U. PORTO

DOUTORAMENTO

BIOLOGIA MOLECULAR E CELULAR

The impact of cell-cell and cell-matrix adhesion on the efficiency of epithelial cytokinesis

Margarida Gonçalves

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2024



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SEDE ADMINISTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

FACULDADE DE CIÊNCIAS



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THE IMPACT OF CELL-CELL AND CELL-MATRIX ADHESION ON THE EFFICIENCY OF EPITHELIAL CYTOKINESIS

Tese de Candidatura ao grau de Doutor em Biologia Molecular e Celular;
Programa Doutoral da Universidade do Porto (Instituto de Ciências Biomédicas Abel Salazar e Faculdade de Ciências da Universidade do Porto)

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This thesis gathers the work performed at the Epithelial Polarity and Cell Division (EPCD) group at the Institute for Research and Innovation in Health (i3S/IBMC), under the supervision of Dr. Eurico Morais de Sá and the co-supervision of Prof. Claudio Sunkel, for the degree of Doctor in Philosophy in Molecular and Cell Biology.

Margarida Gonçalves was awarded a PhD fellowship (SFRH/BD/130708/2017 and COVID/BD/152733/2022) by the Portuguese Foundation for Science and Technology (FCT), and was subsequently funded by a fellowship by IPATIMUP, through the project CANCER_CHALLENGE2022. The research presented in this thesis was supported by National Funds through FCT, under the projects PTDC/BIA-CEL/1511/2021 and UIDB/04293/2020. Margarida Gonçalves also acknowledges the support of the i3S Scientific Platform ALM, member of the national infrastructure PPBI – Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122).

To the three women that made me get this far.

And, as always, to my mom (in memoriam).

Abstract

Epithelial tissues form a biological and mechanical barrier, whose functional properties rely on the positioning of cell-cell and cell-matrix adhesion complexes along its apical-basal axis. Both during development and throughout adult life, epithelial tissues proliferate through cell division. This process, and particularly cytokinesis – the last step of cell division – poses unique challenges for epithelial cells, which have to dynamically coordinate adhesion to themselves and the surrounding matrix with the dramatic shape changes that are necessary to allow an efficient daughter cell separation. To tackle this apparent paradox, several studies have dissected the cytokinetic process in different epithelial tissues. Overall, these studies suggest that cytokinesis is not a strictly autonomous process, as both polarity and adhesion are locally remodeled to accommodate cell division, while simultaneously maintaining tissue integrity. However, the majority of these studies concentrate on Adherens Junctions, an E-cadherin-based apical belt that ensures cell-cell adhesion, remaining unclear the impact of other adhesion molecules on the efficiency of epithelial cytokinesis.

To systematically evaluate how the cell adhesion machinery impacts cytokinesis efficiency *in vivo*, I performed an RNAi modifier screen in a cytokinesis-sensitized background in the *Drosophila* follicular epithelium. In line with previous studies, our findings show that apical cadherin-based adhesion challenges epithelial cytokinesis efficiency. Strikingly, several cell adhesion complexes enriched at the lateral and basal level promote cytokinesis completion, rather than opposing it. One such complex is the Dystrophin-Dystroglycan (Dys-Dg) complex, an extracellular matrix receptor that coordinates basal adhesion with the actin cytoskeleton in epithelial cells. Live imaging uncovered a dynamic spatial redistribution of the Dys-Dg complex during epithelial cytokinesis, with both proteins becoming enriched at the basal side of the ingressing furrow, below the contractile ring, during and after ring constriction. This redistribution accounts for a dual role of the Dys-Dg complex in epithelial cytokinesis, as my work suggests that it promotes ring constriction during the initial phase of cytokinesis and also facilitates daughter-cells separation during the abscission period.

Altogether, these findings pave the way to understand the mechanisms that ensure cytokinesis efficiency in the epithelial tissue, in order to maintain the genetic integrity whilst facing the constant challenge of cell division.

Resumo

Os tecidos epiteliais formam uma barreira biológica e mecânica, cuja função depende do posicionamento de complexos de adesão célula-célula e célula-matriz extracelular ao longo do seu eixo apical-basal. Tanto durante o desenvolvimento como ao longo da vida adulta, os tecidos epiteliais proliferam através de divisão celular. Este processo, e em particular a citocinese - o último passo da divisão celular -, impõe desafios únicos às células epiteliais, que têm de coordenar de forma dinâmica a adesão entre si e à matriz extracelular com as alterações de forma dramáticas que são necessárias para permitir uma separação eficiente das células-filhas. Para descortinar este aparente paradoxo, vários estudos dissecaram o processo de citocinese em diferentes tecidos epiteliais. Em geral, estes estudos sugerem que a citocinese não é um processo estritamente autónomo, uma vez que tanto a polaridade como a adesão são remodeladas localmente para acomodar a divisão celular, enquanto preserva simultaneamente a integridade do tecido. No entanto, a maioria destes estudos foca-se nas Junções Aderentes, uma zona apical baseada em E-caderina que assegura a adesão célula-célula, não sendo claro o impacto de outras moléculas de adesão na eficiência da citocinese epitelial.

Para avaliar de forma sistemática o impacto da maquinaria de adesão celular na eficiência da citocinese *in vivo*, realizei um screen de modificadores de RNAi num contexto sensibilizado para citocinese, no epitélio folicular de *Drosophila*. De acordo com os estudos anteriormente publicados, os nossos resultados mostram que a adesão apical assegurada por caderinas desafia a eficiência da citocinese epitelial. Notavelmente, vários complexos de adesão celular a nível lateral e basal promovem a conclusão da citocinese, em vez de se oporem à mesma. Um desses complexos é o complexo Distrofina-Distroglicano (Dys-Dg), um recetor da matriz extracelular de células epiteliais que coordena a adesão basal com o citoesqueleto de actina. Este complexo é redistribuído espacialmente de forma dinâmica durante a citocinese epitelial, com ambas as proteínas a acumularem na base da invaginação da membrana, abaixo do anel contrátil, durante e após a constrição do mesmo. Esta redistribuição suporta um papel duplo do complexo Dys-Dg em citocinese epitelial, uma vez que este trabalho sugere que, por um lado, o complexo promove a constrição do anel durante a fase inicial da citocinese, e por outro, facilita a separação das células-filhas durante o período de abscisão.

Estes resultados abrem caminho para a compreensão dos mecanismos que asseguram a eficiência da citocinese no tecido epitelial, de forma a manter a integridade genética num contexto de desafio constante devido à frequente divisão celular.

Agradecimentos

Remember who you are.
— Mufasa, *The Lion King*

A Ciência, tal como todas a outras áreas da vida, é particularmente gratificante quando partilhada. E que sorte a minha de a ter partilhado com pessoas que têm tanto de talentosas quanto de amáveis, e sem as quais esta tese não seria certamente possível.

First and foremost – ao Eurico. Obrigada por me teres dado a oportunidade de fazer o doutoramento contigo, pela orientação constante, por nunca me teres feito sentir desamparada. Obrigada por me teres dado espaço para ser eu mesma, enquanto me ajudavas a crescer e a perder medos e inseguranças. Obrigada pelas palavras de ânimo quando os dias eram mais cinzentos e pelas frases futebolísticas motivacionais antes de cada talk que dei. Obrigada por nunca deixares de querer celebrar as pequenas conquistas, e por seres igualmente bom como orientador e parceiro de copos ☺ Obrigada por me permitires conciliar a maternidade com o doutoramento num balanço que dificilmente se encontra hoje em dia, tornando, sem dúvida, ambas as jornadas muito mais leves. E, acima de tudo, obrigada por me mostrares como se faz ciência tão bonita. És a personificação de um verdadeiro mentor.

