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PII: S0012-1606(21)00116-0

DOI: https://doi.org/10.1016/j.ydbio.2021.05.003

Reference: YDBIO 8443

To appear in: *Developmental Biology*

Received Date: 29 January 2021

Revised Date: 28 April 2021

Accepted Date: 5 May 2021

Please cite this article as: Tada, M., The morphogenetic changes that lead to cell extrusion in development and cell competition, *Developmental Biology* (2021), doi: https://doi.org/10.1016/j.ydbio.2021.05.003.

The morphogenetic changes that lead to cell extrusion in development and cell competition

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Key words: cell extrusion, cell competition, cell division, cell intercalation, apoptosis, morphogenesis

5863 words

Highlights:

Tricellular junctions play a pivotal role in coordinating cell extrusion

Differentiation sets up elimination of unfit or mis-specified cells

Geometry and topology of epithelia influence the mode of cell extrusion

Cell extrusion is a morphogenetic process in which unfit or dying cells are eliminated from the tissue at the interface with healthy neighbours in homeostasis. This process is also highly associated with cell fate specification followed by differentiation in development. Spontaneous cell death occurs in development and inhibition of this process can result in abnormal development, suggesting that survival or death is part of cell fate specification during morphogenesis. Moreover, spontaneous somatic mutations in oncogenes or tumour suppressor genes can trigger new morphogenetic events at the interface with healthy cells. Cell competition is considered as the global quality control mechanism for causing unfit cells to be eliminated at the interface with healthy neighbours in proliferating tissues. In this review, I will discuss variations of cell extrusion that are coordinated by unfit cells and healthy neighbours in relation to the geometry and topology of the tissue in development and cell competition.

Global concept for cell extrusion

Cell extrusion is a process by which cells are eliminated from the tissue with high proliferative properties in homeostasis. In homeostasis, crowding-induced extrusion occurs in tissues that are highly proliferative such as apical extrusion in the zebrafish tail fin and basal delamination in the *Drosophila* notum (Eisenhoffer et al., 2012; Marinari et al., 2012). As a result, the extruded cells leave the tissue apically or basally (Fig 1A).

In development, normally a single cell or a group of cells leaves the tissue, internalises and becomes fated to different cell type(s) from their origin. A neuroblast precursor cell delaminates basally from the *Drosophila* notum and subsequently undergoes asymmetric division to produce a neuroblast and a ganglion mother cell (Fig 1B). This process occurs by segregation of cell fate determinants into either daughter cell: for example, Inscrutable in neuroblast or Numb in ganglion mother cell (Knoblich, 2010). In the process of single cell delamination, pulsatile apical constriction mediates this process while maintaining the adherens junctions to the adjacent cells (An et al., 2017; Simões et al., 2017).

Epithelial mesenchymal transition (EMT) is a morphogenetic process in which single cells or a group of epithelial cells become mesenchymal by losing adherens junctions and acquiring actomyosin contractility to migrate to their destination (Yang et al., 2020). This process is often associated with cell fate specification in development. In *Drosophila* gastrulation, apical constriction in presumptive mesodermal cells leads to invagination of epithelial cells, which subsequently undergo EMT (Kölsch et al., 2007; Martin et al., 2009; Weng and Wieschaus, 2016)(Fig1C). The hallmark of EMT is mediated by transcription factors of Snail, Twist and Zeb families that control cell-cell adhesion (Lim and Thiery, 2012). This suggests that apical actomyosin contractility coupled with junctional remodelling is key to mediating group cell extrusion underlying EMT.

If unfit cells arise in development, these cells are eliminated by a mechanism, called ‘cell competition’. Cell competition was first discovered in the *Drosophila* imaginal disc, and is a process through which suboptimal cells survive if they occupy the entire tissue. However if surrounded by normal cells, they undergo apoptosis, referring to the suboptimal cells as ‘losers’ (Morata and Ripoll, 1975)(Fig 1D). This occurs, to some extent, due to the suboptimal cells proliferating lesser than adjacent normal cells. Similarly, cells with more proliferative properties tend to outcompete with surrounding normal cells through a phenomenon, called super-competition (Moreno, 2008). Nevertheless, it appears that differences in cell proliferation alone are insufficient to trigger cell competition (Menéndez et al., 2010; Tamori and Deng, 2013). Since its

discovery, the concept has emerged and been described in excellent review papers and in this issue (Clavería et al., 2013; Di Gregorio et al., 2016). Here, I will focus on cell competition related to morphogenesis and differentiation in developing animals.

Regulation of cell extrusion

Cell extrusion has been initially investigated in cultured cells in the context of UV-induced apoptotic extrusion. During apoptotic cell extrusion in cultured cells, the dying cell produces the bioactive lipid sphingosine1-phosphate (S1P), which activates S1P receptor 2 ($S1p_2$) signaling, thereby inducing actomyosin cable formation basolaterally in neighbouring cells via p115RhoGEF and driving apical extrusion (Gu et al., 2011; Rosenblatt et al., 2001; Slattum et al., 2009). Live imaging of apoptotic extrusion in MDCK cells reveals that UV-induced apoptotic extrusion occurs in two phases; firstly apical contraction, presumably at the level of adherens junctions, removes the apical small part of the dying cell and propagates cortical actomyosin contractility to the neighbouring cells, and secondly the neighbours initiate actomyosin cable basolaterally to squeeze the main part of the dying cell apically (Kuipers et al., 2014). Subsequently, junctional tension of the neighbouring cells in contact with the dying cell relaxes, and the mechanosensitive Src family kinase is activated to coordinate the generation of actomyosin cable (Teo et al., 2020). In this process, E-cadherin couples extruding apoptotic cells and neighbouring cells (Lubkov and Bar-Sagi, 2014; Michael et al., 2016). In addition to actomyosin cable, basal lamellipodial protrusions from the neighbouring cells contribute to apoptotic cell extrusion (Duszyc et al., 2021; Kocgozlu et al., 2016; Le et al., 2021). Recent work in the zebrafish periderm demonstrates that pulsatile medioapical constriction of Myosin II also contributes to extrusion of apoptotic cells (Atieh et al., 2021).

How do suboptimal cells respond to mechanical pressure during morphogenesis?

The capacity of suboptimal cells to respond to the mechanical forces during cell divisions and cell intercalations is the key to determining their fate in growing tissues. In this section, I will discuss the timing of cell divisions, the coordination of junctional remodelling and the signals in response to the changes in the mechanical forces at the interface between the unfit cells and normal cells.

