

Red and green traffic signals

Dual-tagged proteins give a more complete picture of Notch receptor trafficking pathways.

The development of sensory bristles in the *Drosophila* notum begins with the asymmetric division of sensory organ precursor cells (SOPs) to form anterior pIIb and posterior pIIa cells. pIIb cell fate depends on a protein called Numb and its ability to inhibit the Notch signaling pathway. aPKC directs Numb's asymmetric segregation into the anterior pIIb cell, where it is thought to delay the endocytic recycling of Notch and its binding partner Sanpodo to the plasma membrane (1, 2). However, by simultaneously tagging Notch and Sanpodo with red and green fluorescent proteins, Couturier et al. reveal that Numb sorts these signaling proteins to late endosomes and lysosomes (3). The study highlights the limitations of using single fluorescent protein tags and provides a new tool for studying membrane trafficking in general.

Lydie Couturier, François Schweisguth, and colleagues at the Institut Pasteur in Paris had previously studied Numb's effects on Notch and Sanpodo using GFP-tagged versions of these proteins (1). This approach had suggested that all three proteins might accumulate in apically localized endosomes in pIIb cells, but, to check that the proteins colocalized with each other in vivo, Couturier et al. generated a version of the Notch receptor tagged with the red fluorescent protein mCherry (3). To their surprise, the researchers saw that Notch-mCherry didn't even seem to colocalize with Notch-GFP in the notum of *Drosophila* pupae. Whereas GFP-tagged Notch localized to cell-cell contacts and early/sorting endosomes, Notch-mCherry localized in larger intracellular structures that corresponded to late endosomes and lysosomes.

Even flies expressing a version of Notch tagged with both GFP and mCherry showed green and red fluorescence in different parts of their notum cells. Couturier et al. determined that dual-tagged Notch was delivered to cell-cell contacts without being cleaved, but, for some reason,



FOCAL POINT

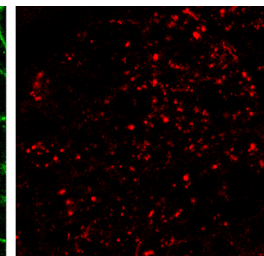
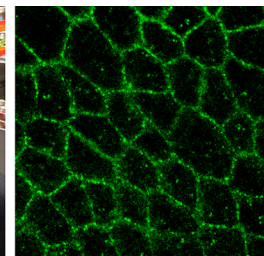


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(Left to right) Mateusz Trylinski, Lydie Couturier, Khallil Mazouni, François Schweisguth, and (not pictured) Léa Darnet reveal that the endocytic regulator Numb down-regulates Notch signaling by sorting the Notch receptor and its binding partner Sanpodo to late endosomes/lysosomes during *Drosophila* sensory bristle morphogenesis. The researchers uncovered this pathway by dual tagging Notch and Sanpodo with both green and red fluorescent proteins after realizing that individually tagged Notch-GFP (middle) and Notch-mCherry (right) showed different fluorescence patterns representing two distinct populations of the Notch receptor. This dual-tagging approach could be useful for following the endocytic sorting of many other membrane proteins.

mCherry wasn't fluorescent at this location. "So the [apparent] differences in localization were due to the properties of the two fluorochromes," explains Schweisguth. "These properties aren't usually taken into account by the many people who use GFP and mCherry."

In effect, GFP and mCherry were revealing two distinct populations of Notch and Sanpodo; one (GFP positive) at the cell surface and in early endosomes, the other (mCherry positive) in late endosomes and lysosomes. Couturier et al. determined that mCherry fluorescence was largely absent from cell-cell contacts because mCherry takes much longer than GFP to fold and become fluorescent. Newly synthesized Notch was transported to the plasma membrane and then removed by endocytosis before mCherry was able to fluoresce.

In contrast, GFP fluorescence was largely absent from late endosomes and lysosomes because of its sensitivity to the acidic pH of these organelles.

Dual-tagged Sanpodo showed a similar separation of green and red fluorescence in living cells, as did another membrane protein, E-cadherin, when it was labeled with both GFP and mCherry. "Each fluorochrome only reveals a subset of membrane proteins in living cells," Schweisguth warns.

"If you just use one tag, you'll miss a fraction of the total population."

Couturier et al. realized, however, that tagging Notch and Sanpodo with both GFP and mCherry would enable them to follow the membrane proteins' transport from one subpopulation to the other. The researchers quantified the fluorescence of dual-tagged Sanpodo in pIIb and pIIa cells over time and found that, specifically in the Numb-containing pIIb cells, Sanpodo was gradually transferred from high GFP/low mCherry early endosomes to low GFP/high mCherry late endosomes. This transfer was blocked in the absence of Numb, suggesting that Numb inhibits Notch signaling in pIIb cells by sorting Sanpodo (and, presumably, Notch) to late endosomes and lysosomes for degradation.

Schweisguth points out that this dual-tagging approach could be used to follow the trafficking of many other membrane proteins in vivo, just as similar sensors have been used to monitor autophagy (4) and cytoplasmic protein turnover (5). Schweisguth himself, however, now plans to investigate how aPKC localizes asymmetrically to the anterior cortex of dividing SOP cells.

1. Couturier, L., et al. 2013. *Curr. Biol.* 23:588–593.
2. Cotton, M., et al. 2013. *Curr. Biol.* 23:581–587.
3. Couturier, L., et al. 2014. *J. Cell Biol.* <http://dx.doi.org/10.1083/jcb.201407071>.
4. Kimura, S., et al. 2007. *Autophagy*. 3:452–460.
5. Khmelinskii, A., et al. 2012. *Nat. Biotechnol.* 30:708–714.

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