Ao professor Claudio, por me ter acolhido no laboratório mais feliz que alguma vez conheci. Obrigada pela co-orientação constante ao longo do doutoramento e por ser um *pain in the neck* durante os lab meetings, porque é isso que nos ajuda a sermos resilientes e metódicos e a crescer enquanto cientistas.

Aos elementos do Epithelial Polarity & Cell Division, passados e presentes. Sou realmente grata por ter partilhado o meu dia-a-dia no laboratório com todos vocês. Obrigada por todas as discussões científicas, por todas as sugestões, pela partilha infindável de papers e conhecimento, pela ajuda com as experiências, pela disponibilidade constante em fazerem troubleshooting em conjunto. E obrigada, acima de tudo, pela parte não-científica – pelas palavras de motivação, pelas mensagens de afecto, pelas jantaradas e convívios, pela celebração das pequenas conquistas ao longo do caminho e pelos abraços que me ampararam nos momentos de desalento. Todos vocês, sem excepção, contribuíram para que eu chegasse até aqui.

Ao Cell Division & Genomic Stability group, os melhores vizinhos de laboratório, com quem formamos o mítico Happy Lab ☺ É difícil transpor para palavras o quão realmente feliz fui durante estes anos em que trabalhámos no mesmo laboratório. Obrigada por serem muito mais do que as pessoas com quem partilhamos o mesmo espaço – obrigada pela forma calorosa como me receberam, pela disponibilidade e prontidão em ajudar, pelas muitas gargalhadas, pela preocupação genuína, pelas infindáveis conversas, e pelos inúmeros momentos felizes que levo no coração.

Um obrigada muito especial à Augusta – amiga de todas as horas, com quem ri e chorei, a quem confiei medos e sonhos, com quem partilhei momentos de pura felicidade e de muita angústia. Obrigada pelo teu apoio inabalável e pela tua preocupação constante. Vivi das melhores fases da minha vida enquanto navegavas pelos dias mais negros da tua e, mesmo então, nunca deixaste de te preocupar comigo. As palavras não chegam para ti.

Um obrigada gigantesco à Catarina e à Sofia, as labmates mais cool que poderia ter! Obrigada pelas infindáveis conversas – sobre tudo e sobre nada –, pelos lanches, pelos concertos, pelas palavras sempre certas, pelas mil mensagens de encorajamento. Obrigada pelas inúmeras vezes em que me desligaram o microscópio e me recolheram virgens para que eu pudesse ir buscar o meu filho mais cedo. Obrigada por todos os pequenos (enormes!) gestos para que eu não falhasse em nenhuma frente. Obrigada por estarem sempre lá, ainda que a muitos quilómetros de distância. *I'll be there for you, 'cause you're there for me too.*

Aos amigos de sempre – pelo tanto que as palavras não conseguem dizer. Obrigada por, no meio da distância, de filhos, de trabalho, do caos, não deixarmos cair esta amizade.

Por último, à minha pedra basilar – a minha família. Obrigada à minha mãe e às minhas irmãs, que me deram o mundo. Nunca conseguirei agradecer-vos o suficiente por tudo o que fizeram. Vocês são a razão pela qual cheguei até aqui. Ao meu companheiro de aventuras e amor de uma (e para a) vida – you are my ride or die. Obrigada por seres calma, colo e casa. Obrigada por me ajudares a pôr sempre tudo em perspectiva, por me fazeres ver sempre o copo meio-cheio, por seres a serenidade na minha tempestade. É realmente maravilhoso partilhar a vida contigo, porque o mundo é muito mais entusiasmante a teu lado. Por fim – ao meu doce Álvaro. Que ingenuidade a minha, a de achar que eu é que tinha tanto para te ensinar. Obrigada por existires, filho. Foste o melhor deste doutoramento, e da vida toda.

Preface & List of publications

It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair.

— Charles Dickens, *A Tale of Two Cities*

This excerpt from a Charles Dickens' novel perfectly describes the roller-coaster that a PhD is (at least, mine was!). But, as all roller-coasters, this PhD finally comes to an end. And, while most of the work done along this ride was performed by myself, the ride only ends due to the contribution of other people. Thus, besides my supervisor – Eurico Morais de Sá – and my co-supervisor – Claudio Sunkel –, I must also acknowledge Catarina Lopes, Carolina Ramos and Mariana Osswald, who directly contributed to this work at the EPCD lab; Hervé Alégot, Graziella Richard and Vincent Mirouse for all the transgenes they nicely shared with me; and Floris Bosveld and Yohanns Bellaiche for their expertise and the experiments performed in the notum.

This thesis firstly focused on the identification of major regulators of cytokinesis efficiency in the follicular epithelium of the fly and then, building on these findings, aimed to dissect the role of the Dystrophin-Dystroglycan complex in ensuring this process in different *Drosophila* epithelia. Thus, my own novel begins with an Introduction (Chapter I) that covers the *Beginning & Foundations* of the mechanisms underlying cytokinesis, the organization of the epithelial tissue and the challenges of cytokinesis in such a complex environment. In Chapter II, I focus on *Crafting the roadmap*, presenting the major aims and the outline of approach used in this work. The *Insights unveiled* with the experimental work of this thesis have been published at EMBO Reports and are transcribed here as a manuscript in Chapter III. This chapter presents a brief Introduction followed by the Results & Discussion and Methods sections, and is preceded by a statement with the detailed contribution of each author. I then present a broader discussion of the main findings of this work (Chapter IV), framing them within the state-of-the-art and making *Reflections on the journey*. Finally, *The curtain falls* in this novel with concluding remarks and the future perspectives.

The following publication is included in this thesis (the original publication can be found in *Appendix C*):

Gonçalves, M., Lopes, C., Alégot, H., Osswald, M., Bosveld, F., Ramos, C., Richard, G., Bellaiche, Y., Mirouse, V. and Morais-de-Sá, E. (2024) 'The Dystrophin-Dystroglycan complex ensures cytokinesis efficiency in *Drosophila* epithelia', *EMBO Reports*, 26(2):307-328. doi: 10.1038/s44319-024-00319-y.

During this time, I also contributed to the following publications within the scope of this thesis (*Appendix B*):

Faria, L., Canato, S., Jesus, T.T., Gonçalves, M., Guerreiro, P.S., Lopes, C.S., Meireles, I., Morais-de-Sá, E., Paredes, J. and Janody, F. (2023) 'Activation of an actin signaling pathway in pre-malignant mammary epithelial cells by P-cadherin is essential for transformation', *Disease Models & Mechanisms*, 16(2):dmm049652. doi: 10.1242/dmm.049652.

Figueiredo, J., Ferreira, R.M., Xu, H., Gonçalves, M., Barros-Carvalho, A., Cravo, J., Maia, A.F., Carneiro, P., Figueiredo, C., Smith, M.L., Stamenović, D., Morais-de-Sá, E. and Seruca, R. (2022) 'Integrin β1 orchestrates the abnormal cell-matrix attachment and invasive behaviour of E-cadherin dysfunctional cells', *Gastric Cancer*, 25(1):124-137. doi: 10.1007/s10120-021-01239-9.

Figueiredo, J., Mercadillo, F., Melo, S., Barroso, A., Gonçalves, M., Díaz-Tasende, J., Carneiro, P., Robles, L., Colina, F., Ibarrola, C., Perea, J., Morais-de-Sá, E., Seruca, R. and Urioste, M. (2021) 'Germline *CDH1* G212E Missense Variant: Combining Clinical, *In Vitro* and *In Vivo* Strategies to Unravel Disease Burden', *Cancers (Basel)*, 13(17):4359. doi: 10.3390/cancers13174359.

