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Mechanisms underlying cell-cell communication and cell function during *Drosophila* development

Research in the lab addresses the mechanisms underlying interactions between cells, that give rise to the formation of elaborate patterns and organs during development. While most of the pathways and genes that participate in patterning have been identified through genetic screens, two central issues in development remain open. One is the interphase between cell and developmental biology. In other words, how do the developmental signaling pathways operate in the context of the cell, its organelles and the extracellular milieu, and what general features of the cell impinge on developmental signaling. Another aspect of the same problem concerns the mechanisms by which basic processes of the cellular machinery, e.g. actin polymerization, are recruited to drive specific morphogenetic events. The second open direction concerns the way in which quantitative fluctuations in cell communication during development are buffered, such that the final outcome is fixed and robust to these fluctuations. Both issues go beyond the classical genetic analysis, traditionally studied by gene loss-of function. In the case of the interphase with cell biology, the challenge is to dissect distinct developmental roles of general cellular components. To study robustness, the systems should be manipulated by altering the levels of their components, but not eliminating them. The ways in which the two frontiers are addressed will be presented through three major research topics in the lab.

The EGF receptor pathway- a conserved cassette dictates diverse cell fates

This pathway is one of a handful of signaling cascades that guide all aspects of embryonic and post embryonic development, and is utilized multiple times during development. The different components comprising this signaling cassette have been identified. In view of a conserved signaling cassette, how is the pathway modified to fit the different biological scenarios in which it operates?

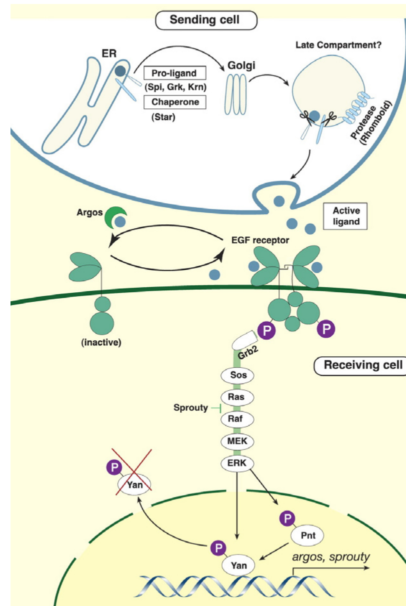

***Fig. 1** The EGF receptor pathway in Drosophila. Three membrane-anchored ligands are retained in the ER, and are processed following trafficking by the chaperone protein Star, which is dedicated to these molecules, to a late compartment in the secretory pathway.*
In the late compartment, the ligands encounter Rhomboid proteins, seven transmembrane intra-membrane serine proteases, which cleave the ligand precursors within the transmembrane domain, to release the active, secreted form.
Rhomboids also cleave and inactivate Star, thus attenuating the level of cleaved ligand that is produced. Within the receiving cells, the ligands encounter the EGF receptor, which upon dimerization triggers the canonical Sos/Ras/Raf/MEK/MAP kinase pathway. The cardinal transcriptional output of the pathway is mediated by the ETS protein Pointed. In addition, the ETS protein Yan provides a constitutive repressor, which competes for Pointed binding sites, and can be removed from the nucleus and degraded upon phosphorylation by MAP kinase. Several negative regulators keep the pathway in check. Especially important is a group of inducible repressive elements, which constitute a negative feedback loop. Argos is a secreted molecule, which sequesters the ligand Spitz, and Sprouty attenuates signaling within the receiving cell.

We focus on the cells that produce the signal in the form of a secreted ligand, since this is where the spatial and temporal features of the pathway are regulated. We explore the role of differential compartmentalization of Rhomboid (Rho) proteases that process the EGF receptor ligands, in modulating the amount of secreted ligand, and consequently the level of EGFR activation. The mSpitz ligand precursor is retained in the ER, and is trafficked by the chaperone Star to a late compartment of the secretory pathway, where Rho-1 resides. Two other Rhomboid proteins, Rho-2 and Rho-3, which are expressed in the germline and in the developing eye, respectively, cleave the Spitz precursor and Star already in the ER, in addition to their activity in the late compartment. This property attenuates EGFR activation, primarily by compromising the amount of chaperone that can productively traffic the ligand precursor to the late compartment, where cleavage and subsequent secretion take place. These observations identify changes in intracellular compartment localization of Rhomboid proteins as a basis for signal attenuation, in tissues where EGFR activation must be highly restricted in space and time.

