Chapter 22. Symmetry breaking mechanisms and the embryonic axial system: intra-cellular gradients and extra-cellular proteolytic cascades.

The initial pre-patterning of the *Drosophila* embryo takes place in the oocyte, and is dependent on the TGFα ligand, Gurken (Grk) [1]. *Grk* is transcribed in the nurse cells, before its mRNA is exported to the oocyte and translated near the posterior pole [2] [3]. As the Epidermal growth factor receptor, Egfr (Torpedo) is only expressed in posterior follicle cells, Grk/Egfr signalling is restricted to the P pole of the oocyte. In turn, this asymmetry initiates the assembly of polarised microtubule growth towards the A pole, with activation of a posterior MTOC by MAP kinase [4]. In general, microtubule growth is nucleated at the cortical F-actin cytoskeletal interface of the oocyte via Shot and Patronin. In this configuration, Patronin stabilises microtubule minus-ends, without the γ-tubulin rings that initiate microtubule extension from centrioles and peri-nuclear MTOCs. The outcome of these cytoskeletal remodelling interactions is determined largely by the topographical shape of the oocyte: the A oocyte pole forms an extended, flat interface with its nurse-cell sheath, while the P pole has a rounded, ovoidal cap. Thus, microtubules tend to be initiated preferentially from the A pole. In addition, Shot is excluded from the P polar cap by Par-1, thus blocking Patronin-initiated microtubule assembly [5]. In consequence, polarised microtubule growth from the flat A cortical interface is given a preferential bias along the A/P axis, allowing active cargo transport in either direction.

Following assembly of a P > A microtubule array, the *bcd* ribonucleoprotein complex (*bcd*-RNP) is transported via the minus-end directed Dynein (Dhc1) motor, to the A pole, where it is anchored to the cortical cytoskeleton and translated. Meanwhile, *oskar*-RNP (*osk*-RNP) particles move towards the posterior pole attached to the plus-end directed Kinesin motor (Khc) [6]. Fluorescently tagged *grk*-RNPs show intermittent pauses and reversals, consistent with transient motor engagement and release [6] [7] [8]. However, the P localization of *osk*-RNPs is also dependent on Dhc1, implying that both plus- and minus-end directed motors are active during *osk* partitioning [9]. An additional contribution to axial setting is that *osk* mRNA may be trafficked by the Myo-V motor and retained on cortical microfibrils. In consequence, the *osk* transcript tends to accumulate at the P pole, where microtubule filaments are depleted [10]. The P > A translocation of *grk*-RNP forms an A ring, as *grk*-RNP is translocated around the L and R flanks of the oocyte towards the D midline. Both stages of *grk*-RNP localisation are Dynein-dependent, with the Dhc1 motor apparently engaging with distinct microtubule arrays [3]. *Bcd*-RNPs are also translocated to an A ring, although not subsequently trafficked towards the D midline [11]. These movements suggest that V > D translocation of *grk*-RNP is coupled to Dhc1 via a linker, to which the *bcd*-RNPs do not become attached. Once *grk*-RNP is concentrated at the anterior dorsal (AD) cortex, the translated Grk protein mandates the fate of adjacent follicle cells. The oocyte nucleus also moves towards the AD cortical boundary, but this movement is independent of Par-1 activity, with the nucleus being pushed by microtubules nucleated from the centriolar MTOCs [11]. Notably, the Par-1 kinase also targets Tau, which cross-links parallel microtubule bundles [12] [13]. In hypomorphic Par-1 mutants, Tau-GFP is distributed uniformly around the oocyte cortex, while Khc-associated *osk* mRNA is located at the centre of the oocyte [11].