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Chapter I



Beginnings & Foundations

Introduction

It is the great prerogative of Mankind above other Creatures, that we are not only able to behold the works of Nature, or barely to sustain our lives by them, but we have also the power of considering, comparing, altering, assisting, and improving them to various uses.
— Robert Hooke, Preface to *Micrographia*

In an era where knowledge is at the distance of a keyboard that allow us to type a few words and search online, it is refreshing to think of how our current knowledge has evolved through centuries. This is particularly impressive in the Cell Biology field, which nowadays takes advantage of state-of-the-art technology to assess the dynamic distribution of a protein, to measure the tension exerted in a certain tissue or to ablate subcellular structures. Nevertheless, this field took its first steps about four centuries ago, when Robert Hooke (1635-1703) analyzed a piece of cork under a microscope he conceptualized himself, and observed box-shaped structures he named cells (from the latin *cella*, due to the resemblance to monastery cells inhabited by monks). Published in Hooke's book *Micrographia* (1665), this was a groundbreaking discovery that set the stage for an era of massive and remarkable findings in the Cell Biology field. Amongst them, is the observation and description of the stages of mitosis and cytokinesis in algae and higher plants by Eduard Strasburger (1844-1912), in the book *Über Zellbildung und Zelltheilung* (*On Cell Formation and Cell Division*, 1876). A few years later, Walther Flemming (1843-1905) published a detailed description of cell division in animal cells, compiled in his book *Zellsubstanz, Kern und Zelltheilung* (*Cell substance, nucleus and cell division*, 1882). This boom of knowledge from the nineteenth century, when curiosity largely surpassed the scarcity of technical means, resulted in a solid foundation for cellular and molecular biology as we know it today.

1. *Divide and conquer* – a general overview on cytokinesis

Almost two centuries after Robert Hooke observation of a cell in a microscope for the first time, Theodor Schwann (1810-1882) and Matthias Schleiden (1804-1881) proposed the Cell Theory, composed by three major assertations. The first two were that all organisms are composed by cells and that these cells are the basic unit of life (1838-1839). The third one, added in 1855 by Rudolf Virchow (1821-1902), stated that all cells derive from preexisting ones, against the commonly accepted concept of spontaneous generation, which assumed that organisms could arise from nonliving matter. This addenda from Virchow, translated in the epigram *Omnis cellula e cellula* ("*All cells come from cells*"), results from one of the first observations on cell division. Although the subsequent 200 years of technically improved research led to a comprehensive knowledge on this topic, several fundamental questions still remain unanswered. One of them is how cells in a tissue cope adhesion to each other and to the extracellular matrix with the need to separate during cytokinesis – the biological question in the basis of this thesis.

Cytokinesis is the final step of cell division, consisting in the physical separation of the two daughter cells derived from one dividing cell (D'Avino et al. 2015, Pollard and O'Shaughnessy 2019, Ozugergin and Piekny 2022). For this cleavage to occur, a physical barrier that bisects the mother cell must be formed – either through the assembly of a contractile ring, as it happens in metazoan and fungi, or through trafficking and fusion of vesicles at the division plane, to form a partitioning membrane, as observed in plants, algae and single-celled organisms ((Muller and Jurgens 2016, Glotzer 2017, Pollard and O'Shaughnessy 2019). In this chapter, I will firstly describe the molecular mechanisms underlying cytokinesis on animal cells, due to the scope of this thesis. I will then present an overview on the numerous roles of a pivotal cytokinetic protein, that is particularly relevant to our work – Anillin.

1.1. Overview on the molecular mechanisms underlying cytokinesis

The word *cytokinesis* derives from a combination of the classical Latin and ancient Greek words *cyto-* (cellular) + *-kinesis* (movement). As its etymology suggests, cytokinesis encompasses a movement of the cell, in which the cytoplasm is bisected to give rise to two physically-independent daughter cells (D'Avino et al. 2015, Pollard and O'Shaughnessy 2019). This movement is driven by an actomyosin contractile ring, which is connected to the plasma membrane and progressively contracts along the cleavage furrow, pinching in the membrane. As constriction progresses, the connection between the daughter cells narrows, until only a thin intercellular bridge connects them. This bridge is eventually cleaved by abscission, a process that originates two physically-independent daughter cells (Figure 1.1).

1.1.1. Positioning of the division plane

The first step of cytokinesis is to set the position of the division plane at anaphase onset, following chromosome segregation (D'Avino et al. 2015, Pollard and O'Shaughnessy 2019). In animal cells, this positioning occurs through the mitotic spindle, which is reorganized to form and array of antiparallel microtubules that interdigitate (named central spindle or spindle midzone), acting as spatial regulators of cytokinesis. From the first evidence of the role of the mitotic spindle in ring positioning – demonstrated by spindle micromanipulation experiments in echinoderm eggs (Rappaport 1985) –, several studies in different animal models (Bement et al. 2005, Miller and Bement 2009) have shown that the signaling pathways driven by the central spindle ultimately target the accumulation and activation of the RhoGTPase RhoA at the cell equator (Figure 1.1), while simultaneously suppressing it at the cell poles, through astral microtubules (Bringmann and Hyman 2005, Murthy and Wadsworth 2008). This equatorial activation of RhoA drives the assembly and constriction of actomyosin filaments that will originate the contractile ring.

The reorganization of the spindle midzone involves an interplay between four major players: the microtubule-crosslinker Protein Regulator of Cytokinesis 1 (PRC1 (*Dm* Fascetto)), the kinesin-4 (KIF4 (*Dm* Kinesin-like protein 3A, Klp3A)) and two major regulatory complexes – the Chromosomal Passenger Complex (CPC) and the Centralspindlin complex (CS) (Fededa and Gerlich 2012, Glotzer 2017, Pollard and

O'Shaughnessy 2019). The CS consists of a homodimer of MgcRacGAP (*Dm* RacGAP50C) and a homodimer of Mitotic Kinesin-Like Protein 1 (MKLP1 (*Dm* Pavarotti)), whose multimerization depends on two rounds of phosphorylation by the Aurora B kinase (Guse et al. 2005, Douglas et al. 2010). In turn, Aurora B is a component of the CPC, together with three regulatory partners: INCENP, Survivin and Borealin (Carmena et al. 2012).

The timing of the interplay between PRC1, KIF4, CPC and CS is tightly regulated by the mitotic kinases Cyclin-dependent kinase 1 (Cdk1)/Cyclin B, Aurora B and Polo-like kinase 1 (Plk1), which act in coordination to suppress cytokinesis until anaphase onset and to trigger it afterwards (D'Avino et al. 2015, Glotzer 2017). Before anaphase onset, Cdk1 is the major active regulator, preventing the assembly of the central spindle through phosphorylation of two targets: 1) PRC1, inhibiting its interaction with KIF4 and, subsequently, preventing it to be transported to the spindle (Zhu et al. 2006, Zhu and Jiang 2005), and 2) MKLP1, inhibiting the affinity of the CS to bind microtubules (Mishima et al. 2004).