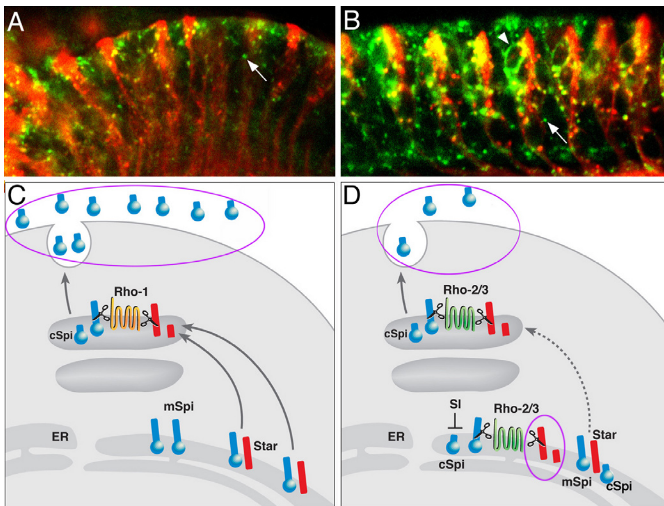

***Fig. 2** ER targeting of Rhomboids reduces active ligand secretion. Rhomboid proteins were expressed in the the epitheium of the larval eye imaginal disc. (A) Rhomboid-1 (green) is detected in punctate structures (arrow) corresponding to a late scretory compartment. (B) Rhomboid-3 (green) is located in punctate structures (arrow), but in addition also shows prominent peri-nuclear distribution (arrowhead) corresponding to the ER. (C) The canonical Spitz processing by Rho-1. Cleavage in the late compartment occurs after the chaperone, Star, has accomplished at least one round of Spitz trafficking, and results in high levels of secreted ligand. (D) ER activity of Rho-2 and -3 reduces the amounts of active, secreted Spitz. Star cleavage in the ER (circled) prevents the chaperone from trafficking Spitz to the late compartment. ER cleaved Spitz is retained by a specific, Sl-dependent mechanism. Thus, only the residual Star molecules, which "escaped" cleavage by the protease in the ER, are capable of trafficking Spitz precursor (and possibly also cleaved Spitz) to the late compartment, where the activity of Rho-2 or -3 produces the secreted ligand, at reduced levels.*

Once the secreted ligand is released, it triggers EGFR in neighboring cells. The extent of activation depends on the amount of ligand released, and on negative feedback loops that attenuate the signal. Graded activation of EGFR is observed, e.g. by following activated MAPK, depending upon the distance from the source of the signal. Yet, induction of target genes, most notably the gene encoding the ETS protein PointedP1, is observed as a sharp threshold. What is the mechanism converting graded EGFR activation to distinct thresholds of target-gene expression? We have obtained an initial glimpse into this problem, by following the EGFR/MAPK-dependent degradation of the ETS transcriptional repressor Yan, which shows a sharp threshold of degradation. By combining experimental and computational tools, in collaboration with the group of Naama Barkai, we have examined different mechanisms of threshold responses. We came to the conclusion that Yan degradation fits the zero-order hypersensitivity model. In other words, Yan shuttles between two reversible states (phosphorylated and dephosphorylated), and is in excess with respect to the kinase and phosphatase enzymes carrying out the opposing activities. Eventually, a sharp switch will occur, where all Yan molecules will be found in only one state, depending simply on the difference between the rates of the two opposing reactions. This mechanism is appealing because it can buffer local temporal fluctuations, and can generate distinct thresholds for different proteins under the same MAPK activation gradient. We are now examining whether it can also apply for the activation of transcription factors which can undergo reversible phosphorylation and dephosphorylation. We have identified a candidate for the transcription factor that is situated between MAPK and induction of *pointedP1* transcription, and will test if the distribution of its MAPK-phosphorylated intermediates agrees with the zero-order hypersensitivity model.

