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Supplemental Data

The Big Brain Aquaporin Is Required for Endosome Maturation and Notch Receptor Trafficking

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Notch signaling in *hrs bib* double mutant tissues

In addition to the EM analysis of the *hrs bib* double mutant clones presented in Figure 3J and 3K, we also examined the relative levels of Notch signaling in clones of this genotype. The *hrs bib* mutant clones display levels of *E(spl)mβ-CD2* expression that are only slightly reduced compared to wildtype cells, consistent with previous findings that *hrs* mutants show little or no effect on Notch signaling (Jékely and Rørth, 2003; Lloyd et al., 2002; Seto and Bellen, 2006) and that *hrs* mutant clones exhibit normal *E(spl)mβ-CD2* expression (Supplemental Figure S1). Considering that other signaling molecules accumulate in *hrs* endosomes and signal persistently, the relatively normal levels of Notch signaling in *hrs* mutants might reflect stalling of Notch in an early compartment that allows prolonged signaling albeit at low efficiency, and hence which is not as strongly impaired by removal of Bib as it is in the invaginating endosomes of *bib* single mutants. In contrast, overactive Notch signaling caused by *lgd* mutants in a later endosomal compartment is strongly dependent on Bib (see Figure 6), revealing a clearer requirement for Bib in Notch signaling from later stage endosomes characterized by acidified, multivesicular structures.

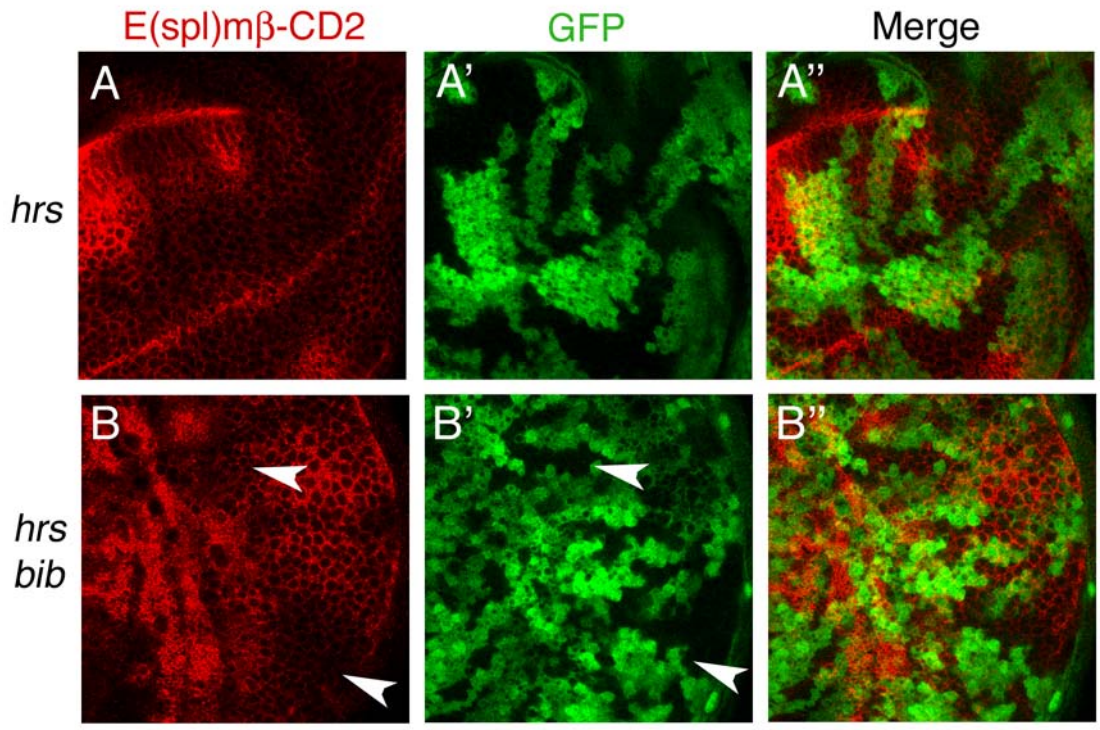

Figure S1. Expression of the Notch reporter *E(spl)mβ-CD2* in *hrs* and *bib* hrs mutant clones

Third-instar *Drosophila* wing imaginal discs bearing *hrs* mutant clones or *hrs bib* double mutant clones analyzed by confocal microscopy. Image triplets depict horizontal confocal optical sections through a single disc quadrant, showing expression of *E(spl)mβ-CD2* in red (A, B), mutant clone locations (A', B'; areas devoid of green GFP signal), and merged images of both signals at right (A", B").