After Cdk1 inactivation by the Anaphase-Promoting Complex (APC) at the metaphase-to-anaphase transition, Plk1 and Aurora B become the major kinases controlling central spindle formation and promoting cytokinesis. The CPC, which localizes at the centromeres during metaphase, is relocated to the spindle midzone after anaphase onset, allowing Aurora B to phosphorylate MKLP1 and promoting CS assembly (Guse et al. 2005). At the same time, the inhibitory phosphorylation of PRC1 by Cdk1 is ceased, allowing PRC1 to be relocated to the spindle midzone via KIF4 (Kurasawa et al. 2004, Zhu and Jiang 2005, Zhu et al. 2006), where they jointly act to regulate the size of the central spindle: while PRC1 promotes the crosslinking of microtubules, KIF4 controls the length of the overlapping microtubules, by inhibiting microtubule polymerization (Bieling et al. 2010, Hu et al. 2011, Nunes Bastos et al. 2013). This restrictive role of KIF4 is, in turn, indirectly regulated by Plk1-mediated inhibition (Hu et al. 2011). Plk1, whose localization was restricted to the centrosomes and kinetochores until anaphase onset, is relocated to the spindle midzone. This accumulation of Plk1 at the central spindle *per se* seems to be crucial for the positioning of the division plane, and has shown to be dependent on its binding to PRC1, which can only occur after dephosphorylation of an inhibitory docking site for Cdk1 (Neef et al. 2007). Additionally, Plk1 also phosphorylates MgcRacGAP, triggering its binding to the Rho-GEF Ect2 (*Dm* Pebble) and subsequently recruiting it to the spindle midzone, where it associates with the plasma membrane. MgcRacGAP then suppresses Ect2 autoinhibition, activating its GEF activity, and leading to the recruitment and activation of RhoA to the central spindle (Kim et al. 2014).

Simultaneously, RhoA activation is modulated at the cell poles through the action of dynamic astral microtubules. RhoA suppression in the non-equatorial cortex of *C. elegans*

embryos (Werner et al. 2007) and vertebrate cells (Murthy and Wadsworth 2008) negatively regulates myosin activation, limiting the contractile activity of the polar cortex – an event termed polar relaxation. In line with this, disruption of astral microtubules induces the formation of a broader zone of active RhoA in echinoderm embryos (Bement et al. 2005) or the formation of a wider contractile ring in *C. elegans* embryos (Werner et al. 2007). Remarkably, astral microtubules alone induce the formation of a cleavage furrow in anucleate cells of sea urchins without a spindle, if the asters are far enough apart (von Dassow et al. 2009).

Altogether, the coordination between spindle microtubules, which promote the equatorial activation of RhoA, and astral microtubules, that prevent its polar activation, allows the assembly of a spatially-restricted and active belt of RhoA at the spindle midzone, which is essential for the next step of cytokinesis – the assembly and constriction of a contractile ring.

1.1.2. Contractile ring assembly and constriction

The formation of an active belt of RhoA at the cell equator is the key event that controls the assembly and constriction of a contractile ring (Bement et al. 2005, D'Avino et al. 2005). This annular structure is mainly composed by filamentous actin (F-actin) and non-muscle myosin motors, which form cross-linked filaments with 0.1-0.2 μm of thickness. The contractile ring also encompasses regulatory and structural proteins that tether it to the plasma membrane so that, when the ring constricts, the cytoplasm of the mitotic cell becomes bisected (Schroeder 1970, Miller 2011) (Figure 1.1).

The assembly of the contractile ring is driven by three parallel signaling pathways, triggered by the equatorial activation of RhoA. The first one occurs through the binding of active RhoA to the diaphanous subfamily of formins (*Dm* Diaphanous), that stimulate profilin-mediated nucleation and assembly of unbranched actin filaments at the spindle midzone (Watanabe et al. 1997, Otomo et al. 2005, Rose et al. 2005). Secondly, RhoA activates two kinases – Rho kinase (ROCK (*Dm* Rok)) and Citron kinase (*Dm* Sticky) – that phosphorylate myosin regulatory light chain (MRLC), activating it (Amano et al. 1996, Riento and Ridley 2003, Yamashiro et al. 2003). Lastly, active RhoA directly binds Anillin (Hickson and O'Farrell 2008a, Piekny and Glotzer 2008), a scaffold protein that, together with septins, anchors the contractile ring to the plasma membrane through a pleckstrin homology (PH) domain in its C-terminal end (Piekny and Maddox 2010). Anillin is able to recruit and interact with a myriad of structural and signaling components involved in the assembly and

constriction of the contractile ring, namely F-actin and myosin, and is also responsible for septins recruitment to the cleavage furrow, which play a pivotal role in ring anchoring (Hickson and O'Farrell 2008a, D'Avino 2009, Piekny and Maddox 2010). Noteworthy, it has been proposed that, while these three signaling pathways are active, MgcRacGAP inhibits Rac GTPase at the midzone (D'Avino et al. 2004, Canman et al. 2008), a negative regulator of cytokinesis. Rac inhibition is thought to inactivate the Arp2/3 complex, which subsequently downregulates the formation of branched F-actin and reduces the cortex stiffness at the cell equator (Canman et al. 2008).

Following its assembly, the actomyosin ring constricts, causing the ingression of the overlying plasma membrane, which is pinched in to bisect the cytoplasm of the dividing cell (Figure 1.1). Although contractile ring forces are crucial for an efficient furrow ingression, the mechanism that drives ring contractility is poorly understood. Ultrastructural analysis and model simulations have led to two major models of force-generating mechanisms, which transversally seem to rely on actomyosin cooperation and on cortical pole relaxation. The classical model is based on a “purse-string” mechanism similar to the one that occurs in muscle sarcomeres, where the myosin motors pull on the actin filaments, sliding them towards their plus-ends and reducing the ring diameter (Fededa and Gerlich 2012, Schwayer et al. 2016, Thieleke-Matos et al. 2017). However, this model implies an alignment of the filaments with the division plane, which does not occur in all the filaments of the contractile ring (Maupin and Pollard 1986, Mabuchi et al. 1988), hence turning the contribution of these non-aligned actin filaments to ring constriction elusive. An alternative and non-exclusive model proposes that the force-generating mechanism that drives ring constriction is the tension generated by the depolymerization of cross-linked actin filaments, triggered by myosin (Zumdieck et al. 2007, Mendes Pinto et al. 2012, Schwayer et al. 2016). Noteworthy, this dynamic contractile network seems to display “security brakes”, that negatively regulate contractility and are essential for its turnover. In *C. elegans*, RhoA activity has been shown to be restricted by two cytokinetic ring components – Germinal Center Kinase-1 (GCK1) and Cerebral Cavernous Malformation (CCM-3) –, which are recruited to the cytokinetic ring by active RhoA and Anillin, where they limit RhoA activity and subsequent ring contractility (Bell et al. 2020).

Studies in *C. elegans* suggest a scalability between the initial ring size and the speed of constriction. The authors propose a “contractile unit model” where the contractile ring consists of blocks of contractile units whose number is proportional to the initial diameter of the ring, and that disassemble during constriction. This means that larger rings are composed by more contractile units than the smaller ones, and thus constrict faster, suggesting the speed of ring constriction scales with the initial ring diameter (Carvalho et al. 2009). A similar correlation was observed in the filamentous fungus *N. crassa*, in which

larger actomyosin rings also constricted faster than smaller rings (Calvert et al. 2011). Nevertheless, this scalability seems to reach an upper limit in vertebrates, when the furrowing speed cannot be further scaled up. For example, the first rounds of division in mouse blastomeres (1-2 to 8-16 cell stage) display a constant speed of constriction, suggesting an insensitivity of the speed of constriction to the cell size. From the 8-16 cell stage onwards, which are more similar in volume to one-cell *C. elegans* embryo, furrowing ingression decreased, suggesting that cell size determines the speed of constriction during the reductive cell divisions of mouse embryos (Paim and FitzHarris 2022).

