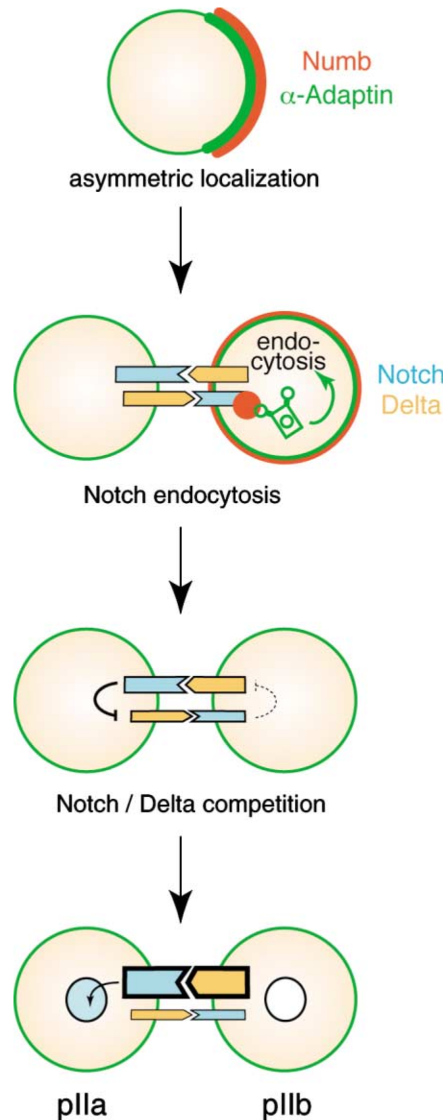


Figure 6. A Model for Cell Fate Specification in the ES Organ Lineage

During SOP division, Numb localizes asymmetrically and is required for the asymmetric localization of α-Adaptin. Simultaneous binding of Numb to α-Adaptin and to the Notch intracellular domain links Notch to the AP-2 complex and targets it for degradation through the endocytic pathway. Small differences in Notch levels between the two daughter cells are amplified by a negative-feedback loop that is known to exist in cells that communicate with each other via the Notch/Delta system (Heitzler et al., 1996).

α-Adaptin and Asymmetric Cell Division

Our results show that α-Adaptin is essential for Numb to repress Notch signaling in the pIIb cell. α-Adaptin is

part of the AP-2 complex that has a well-established function in clathrin-mediated endocytosis of transmembrane receptors (Clague, 1998; Gonzalez-Gaitan and Jackle, 1997; Robinson, 1994; Takei and Haucke, 2001). At the moment, we cannot exclude that the role of α-Adaptin in asymmetric cell division reflects a novel function of the protein that is not related to endocytosis. However, all the known functions of α-Adaptin are connected to endocytic processes, and, therefore, our results indicate that Numb influences cell fate by polarizing endocytosis of a critical component of the Notch pathway. Several results indicate that this critical component is the Notch receptor itself. First, α-*adaptin* functions genetically upstream of Notch, and, therefore, proteins acting downstream of Notch are unlikely to be targets for endocytosis. Second, cell culture experiments have shown that Numb acts in the Notch expressing, signal receiving cell, and it is therefore unlikely to act on Delta or Serrate (Frise et al., 1996). Third, Numb can interact with the intracellular domain of Notch (Guo et al., 1996) in vitro and in two-hybrid assays. This in vitro interaction is supported by the observation that transfection of Numb can recruit the Notch intracellular domain to the cell cortex in tissue culture cells (Frise et al., 1996).

We propose a model in which Numb serves as an adapter that links the AP-2 complex to the intracellular domain of Notch on one side of a dividing SOP cell (Figure 6). This targets the receptor for endocytosis and reduces Notch signaling in the pIIb cell. After division, reciprocal signaling between the two daughter cells can lead to competition, and small differences could be amplified by negative-feedback loops that are known to exist between cells that communicate via Notch and Delta (Heitzler et al., 1996). A slight reduction of Notch levels in one of the two cells should be enough to bias this competition and to establish the pIIb cell fate in the cell that inherits Numb. Consistent with this, we fail to detect differences in Notch protein levels in the two daughter cells by immunofluorescence (data not shown).