Cell cycle progression is a checkpoint for division or extrusion

It has not been investigated whether cell extrusion occurs during cell cycle progression based on live imaging until recently. Using live reporters for cell cycle progression, it was found that in 90% of cases oncogenic Src (vSrc)-driven extrusion in the zebrafish embryonic epithelium, called enveloping layer (EVL) occurs in G2/M phase, whereas such cell cycle-dependent extrusion in epithelial MDCK cells is observed only in 50% of cases (Anton et al., 2018). Importantly, the proliferation rate of vSrc cells is 50% lower than that of neighbours, suggesting that cell extrusion takes place instead of cell division. It appears that the degree to which cell proliferation drives extrusion depends on the proliferative property of the tissue. Consistent with this notion, inhibition of G2/M, but not of G1/S, suppresses vSrc-driven extrusion (Anton et al., 2018). This raises the possibility that cell cycle progression in G2/M phase is a checkpoint for extrusion. It is conceivable that unfit cells preferentially die if they are in G2 during cell competition. It will be important to investigate, using live cell cycle reporters, whether cell extrusion occurs in G2/M phase during cell competition in *Drosophila*.

Tricellular junctions coupling cell divisions with epithelial morphogenesis

During epithelial morphogenesis, tricellular junctions (TCJs), also called vertices, regulate cell geometry and topology in growing tissues by orchestrating cell divisions and cell intercalations at cell-cell junctions, referred to as bicellular junctions (BCJs) (Bosveld and Bellaïche,

2020)(Fig 2A). To understand these processes mechanistically, the vertex model has been used as to how each vertex moves in relation to the relative positions and the apical surface areas of the cells (Fletcher et al., 2014). For example, in order for a cell to divide requires the addition of vertices and reduced apical surface area of each daughter. In contrast, to achieve cell intercalation needs decreasing or increasing distances between the vertices, while maintaining the same apical surface areas. Cell extrusion is mediated by T2 transition in which the apical surface area decreases by shortening the distances of all the vertices in contact with neighbouring cells (Fig 2B). During cell intercalations, shrinking BCJs are mediated by actomyosin contractility, whereas resolving BCJs require fine-tuning of myosin activity at TCJs allowing for E-cadherin-mediated elongation of BCJs (T1 transition; Fig 2B)(Collinet et al., 2015; Lecuit and Yap, 2015; Uechi and Kuranaga, 2019).

TCJs are subdivided into tricellular adherens junctions (tAJ) and tricellular septate junctions (tSJ) in *Drosophila* (Fig 2A). It appears that tAJ is essential for the coordination of tensile forces at BCJs through the cell adhesion molecule Sidekick (Sdk) during *Drosophila* germband extension (Finegan et al., 2019; Letizia et al., 2019; Uechi and Kuranaga, 2019). The actin binding protein Afadin/Canoe is mechano-sensitively localised at TCJs and required for tricellular adhesion during T1 transition (Yu and Zallen, 2020). These findings suggest that TCJs may act as a sensor for tension at BCJs during cell intercalations.

TCJs coordinate cell division by ensuring the position of the mitotic plane with respect to neighbouring cells in the *Drosophila* notum and the *Xenopus* surface ectoderm (Bosveld et al., 2016; Higashi et al., 2016). This function is particularly important in epithelia in which cells undergo mitotic rounding (Matthews et al., 2012; van Leen et al., 2020). If the planar division axis is destabilized, one of the daughter cells could fail to integrate into the epithelium, resulting in basal delamination and subsequently apoptosis (Nakajima et al., 2013)(Fig 3A,B). Moreover, TCJs regulate the apical surface area. Myosin-based apical stress fibres (aSF) in conjunction with adherens junctions are indicative of the response to morphogenetic pulling forces and correlate with cell apical area. The number of TCJs determines aSF number and apical surface area, correlating with cell proliferation in larger cells (López-Gay et al., 2020).

During extrusion of transformed cells, there are two myosin rings; one is a mis-oriented cytokinetic ring in the extruding vSrc cell and the other originates from the TCJs of neighbouring cells during vSrc-driven cell extrusion in the zebrafish EVL (MT, unpublished). This raises the hypothesis that the TCJs act as a pivot for orchestrating cell division and for integrating morphogenetic force at cell-cell junctions during extrusion as has been proposed in development. In support of this notion, depletion of the tSJ transmembrane protein M6 alters the direction of extrusion of Ras cells from basal to apical by regulating actomyosin contractility (Dunn et al., 2018). tSJ might act as a sensor for changes in tension along the apicobasal axis, thus determining the direction of cell extrusion.

In *Drosophila*, septate junctions are important to determine the mitotic plane during cell division. Knock-down, KD, of the apicobasal polarity gene *scribble* (*scrib)*(*scrib*^{KD} cells) leads to randomised mitotic planes during cell division (Nakajima et al., 2013). Furthermore, *scrib*^{KD} cells delaminate basally and undergo apoptosis, but can form a basal mass with EMT-like behaviours if apoptosis is inhibited (Fig 3C). Interestingly, in the presence of apoptotic inhibitors, the cells with randomised mitotic planes also cause the EMT-like phenotypes reminiscent of the *scrib*^{KD} cells (Nakajima et al., 2013). If surrounded by WT cells, *scrib* mutant cells undergo apoptosis by JNK activation in the *Drosophila* imaginal disc (Igaki et al., 2009) or by p38 activation in MDCK cells (Norman et al., 2012). *scrib* mutant cells are also

sensitive to mechanical stress/compaction through p38 activation in MDCK cells (Wagstaff et al., 2016). These results suggest that the *scrib* looser phenotype may be associated with reintegration defects during mitosis. Whether mechanical stress can exacerbate the reintegration defects will require further investigation.

Spontaneous extrusion occurs during mitosis in the *Drosophila* epithelium. One daughter cell can be apically released from the *Drosophila* follicular epithelium, but the popped-out cell can be reintegrated into the epithelium (Bergstralh et al., 2015)(Fig 3D). The process of cell reintegration requires the cell adhesion molecules Neuroglian and Fasciclin 2, which are both localised at the septate junctions and implicated in axon guidance (Bergstralh et al., 2015). Interestingly, cell reintegration is mediated by the septate junctions but not for adherens junctions. Neuroglian exerts the cell adhesive function through the lateral spectrin membrane skeleton at the septate junctions, mediating a traction force during cell reintegration (Cammarota et al., 2020).