(A) *E(spl)mβ-CD2* expression in *hrs* single mutant clones.

(B) *E(spl)mβ-CD2* expression in *hrs bib* double mutant clones. Arrowheads indicate regions of slightly reduced *E(spl)mβ-CD2* expression that overlap with *hrs bib* clones. Note that areas displaying less *E(spl)mβ-CD2* signal do not tightly correspond to exact clone positions and shapes, suggesting that the effects might be due to non-autonomous regulatory crosstalk involving other pathways on Notch signaling, such as Egfr or Wg signaling that is known to be upregulated in *hrs* mutants (Jékely and Rørth, 2003; Lloyd et al., 2002; Seto and Bellen, 2006).

Requirement of γ-secretase activity for internalization of Notch into endosome clusters

Endocytosis of Notch and Delta is a key step in Notch signaling, and triggers the γ-secretase-mediated intramembrane cleavage of Notch to liberate the active signaling fragment NICD (reviewed in Fortini, 2002; Le Borgne et al., 2005). Previous studies have suggested that γ-secretase cleavage of Notch occurs in an endocytic compartment

coincident with or immediately following ligand-induced removal of the Notch ectodomain, a process that might be facilitated by the dynamic forces exerted upon Notch and Delta during the membrane budding events of endocytosis (Brou et al., 2000; Mumm et al., 2000). These considerations, along with the presence of Bib in endocytic Hrs-positive intracellular vesicles and the endosome defects in *bib*-deficient cells, prompted us to investigate the relationship of Bib to γ-secretase activity in the endosomal trafficking pathway.

To eliminate γ-secretase function within *bib* mutant clones, we generated clones that were simultaneously mutant for both *bib* and *aph-1*. Aph-1 is an essential γ-secretase cofactor, with elimination of Aph-1 causing a loss of mature γ-secretase and a failure in Notch intramembrane cleavage (Francis et al., 2002; Hu and Fortini, 2003; Takasugi et al., 2003). Unlike other γ-secretase components, the gene for Aph-1 is located on the same chromosome arm (2L) as *bib*, permitting recombination of both mutants onto one chromosome arm to produce double mutant clones. For these studies, we used the *aph-1* null allele $aph\text{-}1^{D35}$, which leads to a complete absence of mature γ-secretase and no detectable intramembrane cleavage of Notch (Hu and Fortini, 2003). Interestingly, *bib aph-1* double mutant clones do not exhibit an abnormal localization of Notch or Delta in enlarged intracellular puncta (Supplemental Figures 2A and 2B), unlike those detected earlier in *bib* single mutant clones (cf. Figure 1A-D). The finding that the *bib* mutant endosomal accumulation of Delta is suppressed by the *aph-1* mutant suggests that this accumulation might reflect internalization of activated, ligand-bound Notch receptors in a γ-secretase-dependent manner, since *trans*-endocytosis of Delta is associated with Notch receptor signaling (Klueg et al., 1998; Parks et al., 2000).

These results are consistent with at least two alternative roles for γ-secretase in the endosome routing of Notch and Delta. One possibility is that elimination of γ-secretase prevents the endosome maturation defect observed in *bib* mutants, while the other possibility is that it does not prevent the defect from arising, but precludes Notch and Delta from trafficking into the abnormal compartment. To distinguish between these two models, we performed TEM on *bib aph-1* double mutant clone-bearing discs. In the *bib aph-1* double mutant, large endosome clusters consisting primarily of early invaginating endosomes were again observed (Supplemental Figure 2C), indicating that the activities of γ-secretase in Notch trafficking and/or cleavage, and those of Bib in promoting endosome maturation into MVBs, can be genetically uncoupled. Our data are most consistent with a model in which incorporation of ligand-activated Notch into the γ-secretase complex is required for its initial internalization from the cell surface into early endosomes, and that Bib is subsequently required for a separate downstream step in the maturation of signaling endosomes.