This model is plausible; nevertheless, alternative explanations cannot be excluded. For example, Numb could specifically act on the activated form of Notch. Upon ligand binding, proteolytic cleavage removes the extracellular domain of Notch and generates an intermediate fragment that is anchored in the plasma membrane (Fortini, 2001). A second, unregulated cleavage by the transmembrane protease presenilin (Struhl and Greenwald, 1999; Ye et al., 1999) at the plasma membrane (Nowotny et al., 2000) releases the intracellular domain, which translocates to the nucleus to activate gene transcription (Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). α-Adaptin could enhance endocytosis of the intermediate fragment and target it for degradation before the second cleavage can occur. This would reduce Notch signaling without significantly affecting the total amount of Notch protein.

A Conserved Role for Numb and Related Proteins in Endocytosis

A vertebrate homolog of Numb was previously shown to bind α-Adaptin (Santolini et al., 2000). Like in flies, the ear domain is essential for this protein interaction, but the functional significance of this interaction in vertebrates is not completely understood. Fragments of Numb that could act as dominant negatives inhibit both EGF and transferrin-receptor uptake in cell culture (Santolini et al., 2000), suggesting a function for Numb in both constitutive and ligand-induced endocytosis. In *Drosophila*, however, no phenotypes are detected upon overexpression of the analogous domain (data not shown). Furthermore, none of mutant phenotypes described for *numb* so far indicates defects in EGF-receptor signaling or cell lethality, and, therefore, a function in general endocytosis is highly unlikely. Whether these differences reflect inhibition of other pathways by the dominant negative or a true difference across species is not clear.

In *Drosophila* the known phenotypes of *numb* can be explained by a function in asymmetric cell division. One of the mouse Numb homologs, in contrast, is not asymmetrically localized (Zhong et al., 1997) and is unlikely to be involved in asymmetric cell division. Regulation of α-Adaptin-mediated endocytosis is a more likely common task of the Numb protein family. In fact, the function in endocytosis may extend to other more distant sequence homologs: the cell death protein Ced-6 and the adaptor protein ARH are the closely related to Numb (Garcia et al., 2001). The molecular function of Ced-6 is not understood, while ARH is mutated in familial hypercholesterinaemia and involved in endocytic uptake of the LDL receptor upon ligand binding (Garcia et al., 2001). Thus, Numb, Ced-6, and ARH might be members of a protein superfamily involved in endocytosis of transmembrane receptors.

Experimental Procedures

Identification of ada^{ear} Mutations

The *eyeless*-Flp /FRT/cell-lethal system (Newsome et al., 2000) was used to generate large mutant clones on the head of otherwise heterozygous flies. The *eyeless* promoter fragment used in this system is expressed in the whole eye imaginal disc. The adult fly head is derived from the eye imaginal disc (Haynie and Bryant, 1986), and, therefore, mutant clones include most of the micro- and macrochaetae on the head. For a genetic screen, adult male flies carrying an isogenized FRT 40A chromosome were mutagenized by EMS treatment following standard procedures and crossed to females carrying a cell-lethal mutation on FRT 40A. Fifty thousand F1 progeny were screened for bristle phenotypes that indicate cell fate transformations. Details of this genetic screen will be described elsewhere. Among 47 mutants we recovered nine new alleles of *numb* and three alleles of α-*adaptin*. $numb^{15}$ was mapped between 28B and 30F using a recombination strategy (see below) and is lethal over *Df(2L)30AC* (which includes *numb*) as well as the known numb alleles $numb^{1}$ and $numb^{2}$. $numb^{15}$ is a strong apparent protein null allele that causes almost complete transformation of all ES organs into four sockets in clones and complete absence of all neurons in the PNS in germline clones. α-*adaptin* mutants were mapped by recombination (see below) and complementation of known deficiencies and mutants. ada^{ear26} is early larval lethal, while animals homozygous for ada^{ear4} or ada^{ear5} develop into larvae that contain melanotic tumors and die during third instar. For sequence analysis, DNA was isolated from homozygous larvae identified by the absence of a $yellow^{+}$ marker on the balancer chromosome, and the α-*adaptin* coding region was analyzed by PCR sequencing. Mutations described here were the only sequence differences to the paternal chromosome. Other α-*adaptin* alleles used in this study were described previously (Gonzalez-Gaitan and Jackle, 1997).