1.1.3. Abscission – the final cut

The abscission phase corresponds to the latest stage of cytokinesis, when the daughter cells become individualized. For this to occur, contractile ring constriction proceeds until an intercellular bridge (ICB) with about 1–2 µm of diameter connects the two daughter cells (Mierzwa and Gerlich 2014, Rani et al. 2022). At the center of the ICB, a structure named midbody acts as a platform to localize the abscission site (Mierzwa and Gerlich 2014, Thieleke-Matos et al. 2017, Andrade and Echard 2022), recruiting the Endosomal Sorting Complex Required for Transport-III (ESCRT-III) machinery to induce membrane deformation and subsequent scission (Guizetti and Gerlich 2012, Hu et al. 2012, Rani et al. 2022) (Figure 1.1). Abscission encompasses three major steps: midbody formation, stabilization and maturation of the ICB and membrane scission.

Midbody formation

As the contractile ring constricts, spindle microtubules are remodeled to become densely packed and overlapped in antiparallel bundles, forming the core of the midbody (Hu et al. 2012, Mierzwa and Gerlich 2014, Andrade and Echard 2022, Rani et al. 2022). At the same time, spindle midzone proteins are progressively relocated to distinct domains of the midbody, contributing to its formation and maturation. Therefore, besides the array of compacted midzone microtubules, the midbody core also contains PRC1 and KIF4, which remain associated with the midzone microtubules (Hu et al. 2012). The midbody core is encircled by the midbody ring, a contractile ring remnant, where the major ring components accumulate, such as Anillin, Septins, Citron kinase and RhoA (Mierzwa and Gerlich 2014, Addi et al. 2018). Adjacent to the midbody core, microtubule bundles enriched in CENP-E,

MKLP2 and its cargo, Aurora B, form the midbody flanks. Midbody assembly into these three distinct regions (core, ring and flanks) seems to be largely dependent on the kinase activity of Plk1, which is activated following Cdk1 post-anaphase inhibition by the APC. Accordingly, inhibition of Plk1 prevents the relocalization of midzone proteins and the formation of the midbody, which ultimately disrupts abscission (Hu et al. 2012).

Stabilization and maturation of the ICB

Upon full contractile ring constriction, most of the filamentous actin within the ICB disassembles (Guizetti and Gerlich 2012) to allow its maturation and, later, membrane scission (Addi et al. 2018). Low actin levels at the ICB are ensured through RhoA inactivation, which inhibits F-actin polymerization, and through MICAL1-mediated oxidation of actin, that causes F-actin depolymerization (Advedissian et al. 2024). Consequently, the mechanical stability of the ICB becomes independent of the actin filaments and rather relies on non-redundant mechanisms that involve the Centralspindlin complex and the coordinated action of Anillin, Septins and Citron kinase (Mierzwa and Gerlich 2014, Addi et al. 2018).

The motor activity of MKLP1 brings the Centralspindlin complex (composed by MgcRacGAP and MKLP1) to the center of the ICB, where it recruits and interacts with the midbody-adapter protein CEP-55, the Rho-GEF Ect2 and the small GTPase Arf6 (Hu et al. 2012). MKLP1 indirectly links the microtubules within the midbody to the plasma membrane through its binding to Arf6, which contains a membrane-binding PH domain that contributes to the cortical anchorage of the ICB (Makyio et al. 2012). Additionally, the other Centralspindlin component, MgcRacGAP, directly tethers the midbody microtubules to the plasma membrane, through its membrane-binding domain (Lekomtsev et al. 2012). Beyond the midbody core, a pivotal player in ICB stabilization is Anillin, which is recruited and maintained not only at the midbody but also at its adjacent regions by Citron kinase (D'Avino et al. 2004, Piekny and Maddox 2010, Hu et al. 2012). Through its PH domain, Anillin binds phosphoinositides present in the plasma membrane (Liu et al. 2012), while simultaneously interacting with Septins (Maddox et al. 2005, D'Avino et al. 2008). In fact, it was recently highlighted the requirement of the interaction between Anillin and Septins to allow the elongation and maturation of the ICB in HeLa cells (Panagiotou et al. 2022). The inability of Anillin to bind SEPT9, which is required for ICB maturation, was shown to be bypassed through the formation of a complex with an adaptor protein that simultaneously binds Anillin and SEPT9, showing the importance of the interaction between Anillin and Septins for a

successful abscission. Citron kinase further contributes to the anchorage of the ICB to the cell cortex by directly binding MKLP1 and KIF14 (*Dm* Nebbish), another microtubule-associated kinesin, which in turn interact with the PRC1 present at the midbody core (Mierzwa and Gerlich 2014). Interestingly, although PRC1 has been reported to be dispensable for cytokinesis completion in the *C. elegans* early embryo (Verbrugghe and White 2004, Lewellyn et al. 2011, Green et al. 2013), it was recently shown that acute PRC1 inactivation in one-cell and four-cell embryos leads to the formation of an elongated ICB that thins but fails to seal (Santos et al. 2023), demonstrating a requirement for this protein during the abscission phase.

As it matures, the ICB progressively narrows to about half of its initial width (Guizetti and Gerlich 2012), and the cortex adjacent to the midbody (1-2 μm) starts to contract on both sides. These constriction zones have been shown to be encircled by cortical filament helices with 17 nm of diameter, that remain associated to ICB membrane, constricting it until the final cut (Fededa and Gerlich 2012, Mierzwa and Gerlich 2014, Advedissian et al. 2024). Although the identity of these filaments remains unknown, it is likely that they correspond to members of the ESCRT-III complex, since this complex co-localizes at constriction zones and is required for the formation of these filaments (Fededa and Gerlich 2012). The core components of ESCRT-III are four charged multivesicular body proteins: CHMP2 (which has 2 isoforms in humans – (CHMP)2A and 2B), CHMP3, CHMP4 (*Dm* Shrub; human CHMP4 displays 3 isoforms – (CHMP)4A, 4B and 4C) and CHMP6 (Guizetti and Gerlich 2012). ESCRT-III assembly at the midbody is stepwise and triggered by the recruitment of Cep55, a centrosomal-associated protein, to the ICB by MKLP1 (Morita et al. 2007, Lee et al. 2008). In turn, mature Cep55 recruits CHMP4, promoting the assembly of spiral ESCRT-III filaments in one of the midbody arms, via two adaptors – the ESCRT-I subunit TSG101 and Alix (Carlton and Martin-Serrano 2007, Morita et al. 2007). TSG101 and Alix Cep55-dependent recruitment to the midbody require integrin-induced FAK signaling, and does not occur in detached cells (Rani et al. 2022). Interestingly, increasing evidence has been recently uncovering alternative pathways that act redundantly or independently of Alix for the recruitment of ESCRT-III components to the abscission sites in different cell contexts (Goliand et al. 2014, Christ et al. 2016, Gulluni et al. 2021, Hermant et al. 2024). Noteworthy, depletion of the different ESCRT components leads to cytokinesis failure (Elia et al. 2011).

For ICB constriction proceed until completion, the microtubule bundles must be cleared to allow membrane scission, as they obstruct the ICB. Removal of the microtubules is ensured by Spastin, a microtubule-severing AAA-ATPase protein, which is recruited to the abscission site through its binding with the ESCRT-III subunit CHMP1B (Reid et al. 2005, Yang et al. 2008).

Membrane scission

After clearance of the cytoskeleton at the ICB (through F-actin disassembly and microtubule severing), the ESCRT-III depolymerization factor Vps4 accumulates at the ICB, by binding to ESCRT-III subunits (Babst et al. 1998, Saksena et al. 2009). However, despite all the factors known to be involved in abscission and all the signaling cascades discovered so far, the precise mechanism by which membrane scission occurs remains unclear. The most common accepted model is that, after constriction of the helical filaments that progressively close the ICB, Vps4 catalyzes membrane fission (Elia et al. 2011) and the disassembly of ESCRT-III filaments (Wollert et al. 2009) (Figure 1.1).