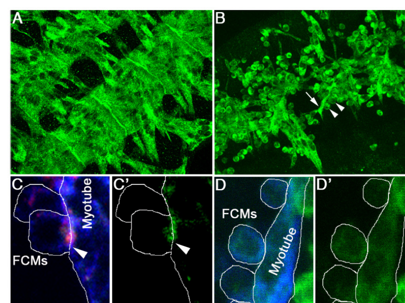

***Fig. 3** WIP recruits WASp to muscle-fusion sites. (A) In wt embryos formation of multi-nucleated muscle fibers is completed by the end of embryogenesis. (B) In mutants for D-WIP, muscle founders undergo a single round of fusion, and most fusion-competent myoblasts (FCMs, arrowheads) remain attached to the myotube (arrow), but fail to complete fusion. (C) The WASp protein (green), activating the actin-nucleating factor Arp2/3, is recruited to the fusion sites between the myotube and FCMs (arrowhead), via the adaptor WIP (red). (D) Recruitment of WASp depends upon WIP, as no recruitment was observed in D-WIP mutant embryos.*

Actin-nucleating factors control muscle fusion

Actin-nucleating factors including WASp, SCAR/WAVE and Dia/Formin control a wide range of cellular functions, such as cell motility and endocytosis. In view of these wide pleiotropic roles, it is challenging to identify distinct morphogenetic functions for these pathways. We have used specific adaptors, as well as the ability to inactivate these pathways in defined tissues, as an entry point to address this issue. These efforts converged surprisingly on a distinct process, namely the fusion of individual myoblasts (muscle cells) to form multinucleated muscle fibers. In the *Drosophila* embryo, muscles are formed by the specification of distinct muscle founder cells in each segment, defining the thirty odd muscle types that will form. In order to gain muscle

mass, each founder fuses to fusion-competent myoblast cells.

Our entry point to the process stemmed from the analysis of WIP/Verprolin, which serves as an anchoring protein for the WASp actin nucleator. WIP is expressed in embryos only in the muscles, before they fuse, and mutants in WIP are defective in muscle fusion. The definitive phenotype of WIP mutants triggered subsequent experiments that demonstrated the role of WASp in the same process. In the absence of WIP or WASp, myotubes associate with myoblasts, small fusion pores are formed, but fail to extend in order to complete fusion. We are exploring the mechanism by which regulated actin polymerization drives the enlargement of the fusion pores.

Interestingly, analysis of mutants and inactivating constructs for members of a second actin-nucleating complex, SCAR/WAVE, also demonstrated a requirement in muscle cell fusion. However, in the absence of this pathway, fusion was arrested prior to the formation of the fusion pores. Thus, the two actin-nucleating factors appear to carry out distinct and consecutive roles during muscle cell fusion.

The process of building muscles by definition of templates and recruitment of myobalsts by cell fusion is repeated at the pupal stage, for the formation of the adult fly muscles. When the effect of WASp on embryonic muscle cell fusion was circumvented, we observed (in collaboration with the lab of Vijay Raghavan, NCBS Bangalore) defects in muscle cell fusion in the pupa, leading to the formation of abnormal, rudimentary muscles in the adult. This is the first identification of a genetic background which arrests adult muscle cell fusion, and provides a new handle to study and dissect this process. In parallel, we are testing whether other elements that are essential for embryonic muscle cell fusion are also operating in the adult.