Thus, the long (A/P) axis of the oocyte is set by the partitioning of *bcd* and *cad* RNPs along polarised microtubules, followed by local translation and diffusion of the Bcd and Cad proteins. The opposing gradients are stabilised by auto-inhibitory feedback loops: with Bcd + Hunchback (Hb) A > P and Cad + Nanos A < P. Nanos blocks translation of *hb* and Bcd blocks translation of *cad,* reviewed in [6]. These mutually inhibitory interactions generate

anterior and posterior signalling centres. Thus, opposing protein diffusion gradients form following a Transport > Anchor > Translate> Release (TATR) mechanism, with the cortically localised mRNAs translated at Golgi outposts. The A > P morphogenetic gradient is also maintained by active degradation of Osk at the posterior pole; mediated via the Par-1 > GSK3 > Slmb ubiquitin-ligase pathway [14]. Critically, a mutated phosphorylation site within the aPKC kinase results in uniform distribution of Par-1, with the failure of cortical microtubule assembly and A/P polarization [15]. At the cellular level, the aPKC kinase is anchored to apical integrin complexes, where it phosphorylates (and excludes) the basal determinants, Miranda (Mir) and Numb [16 17 15]. Similar intracellular TATR gradients may drive embryonic morphogenetic mechanisms, with asymmetrical exchange of MVBs between the lateral boundaries of epithelial cells. On this model, the long axis of the embryo is established via opposing TATR gradients of Cad and Bic, with cytoskeletal remodelling regulated by gap-, segmentation- and Hox-gene functions. By contrast, the short (D/V, L/R) embryonic axis follows an extracellular diffusion gradient in the thin fluid film between the embryonic surface and the vitelline membrane, transduced through the Tl receptor, see above **12**.

In general, the specificity of proteolytic enzymes is dependent on the precise lock-and-key binding of a substrate within the protease cleavage pocket. However, the lysis and release of cleaved peptides may leave the protease free to bind (and cleave) additional substrate molecules. Thus, explosive activation of proteolytic signalling cascades may take place, unless successive protease activities are restrained, or localised. In particular, the D/V (L/R) embryonic axis is dependent the protease signalling cascade being delimited by maternally inherited factors. The initial pre-patterning is dependent on suppression of the Pipe (Pip) sulphotransferase in D follicle cells. In consequence, Pip secretion is restricted to V follicle cells which, in turn, inscribe a sulphated midline on the vitelline membrane surrounding the oocyte. Meanwhile, the Nudel (Ndl) protease is secreted uniformly and remains bound to the cortical surface of the oocyte [18]. Following the mid-blastoderm transition, membrane anchored Ndl activates Gastrulation Defective (Gd) in the perivitelline space; which, in turn, activates the Snake (Snk) and Easter (Ea) proteases. The simplest hypothesis may be that Pip sulphation of extra-cellular matrix components allows anchoring of the Ea and Snk pro-proteases via their N-terminal cysteine-knots [19 20]. On this hypothesis, the Pip-sulphated stripe would anchor pro-Ea and pro-Snk, until the cleavage (and trimming) of their N-termini releases protease activities [21]. Whatever the details, the L/R halves of the embryo are delineated round the ventral midline. Notably, eggs laid by *snake* mutant mothers form a wavy ventral furrow, with disruption of both long (A/P) and short (D/V) embryonic axes [22].

At the end of the extracellular proteolytic cascade, the Tl ligand, Spaetzler (Spz), is cleaved by the Ea to generate activated Spz*, which binds the Tl receptor [23 24]. Residual Ea activity is eliminated by the Serpin-27A inhibitor (Spn27A), which is secreted from the dorsal midline [25 26]. Critically, Serpin family inhibitors form covalent “suicide complexes” with their target proteases, such that both protease and inhibitor activities are destroyed. Thus, the Spn27A/Ea interaction generates a bi-modal gradient of activated Spz* around the left and right flanks of the embryo, reviewed in [27 28]. Signal transduction does not take place at the embryonic surface, but requires endocytosis of the Spz*/Tl complex and formation of Rab5 positive early endosomes [29]. Transduction of the Tl signalling pathway is via the *dorsal* (*dl*) TF, which is translated in the oocyte, and perdures through the syncytial blastoderm stages [30]. After cellularisation, the nuclear localisation of Dl is regulated by its chelation within a cytoplasmic Dl/Cact complex, from which Dl is released by the Pelle kinase [31 32]. Thus, post-translational phosphorylation of Cact regulates the nuclear activity of Dl. Critically, the nuclear Dl gradient is lost and reformed during each division cycle [33]. Cact activity is also regulated by the CalpainA (CalpA) protease, such that CalpA knockdown shifts the Dl