Scission of the ICB occurs sequentially on each side of the midbody, originating two physically independent daughter cells. Membrane scission ends up with the formation of a midbody remnant, whose fate depends on the cell type (Mierzwa and Gerlich 2014, Glotzer 2017, Advedissian et al. 2024). The midbody remnant can be degraded by autophagy, released into the extracellular medium, or be phagocytosed and later degraded. However, it remains unknown how the midbody remnant contributes to the specification of cell fate (Fededa and Gerlich 2012, Glotzer 2017). The inheritance of the midbody is usually asymmetric, and can arise from the preferential accumulation of abscission factors in one of the daughter cells, or as a result of tension release at the ICB (Thieleke-Matos et al. 2017, Andrade and Echard 2022).

Abscission timing is primarily regulated by several mitotic kinases that also coordinate the positioning and the assembly of the contractile ring. Both Plk1 and Aurora B act as negative regulators of abscission – while Plk1 suppresses the polymerization of the abscission machinery through Cep55 phosphorylation (Bastos and Barr 2010), until its degradation upon mitotic exit, Aurora B phosphorylates CHMP4C, an ESCRT-III subunit, most likely inhibiting it (Capalbo et al. 2012, Carlton et al. 2012). On the other hand, abscission is positively regulated by Cyclin B, which seems to act in coordination with Aurora B to regulate the duration of abscission (Mathieu et al. 2013). In epithelial cells, accumulating evidence has been indicating that abscission timing is also regulated by the mechanical tension between the new daughter cells. The current model suggests that the pulling forces exerted by the daughter cells cause tension at the ICB, inhibiting abscission. Upon tension release at the ICB, ESCRT-III polymerization is triggered, leading to cytokinetic abscission (Lafaurie-Janvore et al. 2013, Andrade and Echard 2022).

Contrarily to this classical view, some cells do not complete abscission and become connected to each other through cytoplasmic bridges, a process termed incomplete cytokinesis. These structures are widespread across not only multicellular eukaryotes but also across different cell types, including germ, stem and, more rarely, somatic cells. One

organism displaying incomplete cytokinesis in both germline and somatic cells is *Drosophila*, in which the cytoplasmic bridges are classically termed ring canals (Brown and King 1964). Until stage 6 of oogenesis, the follicle cells that encapsulate the germline in *Drosophila* ovaries undergo cell division. However, abscission is incomplete in the follicular epithelium, forming permeable ring canals that directly interconnect sibling follicle cells, allowing the diffusion of cytoplasmic content through its open lumen (McLean and Cooley 2013). Remarkably, although these ring canals arise from cytokinesis, they are devoid of microtubule bundles (Woodruff and Tilney 1998, Haglund et al. 2011). Stable ring canals display instead an inner rim and a more electron-dense outer rim (Haglund et al. 2011), composed of actin filaments (Woodruff and Tilney 1998). The molecular mechanisms by which cytokinesis halts, in opposition to classical cytokinesis which proceeds to complete abscission, have been more characterized in the *Drosophila* female germline than in the soma, although it remains incompletely understood. Three molecular mechanisms seem to underly incomplete cytokinesis: 1) stabilization of the intercellular bridge, mainly ensured by the thick actin bundles of the inner rim of ring canals, 2) halting constriction of the actomyosin ring, through dephosphorylation of Myosin II, and 3) inhibition of abscission, possibly by inactivation, inhibition or downregulation of abscission factors (Haglund et al. 2011).

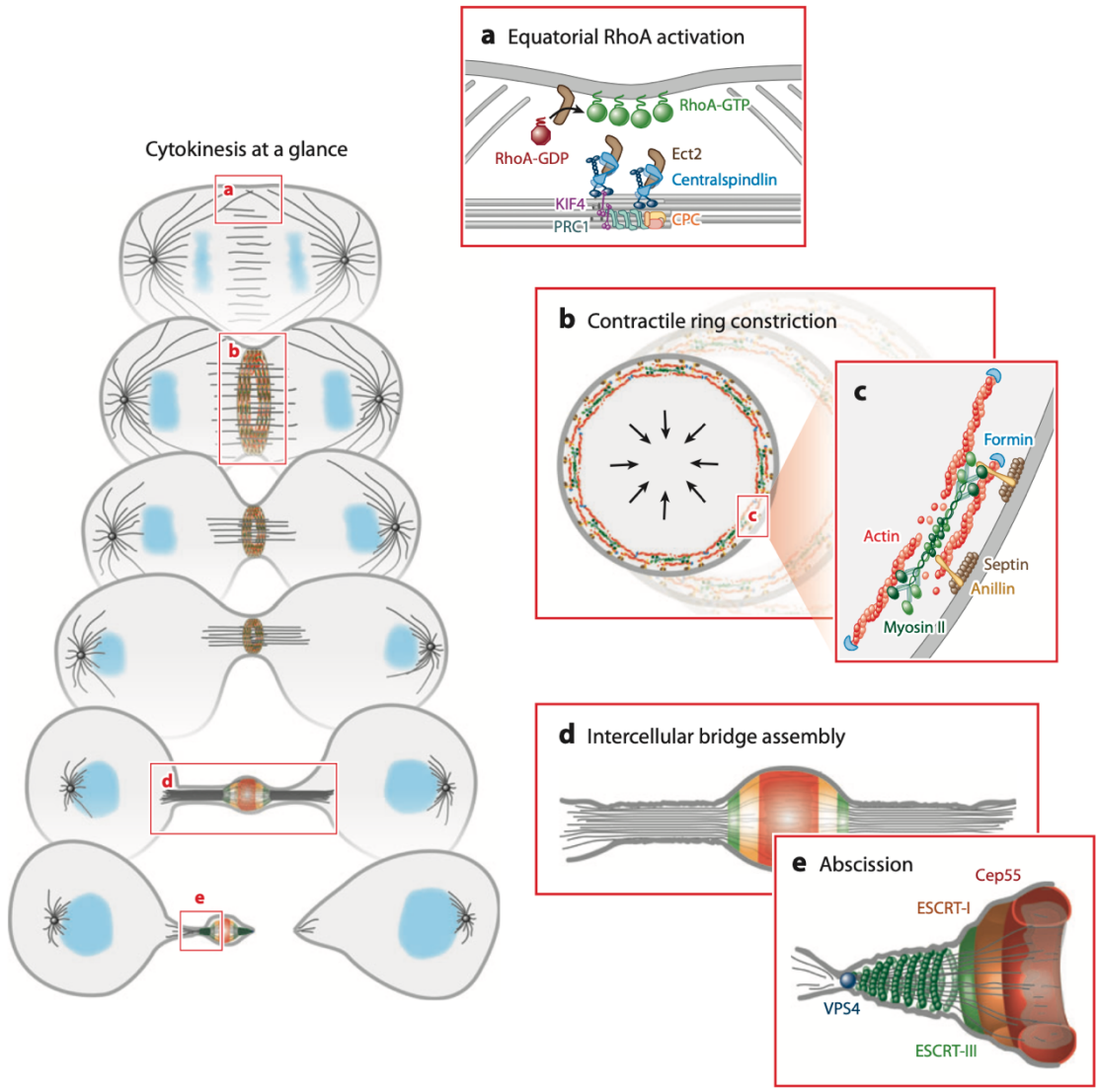


Figure 1. 1. Summary of key events during animal cytokinesis. a) Following chromosome segregation during anaphase, the spindle microtubules overlap and interdigitate to form the central spindle. The central spindle acts as a platform for the equatorial accumulation and activation of RhoA, which is simultaneously suppressed at the cell poles. **b)** Active RhoA directs the assembly of an actomyosin contractile ring, which is anchored to the plasma membrane of cells through Anillin and Septins **(c)**. The contractile ring progressively contracts, bisecting the dividing cell into two daughter cells. **d)** As constriction progresses, the daughter cells become connected through an intercellular bridge, which is then cleaved by abscission, **e)** originating two physically independent daughter cells (adapted from (Green et al. 2012)).