Recently, it was shown that Scrib preferentially localizes to the TCJs and mediates tricellular junction formation through recruiting the core tSJ protein Gliotactin (Gli) (Sharifkhodaei et al., 2019). Considering that loss of Gli can lead to JNK activation and an increase in cell proliferation (Padash-Barmchi et al., 2010), it prompts us to speculate that Gli is key to coupling bSJ with TCJs and to potentially influence *scrib* cell elimination during cell competition. Despite the implication of septate junctions for cell extrusion in *Drosophila*, the roles of the tight junctions in regulating cell extrusion remain to be elucidated, taking into account the topological differences in the septate junctions and tight junctions in the apicobasal axis between *Drosophila* and vertebrates (Furuse and Tsukita, 2006).

What controls the direction of extrusion?

In crowding-induced extrusion in the zebrafish embryonic epidermis, mechanical stress activates the mechanosensitive calcium channel Piezo 1, which in turn mediates $S1p_2$ signalling and triggers live cell extrusion apically (Eisenhoffer et al., 2012; Gudipaty et al., 2017). Similarly, apoptotic cells in cell cultures are extruded apically, as described in the previous section. In contrast to cultured cells, apoptotic cells delaminate basally in the *Drosophila* pupal epithelium (Ohsawa et al., 2018). What determines the direction of extrusion?

In *Drosophila*, apoptotic cells generate apical constriction autonomously, which in turn drives coordinated apical constriction of neighbours, thereby contributing to folding of the epithelium, reminiscent of invagination (Monier et al., 2015). One possible reason why apoptotic cells undergo basal delamination could be due to the differences in cell shape: the shape of the columnar cells in the wing disc (Monier et al., 2015) vs the squamous shape of cultured cells. However, this is unlikely, as there are variations in cell shapes of the *Drosophila* pupal epithelia: cuboidal shape in the pupal notum (Marinari et al., 2012) and squamous shape in the pupal abdominal larva cells (Teng et al., 2017).

Another possibility is due to differences in apicobasal organisation of lateral junctions between *Drosophila* and vertebrates (Furuse and Tsukita, 2006). However, this does not seem to be the case, as apoptotic cells with expressing the death-associated protein kinase 1 (DAPK1) or with aneuploidy undergo basal extrusion in the zebrafish EVL (Anton et al., 2018). This raises the possibility that the fact that the direction of apoptotic cells in tissue cultures is different from that in vivo is due to the lack of basal cues or the presence of rigid matrices in tissue

culture conditions. The fundamental mechanisms for the direction of extrusion will need to be investigated further.

Live apical extrusion normally leads to cell death via anoikis in cultured cells and in the zebrafish embryonic epidermis, called periderm (Eisenhoffer et al., 2012). In the case of vSrc cell extrusion in the zebrafish EVL, vSrc cells orient their division plane and retain the connection through the adherens junctions with neighbouring cells until eventually they are completely out of the epithelium (Fig 3E). The part of the vSrc cell that inherits the nucleus is extruded apically and often undergoes anoikis, presumably due to the fact that the apical side faces outside of the embryo. However, apically delaminated *scrib* mutant cells proliferate and form tumours (Tamori et al., 2016). One possibility is that the apical surface of the *Drosophila* pupal epithelium is covered by the squamous epithelium, called peripodial epithelium, which could protect the delaminated cells from death physically or through secretion of survival factors (Gibson et al., 2002; Moreno et al., 2002).

If surrounded by wild-type cells, *scrib* mutant clones are eliminated basally by JNK activation. However, expression of the axon guidance receptor Robo2, a downstream target of JNK signalling, converts the fate of *scrib* mutant cells from basal apoptotic extrusion to apical extrusion with proliferative properties (Vaughen and Igaki, 2016). It needs to be investigated whether Robo2 modulates the signal that promotes apical extrusion or cell survival.

It will be informative to test whether apically extruded cells survive in the vertebrate epithelium, as apically extruded cells from 3D epithelium of mammary acini have the potential to survive and grow (Leung and Brugge, 2012). It will be important to test it in epithelia with different topology, in which the apical surface faces another epithelium and the apical area is smaller than the basal surface, such as in the retinal pigment epithelium (Bazin-Lopez et al., 2015). It also remains to be elucidated what instructs apoptotic cells to undergo apical extrusion in cultured cells.

Asymmetric cell division and cell extrusion: alive or dead

Further analysis in the *Drosophila* notum revealed that the mechanical stress due to crowding in the space constraints of the proliferating tissue induces Caspase activation prior to basal delamination (Levayer et al., 2016). Together with the fact that the mode of extruding vSrc cell is reminiscent of asymmetric cell division (Fig 3E), these observations raise the hypothesis that cell competition encourages divisions to be asymmetric and/or apoptotic.

Apoptotic asymmetric cell divisions occur naturally during *C. elegans* development. For example, the neurosensory motoneuron (NSM) neuroblast divides to produce a large and a small daughter cell (Fig 3F). While the larger daughter cell becomes the NSM neuron, the smaller daughter cell undergoes apoptosis (Conradt, 2009). In this process, the two opposing gradients contribute to the fates of the daughter cells: a gradient of the pro-survival EMT transcription factor Snail/CES-1 being higher in the larger NSM and a gradient of Caspase 3 being higher in the smaller daughter cell (Mishra et al., 2016; Thellmann et al., 2003; Wei et al., 2017; Wei et al., 2020). Moreover, Snail/CES-1 controls cell cycle progression and cell polarity in the NSM neuroblast (Hatzold and Conrart, 2008; Yan et al., 2013). These findings are consistent with the notion that Snail acts primarily as a survival factor (Barrallo-Gimeno and Nieto, 2005). One intriguing feature potentially related to cell competition, albeit with the stereotypic lineage-specific process in *C. elegans*, is that there is the interplay between the NSM neuroblast and neighbouring cells in metaphase of the cell cycle. The gradient of Caspase 3 from the NSM neuroblast sets up a signal to adjacent cells, which in turn promote

apoptotic death of the smaller daughter cell through engulfment pathways (Chakraborty et al., 2015).

Apoptotic asymmetric cell division is also described in *Drosophila* neurogenesis. The ventral multidendritic neuron (vmd1a neuron) is generated by two-sequential asymmetric divisions, in which one of the daughter cells undergoes apoptosis and the other differentiates to a neuron. The asymmetric inheritance of Numb prevents the latter daughter cell from triggering apoptosis, thereby allowing for neuron differentiation (Orgogozo et al., 2002). How Numb acts as a survival factor remains elusive, but it is feasible that Numb antagonises Notch, as Notch activation in the vmd1a neuron lineage leads to apoptosis.