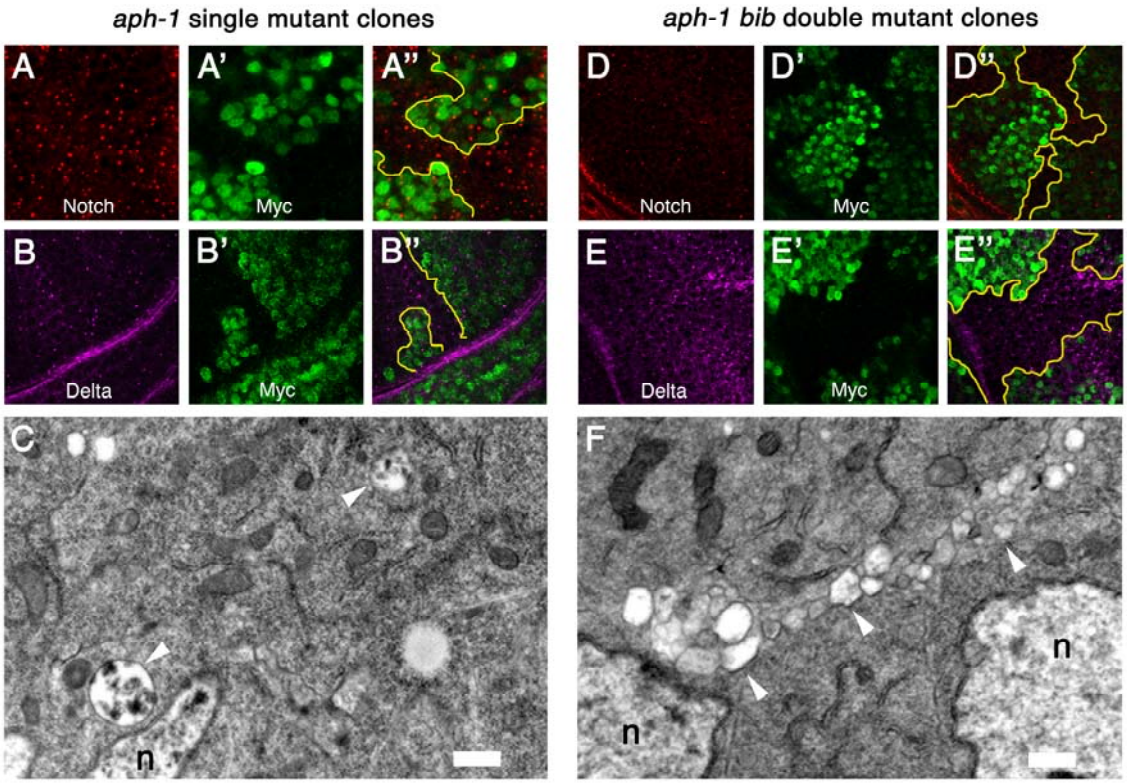

Figure S2. Relationship of the *bib* endosome phenotype to γ-secretase activity
Third-instar wing imaginal discs bearing homozygous mutant tissue deficient for either *aph-1* alone (A-A'', B-B'', C) or *aph-1* and *bib* together (D-D'', E-E'', F). For the confocal analyses, each triplet of images represents a single disc quadrant, showing the subapical distribution pattern of Notch (red) or Delta (magenta) at left, the clone locations (absence of green Myc signal) at center, and the corresponding merged images at right with approximate clone borders indicated by yellow tracings.
(A-A'') Subapical distribution of Notch in *aph-1* mutant clones using an antibody that recognizes the Notch intracellular domain.
(B-B'') Delta protein distribution in *aph-1* mutant clones.
(C) Representative transmission electron micrograph (TEM) of *aph-1* mutant discs, showing absence of large endosome clusters and readily detectable MBVs (arrowheads). Scale bar, 500 nm; n, nuclei.
(D-D'') Subapical distribution of Notch in *aph-1 bib* double mutant clones.
(E-E'') Delta protein distribution in *aph-1 bib* double mutant clones.
(F) Representative TEM of an *aph-1 bib* double mutant clone, revealing large endosome clusters similar to those seen in *bib* mutant cells (arrowheads; cf. Figure 3). Scale bar, 500 nm; n, nuclei.

Bib activity requires a functional pore domain
Members of the aquaporin protein family exhibit selective permeability to water, ions, or other small solutes depending upon the structural characteristics of their membrane-

spanning pore domain (reviewed in King et al., 2004). In *Xenopus* oocytes, heterologously expressed *Drosophila* Bib has been shown to function as a nonselective monovalent cation transporter, and mutagenesis of the highly conserved channel residue glutamate-71 to asparagine (E71N) causes a bidirectional block in ion conductance without affecting the membrane localization of Bib (Yanochko and Yool, 2002; Yanochko and Yool, 2004; Supplemental Figure 3A).