1.2. Anillin – a multitasking protein during cytokinesis

Anillin is a transversal component of all eukaryotic cytokinetic contractile rings, a peculiarity that originated its name, which derives from the Spanish word for ring – *anillo* (Field and Alberts 1995). Its sub-cellular localization varies with the cell cycle progression: in both flies and vertebrates, Anillin localizes to the nucleus during interphase and to the cortex after nuclear envelope breakdown; during anaphase, it accumulates at the equatorial cortex and, from telophase onwards, it is subsequently relocated to the cleavage furrow/contractile ring, being also observed in the midbody ring after full constriction (Field and Alberts 1995, Oegema et al. 2000).

Although originally identified in *Drosophila* embryos (Field and Alberts 1995), subsequent studies showed that Anillin and Anillin-related proteins are conserved amongst eukaryotes, from yeast to humans (D'Avino 2009, Piekny and Maddox 2010, Thieleke-Matos et al. 2017, Naydenov et al. 2021). The structural domains of Anillin have been mapped according to the binding motifs required for its interaction with different binding partners (Figure 1.2). The N-terminal domain of Anillin harbors a Myosin II-binding domain followed by an actin-binding domain (Straight et al. 2005). In addition, the N-terminal region of Anillin contains binding sites for other binding partners, such as the Diaphanous-related Formin 3 (DIAPH3), the cytokinesis regulator Citron kinase and the actin filament capping protein CD2AP, as well as several nuclear localization sequences (NLSs), necessary for the nuclear localization of Anillin during interphase (Field and Alberts 1995, Oegema et al. 2000). Downstream of the actin-binding domain, there is a central domain with intrinsically disordered regions, that has been recently showed to facilitate the linear structure formation of Anillin (Lebedev et al. 2023).

The C-terminal half displays a unique Anillin homology domain (AHD), essential for the Anillin-dependent regulation of the Rho GTPases family (Piekny and Glotzer 2008), and also seems to harbor a microtubule-binding site (Sisson et al. 2000, Hickson and O'Farrell 2008b). Downstream of the AHD, a pleckstrin homology (PH) domain, together with the adjacent terminal part of the AHD, mediates the interaction of Anillin with Septins in different systems (Maddox et al. 2005, D'Avino et al. 2008, Hickson and O'Farrell 2008b, Goldbach et al. 2010, Kinoshita et al. 2002), which, in turn, can also directly bind membrane lipids (Zhang et al. 1999, Casamayor and Snyder 2003). Additionally, the PH domain of Anillin can also directly interact with the phosphatidylinositol phosphate lipids (PIPs) present in the furrow of mammalian cells (Logan and Mandato 2006, Liu et al. 2012). Due to these membrane-interacting abilities of both Anillin and Septins, these proteins have been

proposed to act as a scaffold platform linking the contractile ring to the plasma membrane. Altogether, the interaction of Anillin with this myriad of contractile ring components and cytokinesis regulators place this protein as a master organizer of cytokinesis.

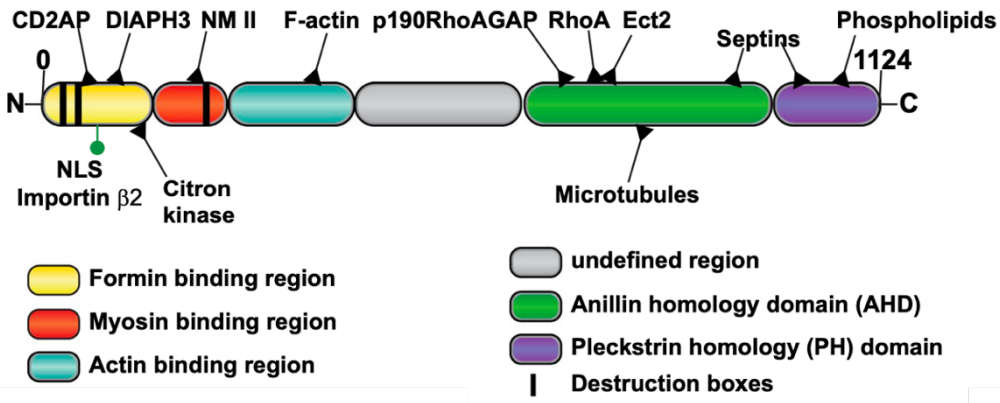


Figure 1. 2. Schematic representation of the different domains/binding partners of Anillin. The N-terminal domain of Anillin displays a formin- (yellow), myosin- (red) and actin (blue)-binding region, which seems to be responsible for the organization of actomyosin structures and for driving ring contractility during cytokinesis. Downstream of the central disordered domain (grey), an Anillin-homology domain (green) harbors binding sites for different cytokinetic regulators, as Septins, RhoA and its activator, Ect2. The last half of the C-terminal domain comprises a pleckstrin-homology domain (purple), that also contributes for binding Septins and membrane phospholipids (adapted from (Naydenov et al. 2021)).

The Myosin II-binding domain was initially identified in *Xenopus* and contains several amino acids highly conserved in humans and *Drosophila* (Straight et al. 2005). The direct interaction between Anillin and Myosin seems to be independent of F-actin – although they also indirectly interact via F-actin (Piekny and Maddox 2010) – and is potentiated by the phosphorylation, and subsequent activation, of non-muscle Myosin II via Myosin Light Chain Kinase (MLCK) (Straight et al. 2005). Anillin and Myosin are recruited to the contractile ring independently of each other in both mammalian and *Drosophila* S2 cells (Straight et al. 2005, Hickson and O'Farrell 2008b), as well as in *C. elegans* (Maddox et al. 2005), suggesting this interaction is not required for their recruitment to the cleavage furrow. Instead, Anillin seems to act as a key organizer of Myosin II, being essential for the proper organization of actomyosin-based contractile structures. Depletion of Anillin in dividing mammalian (Straight et al. 2005, Zhao and Fang 2005, Piekny and Glotzer 2008) and *Drosophila* S2 (Straight et al. 2005, Hickson and O'Farrell 2008b, Kechad et al. 2012) cells does not abrogate Myosin recruitment to the furrow nor furrow initiation or positioning, but perturbs the spatial stability of myosin at the cell equator during cytokinesis. These cells ultimately fail cytokinesis due to oscillating furrows that displace back and forth during constriction and/or due to furrow failure at late cytokinesis. In *C. elegans*, Anillin (*Ce* ANI-1)