In addition to neurogenesis in invertebrates, potential apoptotic asymmetric divisions have been described in vertebrates. In the expanding roof plate of the zebrafish embryo, roof plate progenitors, called veil cells, undergo asymmetric cell division into two daughters: a veil cell and a roof plate cell. Interestingly, based on time-lapse movies, veil daughter cells are occasionally observed to be extruded and to undergo apoptosis and this extrusion behaviour increases when epithelial space is limited (Campo-Paysaa et al., 2019). One possible explanation for this phenotype is that if a prospective roof plate cell fails to integrate into the roof plate for any reasons, it will be fated to die, however this needs to be investigated further.

Non-cell autonomous response from neighbours

The fate of extruding cells is also determined non-cell autonomously by the properties of neighbouring cells within the tissue. In this section, I will discuss the influences of neighbours in different contexts.

Mechanical competition

Unfit cells are more sensitive to mechanical strains than normal cells and become apoptotic, while the normal cells can expand their territory towards the loser cell population via a phenomenon, called mechanical competition (Brás-Pereira and Moreno, 2018; Matamoro-Vidal and Levayer, 2019). In limited space, differential proliferation rates in two populations can cause the cells with lesser proliferative property to be eliminated upon mechanical strain (Shraiman, 2005). Live imaging in the *Drosophila* notum revealed that Caspase activation precedes basal delamination in crowding induced extrusion (Levayer et al., 2016). Consistent with the fact that apoptotic cells facilitate morphogenetic processes (Monier et al., 2015), faster-growing cells undergo perpendicular intercalations in relation to the apoptotic cell, reminiscent of combined T1 and T2 transitions (Fig 2B), allowing winner cells to effectively expand towards the loser territory (Tsuboi et al., 2018). Oncogenic Ras clones are capable of compressing neighbouring wild-type cells non-cell-autonomously in the *Drosophila* notum, thereby expanding the territory (Levayer et al., 2016).

Mechanical cell compaction results in down-regulation of EGFR/ERK signalling in looser cells, leading to elimination of the looser cells via apoptosis, which is dependent on the proapoptotic gene *hid* (Moreno et al., 2019). Interestingly, compression in the *Xenopus* embryonic epithelium by high gravity causes the cells to activate FGFR/ERK signalling by sensing tensile forces at the adherens junctions and tight junctions (Kinoshita et al., 2020). The Hippo pathway regulates the nuclear translocation of Yap in mechanical strain (Codelia et al., 2014), and Yokie/Yap acts as a super-competitor in the *Drosophila* pupal epithelia (Neto-Silva et al., 2010; Ziosi et al., 2010). It appears that p53 plays a pivotal role in sensing for mechanical competition in loser cells in MDCK cells (Wagstaff et al., 2016). It will be important to further investigate what signals are responsible for mechanical competition in an integrated manner.

Differentiation sets up cell elimination

In development, the onset of differentiation from a homogenous pluripotent cell population is a checkpoint for elimination of sub-optimal cells. During epiblast formation from the inner cell mass of the mouse embryo, Yap/TEAD activity and pluripotency factor expression become heterogeneous, leading to selection for high-quality epiblast cells by cell competition (Hashimoto and Sasaki, 2019). The cells with low Yap activity remain unspecified, which causes their elimination through apoptosis or their differentiation into the primitive endoderm (Hashimoto, 2019). In the mouse epiblast, in which pluripotent cells are all differentiated into the ectoderm, mesoderm or endoderm, the cells with low Myc levels tend to undergo apoptosis (Clavería et al., 2013). Likewise, mis-specified cells are eliminated by cell competition at the onset of neural differentiation from pluripotent epiblast in mice (Sancho et al., 2013). This process is regulated by the mechanistic target of rapamycin (mTOR) pathway, which regulates cell growth, and the tumour suppressor protein p53 (Bowling et al., 2018). It is unclear whether cell proliferation promotes cell competition.

It appears that spontaneous basal delamination occurs in development but that delaminated cells can adapt to a new environment (Solnica-Krezel and Sepich, 2012), indicating that the delaminated cells have plasticity in adapting their fates. However, in response to external signals, the cells can be mis-specified and subsequently eliminated from the tissue. Increased actomyosin contractility at the interface between normal and mis-specified cells in the *Drosophila* wing disc drives extrusion. If a single cell is mis-specified, it delaminates basally and undergoes apoptosis, whereas if a large group of cells are mis-specified they forms basal cysts, reminiscent of the EMT phenotype (Bielmeier et al., 2016; Klipa and Hamaratoglu, 2019). Interestingly, in this process, misspecified cells can recruit surrounding normal cells into the cysts, implying a non-cell-autonomous effect (Bielmeier et al., 2016). Mis-specified cells in the *Drosophila* epidermis exhibit aberrant EGFR signalling with ERK activation, leading to *hid*-dependent apoptosis, whereas endogenous ERK activation apparently promotes cell survival (Crossman et al., 2018). Aberrant Wnt activity can mis-specify the epiblast of the early zebrafish embryo but small clones with increased or decreased Wnt levels are eliminated by apoptosis through non-cell-autonomous E-cadherin-mediated mechanisms in normal neighbours (Akieda et al., 2019). These findings imply that plasticity of the cells under pressure determines the fate of the extruded cells; survive if able to adapt to a new environment or die if unable to do so.

Stem cell competition

Stem cells reside in the basal layer of stratified epithelia of the mammalian skin, which is in contact with the basal lamina. The loser cells are eliminated from the basal layer by asymmetric cell divisions and differentiate to be destined to die at the apical surface (Ellis et al., 2019; Liu et al., 2019). This suggests that the basal lamina provides the stem cells with survival signals.

What determines which cells become winners in stratified epithelium? Activation of Notch is associated with differentiation in many different contexts, and clonal inhibition of Notch in the basal layer of the mouse esophageal stratified epithelium leads to expansion of Notch-abrogated clones (Alcolea and Jones, 2015). Importantly, the degree of expansion of Notch mutant clones is determined by the relative proliferative properties of neighbours (Colom et al., 2020).