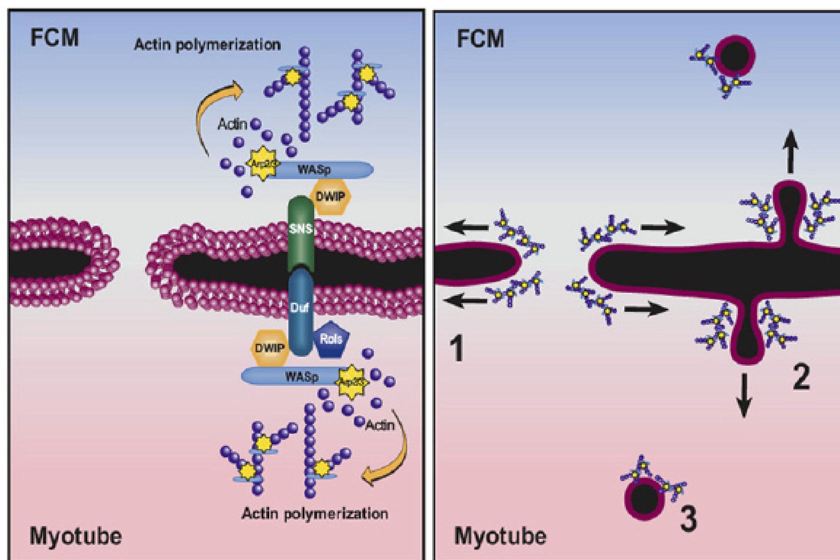

Fig. 4 *Models for recruitment of the Wsp-Arp2/3 system to myoblast attachment sites. (Left) D-WIP localizes to cell-surface regions of myotube-FCM contact and adhesion, via association with the transmembrane myoblast adhesion proteins Duf and SNS. D-WIP recruits Wsp to the myoblast contact area, providing for localized nucleation of actin filaments via the Arp2/3 complex in both myoblast types during fusion. Force generated by localized, Arp2/3-based actin polymerization is utilized after pores have formed in the apposed membranes. (Right) This force can be used for (1) enlargement of the nascent pores via interaction of branched actin networks with the perforated membranes, (2) breakdown of membranes via endocytosis, or (3) propulsion of vesiculated membrane particles, formed during the final stages of fusion, away from the myoblast attachment sites.*

Given the evolutionary conservation of the WASp and SCAR/WAVE actin nucleators, it is tempting to speculate that these elements are involved in mammalian muscle fusion, a process whose molecular basis remains mostly unknown. We plan to address this issue directly, by studying muscle development in *WASp* mutant mice.

Robust developmental patterning

Since development of multicellular organisms is dictated by cell-cell interactions mediated by conserved signaling pathways, how are reproducible patterns obtained despite fluctuations in the doses of the signaling components. To address these questions, we are combining experimental and computational approaches, in collaboration with the lab of Naama Barkai. We employ a systems-level approach to a restricted signaling module, where we assume that all the components within the module are known. The challenge is to obtain a quantitative understanding of the design principles of the module, in a situation where most of the quantitative *in vivo* parameters (e.g. concentration, affinity, diffusion rates) are not known.

We find that robustness of a pathway to fluctuations in the levels of its components provides a major restriction, and significantly limits the number of potential mechanisms that generate pattern. Thus, we initially check the pathway for its robustness to heterozygosity for mutations in the different components. If the pathway is robust, we describe by equations the possible interactions between components, and are liberal in giving values that span several orders of magnitude. For each of the cases the outcome is solved, and subsequently examined under conditions where ligand levels are halved. Only a very small fraction of solutions shows robustness. The restricted values of these solutions guides us towards the mechanism of robustness, and allows to design experiments that will indeed examine these predictions.

This approach was utilized to address the patterning of early embryos by BMPs. Ligand levels are uniform in the dorsal part of the early *Drosophila* embryo, yet signaling is graded, and peaks in the dorsal midline. The key to this pattern is a secreted inhibitor of BMP, termed Sog, which is expressed in a lateral pattern flanking the region expressing the ligand. By using the computational screen and experimental verification, we demonstrated an active ligand shuttling mechanism, where the inhibitor traps the ligand, translocates it, and physically releases it in the dorsal-most region. This mechanism provides robust patterning. We have recently shown that an elaboration on this conserved mechanism in *Xenopus*, allows not only to obtain robust dorso-ventral patterning, but also to scale patterning with modulations in embryo size. This mechanism may explain some of the cardinal outcomes of the classical Spemann experiments.

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