Recombination Mapping of Mutations

The following recombination strategy was used to map mutations isolated in the screen: females carrying the mutation on *FRT 40A* over a mapped homozygous viable P element containing a *[w+]* marker were crossed to males carrying *eyeless-Flp* and a cell-lethal mutation on *FRT 40A*. In the next generation, mutations become homozygous by mitotic recombination, and mutant phenotypes can be scored on the head. If the mutation lies proximal to the P element, recombinant chromosomes carrying *FRT 40A*, the mutation, and *[w+]* can be recovered, while recombinants carrying *FRT 40A* and neither the mutation nor *[w+]* indicate location of the mutation distal to the P element. Recombination with 20 evenly spaced P elements (list available on request) maps mutations within one cytological division.

Immunofluorescence

Pupae were aged for 24–26 hr (for lineage) or 15–17 hr (for Numb and α-Adaptin localization) after pupariation, dissected in 5% paraformaldehyde, fixed for an additional 20 min at room temperature, and stained, essentially as described (Rhyu et al., 1994). Note that the asymmetric distribution of α-Adaptin cannot be observed when pupae are dissected and fixed on ice. Antibodies were mouse anti-Elav (1:30, mAb9F8A9; Developmental Studies Hybridoma Bank), rabbit anti-Prospero (1:1000) (Vaessin et al., 1991), rat anti-Su(H) 1:2000 (gift from Francois Schweisguth), rabbit anti-Numb (1:100) (Schober et al., 1999), guinea-pig anti-Asense (1:5000) (Brand et al., 1993), mouse anti-Numb (1:200) (Schaefer et al., 2001) and rabbit anti-α-Adaptin (1:100) (Gonzalez-Gaitan and Jackle, 1997). Images were recorded on a Zeiss LSM510 confocal microscope and processed with Adobe Photoshop.

Epistasis Experiments

For *numb* epistasis, an *hs-numb* transgene (Rhyu et al., 1994) was mobilized and reinserted on the X chromosome. In the experiment, flies of the genotype *ey-Flp/hs-numb; adaear4, hs-numb, FRT 40A/l(2)cl-L3^1, P[w+] 30C FRT 40A* and their balancer siblings of the genotype *ey-Flp/hs-numb; adaear4, hs-numb, FRT 40A/CyO* were used with and without heat shock. For heat shocks, pupae were collected at 25°C, aged to 16–18 hr APF, subjected to two 45 min 37°C heat shocks separated by a 30 min 25°C recovery period, and allowed to hatch at 25°C. Adult flies were dissected in isopropanol and mounted in Hoyer's mount. For *Notch* epistasis, flies of the genotype *N^{ts}, ey-Flp/Y; adaear4, FRT 40A/l(2)cl-L3^1, P[w+] 30C, FRT 40A* and their balancer siblings of the genotype *N^{ts}, ey-Flp/Y; adaear4, FRT 40A/CyO* were used at the permissive and the restrictive temperature. The permissive temperature is 18°C. For heat treatment, flies were raised at 22°C, and pupae were collected for 2 hr, aged to 16–18 hr APF, and heat treated at 29°C for 6 hr, after which they were returned to 22°C and allowed to hatch.

Binding Experiments

For in vitro binding experiments, α-Adaptin was translated in vitro from the EST SD07441 (Berkeley Drosophila Genome Project) using the TNT kit (Promega). α-Adaptin-ΔC was generated by introduction of a stop codon after amino acid 841 via PCR (identical to *ada^{ear4}*). The full-length *numb* cDNA (Rhyu et al., 1994) and the deletion construct *numbΔ5* (Frise et al., 1996) were cloned in-frame into pGEX4T-1 (Pharmacia) to express a full-length GST-Numb fusion protein or a fusion protein lacking amino acids 426–546. Fusion proteins were bound to GST according to the manufacturer's instructions, and binding assays and immunoprecipitations were performed as described (Schober et al., 1999). To express truncated α-Adaptin, *adaptin-ΔC* was cloned into *pUAST* and expressed in transgenic flies using maternal *GAL4 V32* (D. St. Johnston and J.P. Vincent, unpublished data). For immunoprecipitations from *ada^{ear}* mutants, *ada^{ear4}/ada^{ear5}* mutant larvae were identified by absence of a *yellow$^+$* gene on the balancer chromosome. Protein extracts were made after removal of gut tissue from 100 larvae. Numb was immunoprecipitated using a rabbit peptide antibody (Schober et al., 1999), and α-Adaptin was identified by immunoblotting using rabbit anti-α-Adaptin (1:100) (Gonzalez-Gaitan and Jackle, 1997).

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