In intestinal homeostasis, E-cadherin loss at cell-cell contacts precedes apoptosis due to anoikis in mouse intestinal enterocytes, and this implies that E-cadherin acts as a survival factor (Fouquet et al., 2004). Similarly, loss of E-cadherin activates the EGF processing enzyme Rhomboid in apoptotic enterocytes of the *Drosophila* gut, secreting EGF ligand, which in turn activates EGFR signalling in adjacent stem cells (Liang et al., 2017). Loss of *Apc* in stem cells leads to tumour growth by out-competing normal neighbours (Suijkerbuijk et al., 2016) and/or constitutively activating EGFR signalling which can non-cell autonomously activate JNK in neighbouring cells (Ngo et al., 2020).

Yap regulates asymmetric division of hematopoietic stem cells (HSC), which is modulated by Scrib along with the Hippo kinase Lats and the polarity protein Cdc42. Scrib deficiency unbalances cytoplasmic Yap and increases apoptosis of daughter cells, eventually leading to increased symmetric division of HSC, compared to asymmetric division of normal HSC (Althoff et al., 2020). This implies that one of the daughter cells, which is fated for differentiation, predominantly dies in Scribble deficient HSC. It appears that fine-tuning of the Scrib polarity complex is key to determining the mode of asymmetric cell division in HSC.

Can planar cell polarity modulate cell competition?

The genetic system that controls differential cell behaviours at the interface of the two populations is planar cell polarity (PCP), such that mutant clones can instruct cell bahaviour of neighbours non-cell-autonomously in the *Drosophila* wing (Lawrence and Casal, 2018). PCP can orient cellular force generation and directionally coordinate mechanical properties of cells in a long-range, including cell divisions and cell intercalations (Jülicher and Eaton, 2017; Tada and Kai, 2012; Vichas and Zallen, 2011). These properties of PCP prompt me to discuss the potential roles of PCP in modulating cell competition.

The atypical cadherins Fat (ft)/Dachsous (Ds) pathway of PCP can coordinate the local changes in mechanical properties of the cells during the establishment of PCP (Herszterg et al., 2013). It was shown that *ft* null clones are capable of rescuing the loser death phenotype in the *Drosophila* wing disc (Tyler et al., 2007). However, none of the other core PCP mutant clones such as Frizzled (Fz) or Ds is able to rescue the loser phenotype (Tyler et al., 2007). This suggests that *ft* is involved in cell competition, because the loss of *ft* presumably results in activation of Yki/Yap via the Hippo pathway, independently of the PCP pathway (Fulford and McNeill, 2020). Interestingly, *ft* hypomorph clones undergo apoptosis in the *Drosophila* eye disc (Domingos et al., 2019); however, it is unclear whether this phenotype is related to the PCP pathway.

One might speculate that the genes that mediate PCP during *Drosophila* germband extension have crucial roles in cell competition. Three different Toll receptors with Leucine-rich repeats in different stripes regulate cell intercalation through their heterophilic interactions and thus integrate directional mechanical forces during PCP (Paré et al., 2014). Unfit cells are eliminated by Toll activation, which mediates NF-kB-dependent activation of proapoptotic genes (Meyer et al., 2014) but activation of the ligand Spätzle (Spz) is regulated by fit neighboring cells during cell competition (Alpar et al., 2018; Katsukawa et al., 2018). In parallel with the Toll system, it has been recently shown that the leucine-rich repeat receptor Tartan and the teneurin Ten-m also mediate integration of mechanical forces during *Drosophila* germband extension (Paré et al., 2019). It will be interesting to determine whether the Toll or Tartan system is involved in mechanical competition.

How does mechanical signal propagate to neighbours?

Long-range calcium waves propagate from extruding Ras cells across normal neighbouring cells and facilitates Ras cell extrusion in MDCK cells and in the zebrafish EVL (Takeuchi et al., 2020). The mechanosensitive calcium channel TRPC1 mediates the generation of calcium waves and drives extrusion. Mechanical stretch activates ERK downstream of EGFR signaling and the ERK wave propagates in a long range in collectively migrating MDCK cells (Hino et al., 2020). Moreover, ERK waves coordinate convergence movements of neighbours during oncogenic BRAF-driven extrusion (Aikin et al., 2020). Considering the facts that ERK is activated in Ras cells during extrusion (Hogan et al., 2009) and that ERK signalling is responsible for mechanical competition (Moreno et al., 2019), it will be intriguing to explore whether calcium and ERK waves are propagated independently or interdependently. Apoptosis-induced apical extrusion is also associated with the calcium waves in both MDCK cells and in the zebrafish EVL but does require the calcium wave for extrusion to a lesser extent, compared to Ras-induced extrusion (Takeuchi et al., 2020). Although calcium waves are also observed in the *Drosophila* imaginal disc, it will remain to be investigated whether calcium waves are responsible for cell extrusion (Balaji, 2017).

In addition to calcium waves orienting neighbouring cells towards Ras-extruding cells, the death of unfit cells induces oriented cell division during cell competition in *Drosophila* wing disc, which depends on Ft and Ds (Li et al., 2009). Consistent with this, atypical myosin Dachs, a downstream effector of Ds, orients cell division in the *Drosophila* wing disc (Mao et al., 2011). Oriented cell division is also mediated by the Fz system of PCP proteins in the zebrafish embryo and in *Drosophila* in a manner dependent on NuMA/Mud, a factor responsible for mitotic spindle orientation at the cortex (Ségalen et al., 2010). Importantly, anisotropic tension orients cell division axis in the early zebrafish embryo (Campinho et al., 2013; Castanon et al., 2013), suggesting that PCP is part of the tension sensing mechanism during cell extrusion.

In MDCK cells, Ras-driven extrusion requires forces from neighbouring cells through the actin-crosslinking protein Filamin and the intermediate filament Vimentin (Kajita et al., 2014). Interestingly, Vimentin, also known as a marker for EMT in wound healing (Cheng et al., 2016), is accumulated in neighbouring cells in a way that resembles a constricting ring (Kajita et al., 2014). This behaviour in the neighbouring cells might reflect the acquisition of mechenchymal properties in neighbouing cells, or epithelial-mesenchymal plasticity (EMP), in which the cells have adapted to mixed properties in an intermediate state (Yang et al., 2020).

Little is known about how mechanical signals propagate to neighbours and in turn how neighbours respond to the cells to be extruded. To explore these questions awaits further investigations in the future.