To determine whether the function of Bib in endosome biogenesis and Notch trafficking depends upon the ability of Bib to conduct ions, we tested whether transgenically-expressed wildtype Bib and the E71N mutant variant were able to rescue the *bib* loss-of-function mutant phenotypes. An ~16.5 kb genomic rescue fragment encompassing the intact *bib* transcribed region (Bib-WT; see Experimental Procedures) fully rescued the *bib* mutant embryonic lethality and neurogenic phenotypes to produce viable, morphologically normal, fertile adults (Supplemental Figure 3B). As noted earlier, Bib-WT also rescues the Notch endosome accumulation phenotype of *bib* homozygous mutant clones (Supplemental Figure 3D). In contrast, the equivalent Bib E71N mutant construct (Bib-E71N) failed to rescue these *bib* mutant phenotypes (Supplemental Figures 3B and 3E). As in *Xenopus*, the E71N mutant form is properly localized in *Drosophila* cells; an epitope-tagged Bib E71N mutant is targeted predominantly to the Hrs-positive endosome compartment (Supplemental Figure 3C).

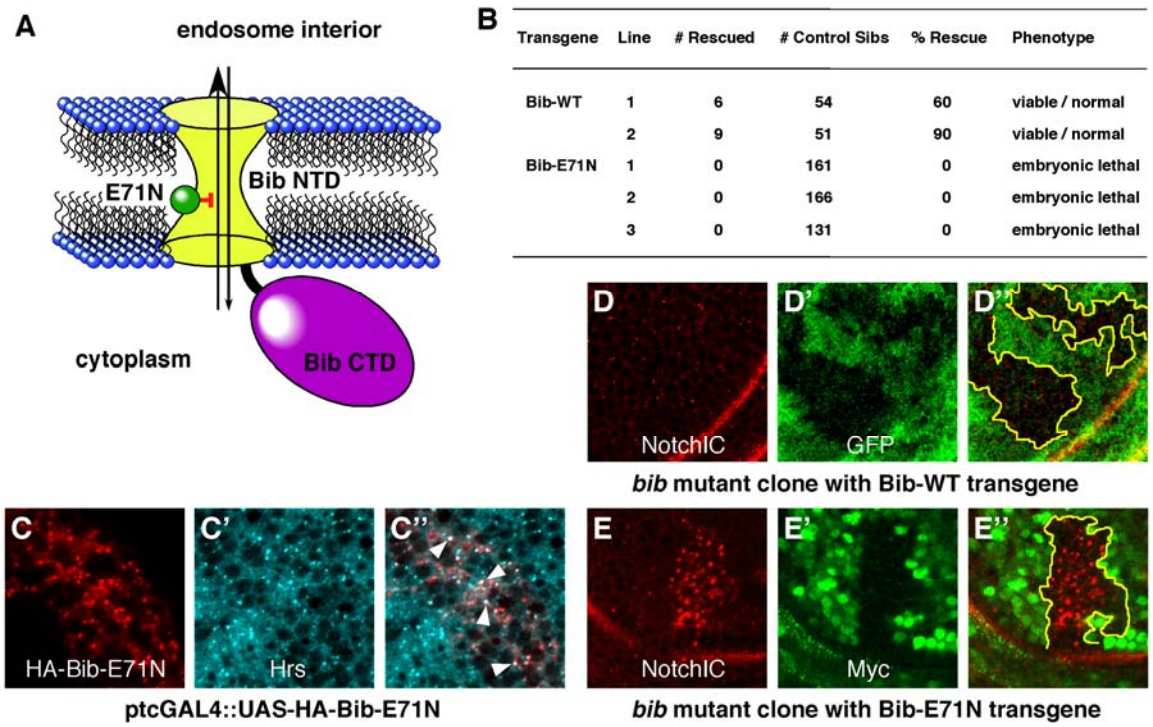

Figure S3. Requirement for Bib pore domain function in endosome maturation and developmental patterning