gradient, like the *dpp* mutant phenotype [34]. Notably, lack of Dpp blocks the polar/equatorial mitotic waves of the syncytial blastoderm, with ventralisation of the cellular blastoderm [35 36]. CalpA also targets CycB and is required for cell-cycle progression at the metaphase/anaphase transition [34 37]. Within this complex network of interactions, the GOF Tl^{10b} mutation gives an unsegmented block of mesodermal tissue. The mutant Tl^{10b} protein accumulates in cortical punctae, consistent with a blockage in its export. However, when coupled to the *bic* 3'UTR, transgenic $Tl^{10::bic}$ expression produces an ectopic A > P gradient of Dl, superimposed on the endogenous Tl-dependent pattern; with rotation of the *sna*, *sim*, *vnd*, *ind* and *sog* domains around the long (A/P) embryonic axis [30 38].

Consistent with these morphogenetic alterations, the binding affinity of Dl to the promotors of target genes varies inversely with their distance of expression from the ventral midline: *zen* > *dpp* > *sog* > *sna* > *twi* [30 39]. The homeobox TFs *vnd*, *msh* and *ind* (*ventral nervous system defective, muscle specific homeobox* and *intermediate neuroblast defective*) are also expressed in lateral stripes parallel to the V midline [40 41]. In particular, the *vnd* embryonic promoter carries multiple Dl binding sites, as well as Med, Sna and Twi sites [39]. These TFs form extended TFBS footprint chains across the promoters and intragenic regulatory regions of *vnd*, *msh* and *ind* [44 45 46]. Taken together these studies imply that the Toll signalling pathway sets all three embryonic axes, long A/P, short (D/V, L/R) and radial (Ap/Ba), via post-translational regulation of Dl activity.

Summary:

Asymmetric localisation of *grk* initiates cytoskeletal remodelling and polarised cargo transport, with microtubules preferentially aligned along the A/P axis of the oocyte. Subsequent transport around the D/V (L/R) axis concentrates grk-mRNA to the AD cortex, where it is anchored, translated and released, to form a gradient of Grk protein. Similar TATR gradients of Bcd and Cad set the A and P signalling centres, respectively. Morphogenetic cargos may also be partitioned, and transported, along actin microfilaments. Remodelling of the microtubule cytoskeleton minus takes place from the Actin/Shot/Patronin cortical interface, where microtubule minus ends are anchored, in the absence of γ-tubulin rings. In consequence, the flattened A boundary of the oocyte imposes a preferential A > P bias on microtubule assembly. Similarly, the embryonic surface topography constrains the progression of polar mitotic waves during the syncytial blastoderm. Following cellularisation, a V > D gradient of nuclear Dl (NF-κβ) TF is formed, and lost, during each division. An extracellular proteolytic cascade generates a Tl signalling gradient of around the L and R flanks of the embryo. Activation of the Tl signalling cascade is constrained around a sulphated, ventral stripe on the vitelline membrane; with explosive activation of Ea prevented by an opposing D > V gradient of the protease inhibitor, Spn27A. Signal transduction, does not take place directly from the embryonic epithelial surface but requires endocytosis of the Tl/Spz* complex. Disruption of the Tl > Dl signalling cascade can rotate all three embryonic axes, which are normally aligned with respect to the ventral furrow.

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