Concluding remarks

In this review, I have focused on how cell extrusion can occur in developmental processes and in cell competition in global definitions. The key parameters that control extrusion are the phase of the cell cycle that the cells are in, the extent to which the junctional tensions are integrated at the interface with distinct cell populations and the survival signals that feed into the cells to be extruded.

The G2/M transition is presumably a checkpoint for ensuring healthy daughter cells, but one of the daughter cells with inheriting damaged DNA or organelles could undergo apoptosis in asymmetric cell divisions, as in the case of Src cell extrusion. This could explain why extrusion of unfit cells or mis-specified cells tends to occur during differentiation, such that

differentiation of neurons occurs after mitosis. In some contexts, extrusion or delamination can be a consequence of failed reintegration of one daughter cell into the epithelium after division, if the division plane is altered or the lateral junctions with neighbours/TCJs are altered. Thus, TCJs may play a pivotal role in regulating reintegration, and hence abrogation of TCJs can result in cell extrusion.

The integration of mechanical forces at the interface between unfit cells and healthy neighours are the key for determining the mode of extrusion through the coordination of cell divisions and cell intercalations. These processes are coordinated by a variety of specific RhoGEFs both in the apicobasal axis and in the plane of the proliferating epithelia. Recent work demonstrates that apical medial actomyosin contractility and junctional actomyosin contractility of intercalating cells are regulated by RhoGEF2 and Dp114RhoGEF/ARHGEF18, respectively (Garcia De Las Bayonas et al., 2019). It will be necessary to tease apart which RhoGEF mediates the direction of extrusion in extruding cells or neighbours with respect to their geometry and topology in different types of epithelia.

Extruded cells undergo anoikis primarily due to loss of the connection with healthy neighbors through E-cadherin unless survival signal(s) are provided. This implies that E-cadherin might act as a bridge for survival signal(s). The survival signals are EGFR/ERK, PI3K, anti-apoptotic proteins, Snail family proteins. In development, EMT is associated with cell fate specification in conjunction with survival factors provided (e.g. mediators of EGF/FGF/TGF-β signalling). In cancer, tumour progression is highly correlated with the extent to which these EMT factors are provided.

Perspectives

To better understand cell extrusion in development and cell competition, several issues remain elusive.

How does the geometry of the epithelia determine the mode of extrusion? To better understand what determines the direction of cell extrusion (apical or basal) and the fate of extruded cells in vivo, we will need to apply 3D-based models such as the 3D apical vertex model (Alt et al., 2017), taking into account the curvature of epithelia (evagination or invagination) and the positioning of the lateral junctions (adherens junctions and septate/tight junctions) relative to the height of epithelia, as in the case of the mechanical aspect of epithelial folding formation (Tozluoğlu et al., 2019). The curvature of epithelia determines tumour hot spots, where *scrib*^{KD} cells in the bottom of invaginated epithelium (the hinge region of the *Drosophila* pupal wing) are in large contact with the basal lamina, undergo apical delamination followed by hyperproliferation (Tamori et al., 2016). Interestingly, depletion of RhoGEF2, which mediates apical constriction (Kolsch, 2007), is sufficient for *scrib*^{KD} cells to undergo apical delamination but not to survive unless JAK/STAT signallling is activated (Tamori et al., 2016). It also remains to be investigated how the spatial bias occurs as to whether eliminated cells survive or die, as in the case of basally eliminated mis-specified cells (Klipa and Hamaratoglu, 2019). It remains to be elucidated how morphogenetic movements on the basal surface of the epithelium attribute to cell extrusion. During *Drosophila* germband extension, basolateral protrusions rosette, cooperate with apical contraction and drive the morphogenetic process (Sun et al., 2017). Together with the 3D vertex model, high-resolution live imaging will uncover a novel mode of cell extrusion in growing tissues.

Can we predict which cell will be extruded based on cell behaviours? Modelling of mechanical competition revealed new aspects of morphogenetic movements at the interface between

winners and losers in vivo (Lee and Morishita, 2017; Moreno et al., 2019; Tsuboi et al., 2018). Live-imaging approaches in cultured cells uncovered that the cells to be extruded are determined by stereotypic topological defects (Saw et al., 2017). Similarly, high throughput live-imaging of cell competition has been undertaken in cultured cells (Gradeci et al., 2020). In addition to the classical genetic analysis, live-imaging using a variety of fluorescently labelled markers to visualise cellular events has recently revealed the novel mechanisms for cell extrusion as part of morphogenesis. Quantitative analysis along with computational modelling will further elucidate the fundamental mechanisms underlying cell extrusion in the future.

Acknowledgements

I would like to thank Kasia Anton, Barbara Conradt and Jon Clarke for critical reading of the manuscript. This work is supported by the Cancer Research UK, A15936 and the BBSRC, BB/N014855/1.

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Figure legends

Figure 1. Cell extrusion in development and cell competition. A) Cell extrusion. **B)** Neuroblast delamination in *Drosophila*. Neuroblast undergoes basal delamination, followed by asymmetric division to produce a neuron. **C)** Invagination and epithelial mesenchymal transition (EMT) during *Drosophila* gastrulation. The mesoderm cells (green) undergo apical constriction, leading to invagination and subsequently EMT. The mesoderm expresses the EMT transcription factor Snail. **D)** Cell competition in *Drosophila*. A suboptimal cell undergoes basal delamination in association with death.

Figure 2. Cell extrusion during morphogenesis. A) A cell to be extruded is surrounded by normal neighbours through lateral cell junctions (Adherens junctions and septate junctions) in Drosophila and tricellular junctions (or vertices). **B)** Cell interactions are mediated through T1 transition, whereby a shrinking junction by actomyosin contractility until two vertices meet, then the two vertices resolve in a perpendicular direction. Cell extrusion is mediated by T2 transition, whereby several vertices meet to generate a single vertex.

Figure 3. Variations in cell extrusion. A) Cell division occurs in simple epithelium. **B)** Cell extrusion can occur if the mitotic plane is unstable in the apicobasal axis, in that one of the daughter cells delaminates basally, followed by apoptosis. **C)** If apoptosis is inhibited, cell extrusion leads to a mass of cells underneath the epithelium, reminiscent of EMT. **D)** Cell reintegration can occur, in that one of the daughter cells apically leaves the epithelium then reintegrates in the epithelium. **E)** vSrc cell orients the mitotic plane perpendicular to that in normal division and coordinates with the lateral junctions of neighbour. One large cell leaves apically and the other small part goes basally. **F)** Asymmetric cell division occurs in neurosensory motoneuron (NSM) neuroblasts in *C. elegans*, in that the larger daughter cell becomes NSM neuron while the smaller daughter cell undergoes apoptosis. The apoptotic cell is engulfed by neighbouring cells.