(A) Diagram of the Bib protein, showing the membrane-spanning pore formed by the Bib N-terminal domain (Bib NTD in yellow), as well as the cytoplasmically exposed Bib C-terminal domain (Bib CTD in purple). The E71N mutation that has been demonstrated to

impede bidirectional cation flow across the *Drosophlia* Bib pore domain (Yanochko and Yool, 2002; Yanochko and Yool, 2004) is indicated in green.
(B) The lethal developmental phenotype of *bib* loss-of-function mutants is rescued by a wildtype ~16.5 kb bib^{+} genotype transgene (Bib-WT), but not by an equivalent genomic transgene expressing the E71N mutant variant of Bib (Bib-E71N). *CyO* (Control Sibs) and *non-CyO* (Rescued) progeny were scored from crosses of bib^{1}*/CyO; transgene/+* x *Df(2L)BSC50/CyO* (which uncovers *bib*, see Experimental Procedures), as shown in columns 3 and 4. Assuming Mendelian segregation, a trangene exhibiting full rescuing activity would be expected to yield 1 *non-CyO* survivor for every 5 *CyO* siblings, producing a survival rate of 1/6. Column 5 shows the measured activity of each transgene expressed as a percentage of the expected 1/6 rate. For two independent insertion lines of the Bib-WT transgene, all survivors recovered were morphologically normal adults with no apparent Notch pathway-related developmental defects. For three independent insertion lines of the Bib-E71N transgene, representing two independent PCR-generated mutant constructs, no survivors beyond the embryonic stage were observed despite >100 control *CyO* siblings scored for each test.
(C-C'') Subcellular distribution of HA-Bib-E71N expressed using *patched* (*ptc*)-*GAL4* (C) in a wing imaginal disc also labeled for Hrs (C') showing colocalization in some intracellular puncta (C'').
(D) Rescue of the endosome phenotype of *bib* mutant clones by the Bib-WT transgene, as determined by immunolabeling for the Notch intracellular domain (NotchIC) as in Figure 1 (Notch signal in D; GFP labeling of clones in D'; approximate clone borders traced in yellow in the merged image in D''). Clones in three separate discs were optically z-sectioned from their apical to basal surfaces (16 sections per disc in 2 μm increments), and no difference in Notch distribution was detected between mutant and wildtype regions. Both insertion lines of Bib-WT exhibited full rescue in this assay.
(E) The Bib-E71N genomic transgene fails to rescue the *bib* endosome phenotype when assayed as above (Notch signal in E; Myc labeling of clones in E'; approximate clone borders traced in yellow in the merged image in E'').

Supplemental Experimental Procedures

Wildtype and Bib mutant E71N transgenes The Bib-WT transgene was constructed by subcloning a 20.0 kb *KpnI-SpeI* fragment corresponding to *Drosophila* genomic sequences between map coordinates 2L:9,980,096 and 2L:10,000,095 from BAC clone RP9806G16 (cf. FlyBase; Crosby et al., 2007) and trimming it to an ~16.5 kb *NruI-SpeI* fragment for subcloning into the *Drosophila* germline transformation vector pCaSpeR-4 (Thummel et al., 1988). The E71N genomic mutant was made by subcloning an ~11 kb *NheI-SpeI* fragment and introducing the E71N mutation by a three-way ligation using PCR products from primer pair 5'-attgactatggttttg**gctagc**tggtgtgcg-3' and 5'-cgtatccac**ggatcc**atcatcagcaactgtctggcctcc -3' with *NheI* and *BamHI* sites (in bold) respectively and bearing the E71N mutation (underlined above), and the primer pair 5'-gaaggaggccagacagttgctgatgatg**gatc**-3' and 5'-ttaattgccccct**agagctc**ccccatccg-3' with *XhoII* and *SacI* sites respectively (in bold). The resulting *NheI-SacI* fragment was used to replace the equivalent fragment of the Bib-WT construct, thus creating Bib-E71N. The HA-tagged Bib expression constructs HA-Bib and HA-Bib-E71N were made by inserting

HA epitope-encoding sequences at the 3' end of the *bib* cDNA (LD45157) and subcloning the resulting fragment into pUAST. The E71N mutation was introduced by site-directed mutagenesis using oligonucleotides 5'-ccatcatcagcaactgtctggcctcc-3' and 5'-ggaggccagacagttgctgatgatgg-3'. All PCR products were confirmed by sequencing.

Transgenic flies were generated by microinjection of *Drosophila* embryos with the above constructs together with transposase helper plasmids pP{Δ2-3} or pπ25.7wc using standard methods (Rubin and Spradling, 1982). For Bib-E71N, transgenic flies were produced by the CBRC Transgenic *Drosophila* Core (Harvard Medical School).

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