Figure 4. Possible mechanisms for long range propagation during cell extrusion. An extruding cell sends Calcium wave (in yellow) to neighbours in a long range. A neighbouring cell can orient division axis to the extruding cell. Potentially, mechanical stretch can activate ERK downstream of EGFR signaling and ERK wave can propagates in a long range.

Figure 1

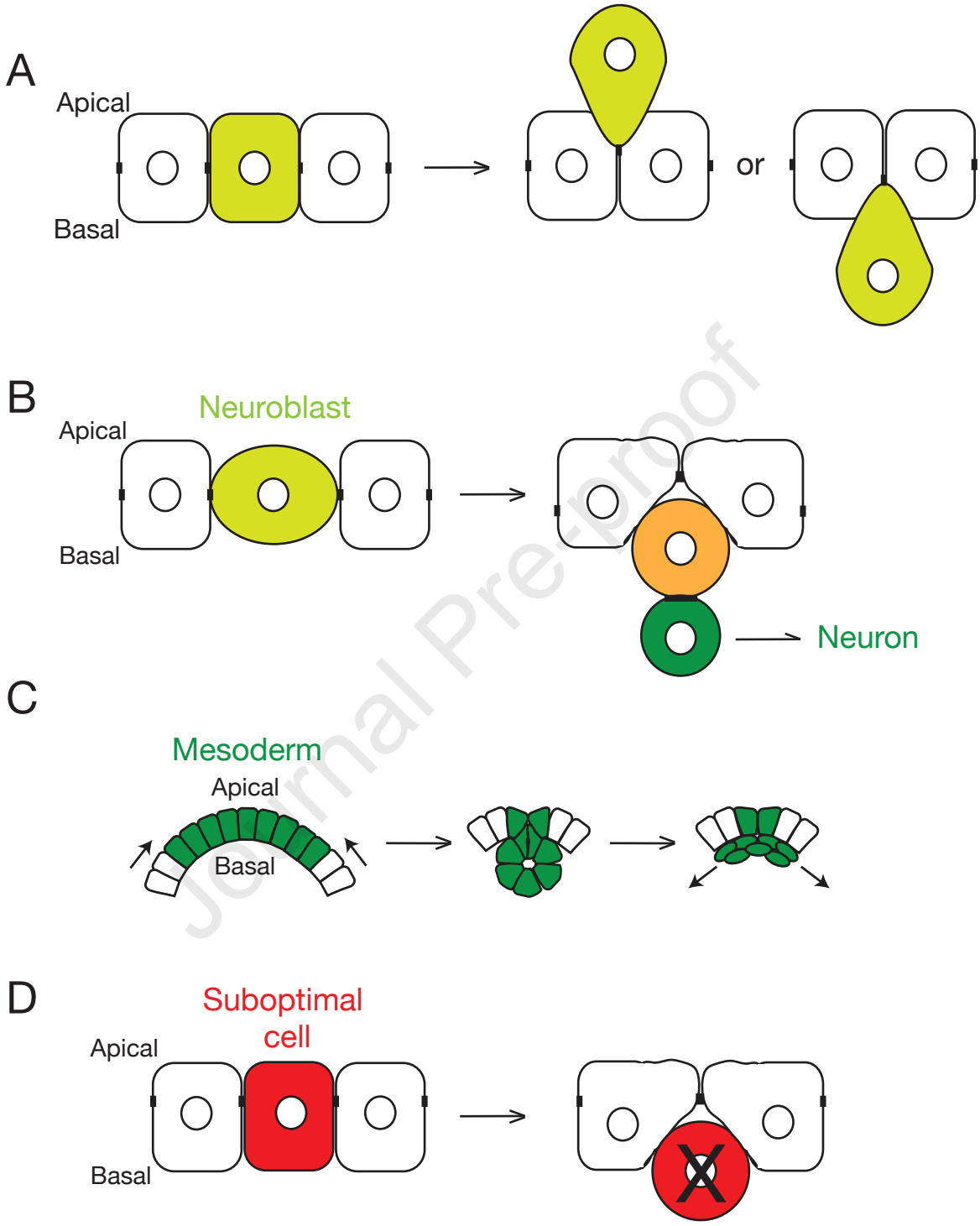
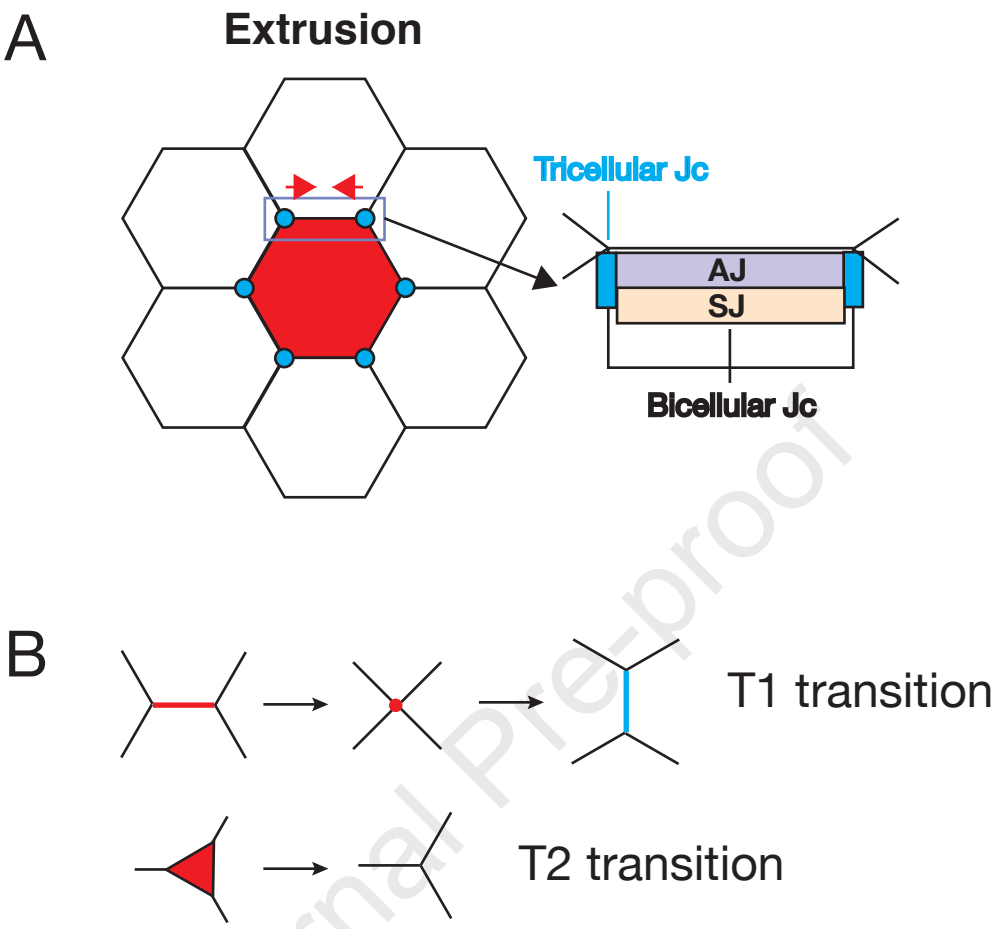


Figure 2



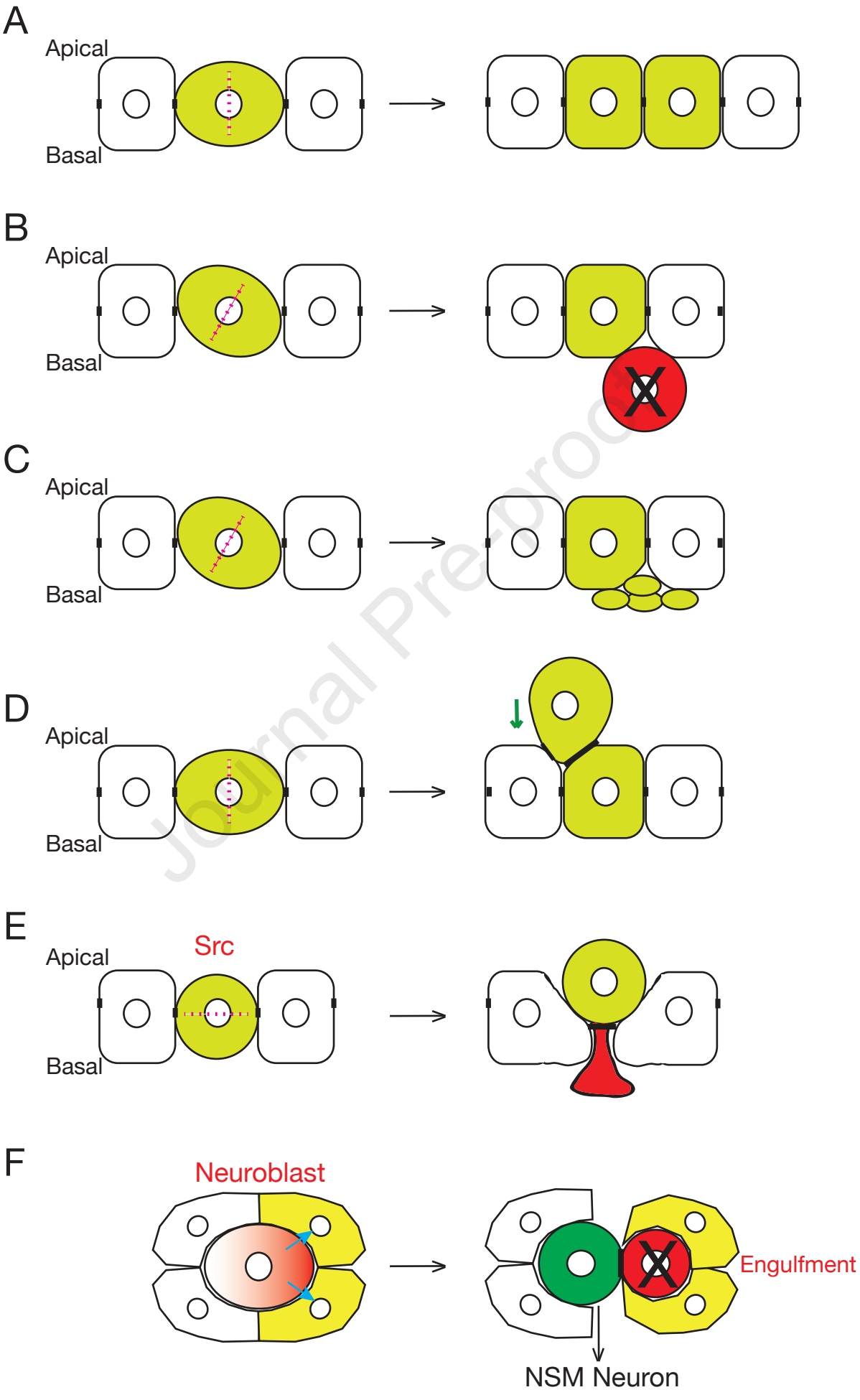
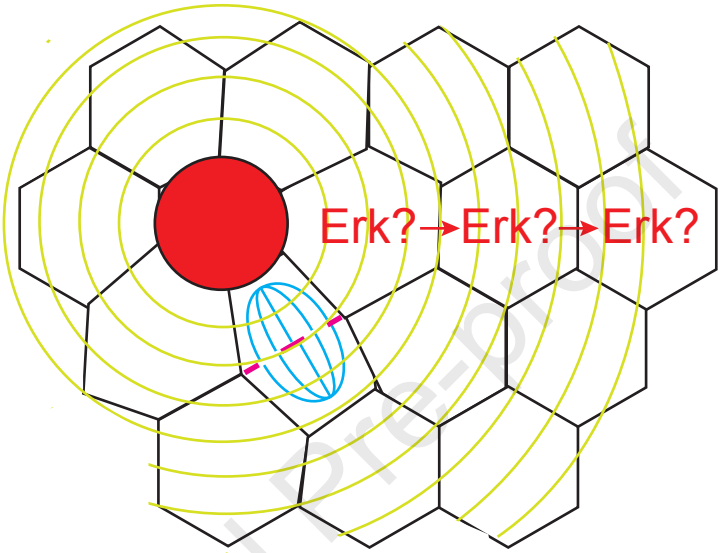


Figure 4



Highlights:

Tricellular junctions play a pivotal role in coordinating cell extrusion

Differentiation sets up elimination of unfit or mis-specified cells

Geometry and topology of epithelia influence the mode of cell extrusion