was shown to be required for pre-embryonic contractile events that culminate with the extrusion of polar bodies (actomyosin-based rings), after two rounds of meiotic divisions (Maddox et al. 2005). Strikingly, ANI-1 seems to be dispensable for cytokinesis in this system, not affecting neither the asymmetric positioning of the furrow within the anterior-posterior axis of the dividing embryo, nor cytokinesis completion (Maddox et al. 2005, Maddox et al. 2007). Nevertheless, Anillin-depleted embryos displayed a symmetric and slightly delayed ring furrowing, in contrast to wild-type embryos that constrict asymmetrically (Maddox et al. 2007, Hsu et al. 2023, Santos et al. 2023). This asymmetric furrowing in *C. elegans* is partially ensured by an asymmetric distribution of Myosin around the contractile ring, which is lost upon Anillin depletion (Maddox et al. 2007). Recently, it was further shown in the same system that inhibition of ANI-1 strikingly increases the cytokinesis defects caused by acute inhibition of PRC1, a microtubule-bundler (Santos et al. 2023). Additionally, it was shown that these defects were due to a failure in maintaining Myosin at the contractile ring, which stalled constriction and led to a regression of the furrow. Interestingly, if ANI-1 is depleted along with a partial PRC1 inactivation, the furrow ingresses until late cytokinesis (about 97% ingression), but Myosin progressively disappears from the intercellular bridge (ICB), impairing its ability to thin and elongate, and ultimately leading to late-furrow regression. Altogether, this suggests a jointed action of ANI-1 and PRC1 in ensuring proper Myosin localization at the contractile ring both during its constriction and also at the ICB, during the abscission phase, so the ICB can elongate and seal to drive a faithful cytokinesis (Santos et al. 2023). Furthermore, it has been shown in the same system that the assembly of the contractile ring is facilitated by a cortical flow – a concerted movement of cell cortex components at the cell surface – oriented from the poles towards the contractile ring (Reymann et al. 2016, Li and Munro 2021). A recent study reports that the mechanical inhibition of this ring-directed flow is mechanosensed by the contractile ring, driving asymmetric ring furrowing. The mechanosensitivity of the contractile ring is specifically fine-tuned by Anillin (Hsu et al. 2023), suggesting a novel role for this protein in driving asymmetric ring closure. Interestingly, it was also recently reported a role for Anillin in organizing the basal array of Myosin that is formed during *Drosophila* cellularization, an atypical form of cytokinesis, through the upstream regulation of Dunk, a cellularization-specific protein (Chen et al. 2023).

Just downstream of the Myosin II-binding domain, the actin-binding domain harbors several actin-binding sites (Field and Alberts 1995, Straight et al. 2005) – three in *Drosophila* (Jananji et al. 2017) and at least two in human cells (Matsuda et al. 2020). These multiple actin-binding sites sustain the preferential interaction of this protein with filamentous actin and its actin-bundling activity (Naydenov et al. 2021). The direct requirement of actin for Anillin localization to the cleavage furrow seems to be partially dispensable, at least in some

cell-contexts. The recruitment of Anillin to the cell equator or to the cleavage furrow in hamster fibroblasts (Oegema et al. 2000) and *Drosophila* S2 (Hickson and O'Farrell 2008b, D'Avino et al. 2008) cells, as well as to the meiotic rings formed during *Drosophila* male meiosis (Giansanti et al. 1999, Goldbach et al. 2010), seems to be able to occur, at least in a partial extent, independently of F-actin. Nevertheless, the interaction with F-actin promotes a more efficient and both timely- and spatially-restricted recruitment of Anillin to the cell equator and to the cleavage furrow, hinting an F-actin-dependent regulation of Anillin (Oegema et al. 2000, Hickson and O'Farrell 2008b, D'Avino et al. 2008). In contrast, F-actin depolymerization with Latrunculin disrupts Anillin localization in mitotic HeLa cells (Straight et al. 2003), suggesting that the extent of actin requirement for proper Anillin localization might vary with cell type.

Although Anillin is largely localized at the nucleus during interphase, there is also increasing evidence for a pool of Anillin at cell-cell junctions in interphasic cells, such as in *Xenopus* embryos (Reyes et al. 2014, Arnold et al. 2019, Craig et al. 2024) and in human mammary gland cell lines (Budnar et al. 2019). At the junctions, Anillin regulates junctional and medial-apical actomyosin networks (Reyes et al. 2014, Arnold et al. 2019), which are remodeled upon Tight Junctions repair to maintain its integrity (Craig et al. 2024). Despite this junctional interaction between Anillin and actin, a direct binding between these proteins has also been frequently observed in contractile structures involved in cell division. During dorsal closure in *Drosophila* embryos, Anillin is absent from the apically-constricting cells at the leading edge, but present in small spots scattered throughout the late embryo, which most likely correspond to remnants of the cytokinetic ring (Field and Alberts 1995). In line with this, Anillin localizes to ring canals, mitotic and meiotic contractile rings and also appears in puncta in the vicinity of the actin cones formed during spermatids individualization during *Drosophila* spermatogenesis; in contrast, in actin cones ready to move away from the mature sperm nuclei, these Anillin puncta coalesce (Goldbach et al. 2010). Similar to its aforementioned role on organizing Myosin, Anillin has been implicated in F-actin organization, namely through its ability to regulate actin bundling, which seems to be conserved amongst *Drosophila* (Field and Alberts 1995) and vertebrates (Kinoshita et al. 2002). In fact, more than “simply” organizing filamentous actin into bundles, Anillin also has the ability to autonomously propel the contractility of these actin bundles, by generating contractile forces of tens of pico-Newtons that maximize the overlapping between the bundled actin filaments (Kucera et al. 2021). When these multiple actin filaments are organized into a ring – as in the cytokinetic ring –, the contractile forces generated by Anillin, rather than the activity of molecular motors, can autonomously propel ring constriction.

Anillin also interacts indirectly with F-actin through its binding to formin DIAPH3 (also known as mammalian Dia2 – mDia2) (Watanabe et al. 2008), which catalyzes actin

nucleation and polymerization, namely to form the contractile ring (Miller 2011). Expression of an Anillin isoform lacking the formin-binding domain in mammalian cells disrupts DIAPH3 localization at the cleavage furrow and induces its accumulation at the central spindle (Watanabe et al. 2010). Furthermore, depletion of DIAPH3 disrupted the cortical localization of Anillin, Myosin and RhoA, inducing oscillatory contractions at aberrant sites of the cell (Watanabe et al. 2008), similar to the ones described upon Anillin depletion in other systems (Straight et al. 2005, Zhao and Fang 2005, Piekny and Glotzer 2008, Hickson and O'Farrell 2008b, Kechad et al. 2012), and, thus, most likely explained by the abrogation of the Anillin-DIAPH3 interaction. Moreover, cytokinesis failure induced by Anillin depletion is efficiently rescued upon expression of full-length Anillin, but not when this truncated isoform is expressed, reflecting the requirement of the formin-binding domain for the localization of DIAPH3 at the cleavage furrow and, consequently, for its role in ensuring a successful cytokinesis (Watanabe et al. 2010). The Anillin-DIAPH3 binding was shown to be competitive with the diaphanous auto-regulatory domain (DAD) of DIAPH3 in its auto-inhibitory interaction (Watanabe et al. 2010). Recently, a compensatory mechanism of cleavage furrow ingression upon F-actin disruption in formin mutants was reported in the *C. elegans* embryo, ensured by Anillin and non-muscle Myosin II (Lebedev et al. 2023). Co-depletion of septins and the CYK-1 formin leads to the formation of a meshwork of circumferentially aligned linear Anillin structures, which is also enriched in membrane-bound Myosin II that orients these Anillin structures. This Anillin/Myosin-based meshwork is able to promote furrowing when unbranched F-actin polymerization is compromised, allowing cells to successfully complete cytokinesis (Lebedev et al. 2023). Strikingly, the formation of Myosin II and/or Anillin-enriched circular foci that evolve into linear structures upon F-actin depolymerization has also been described in *Drosophila* S2 cells (Oegema et al. 2000, Hickson and O'Farrell 2008b, D'Avino et al. 2008, Carim and Hickson 2023), suggesting that this mechanism might be conserved amongst animal cells. Taken together, these results pinpoint an active role of Anillin in organizing actin (and myosin), promoting actin polymerization and, ultimately, driving ring contractility.

Anillin also interacts with Septins, a conserved family of GTP-binding proteins that was first identified as the main components of the filamentous ring associated with the plasma membrane within the bud neck of dividing *S. cerevisiae* (Byers and Goetsch 1976). Septins can heteroligomerize into apolar rods, and form hexamers in flies and mammals and octamers in mammals and yeast. Septin complexes can further associate with each other to generate membrane-associated filaments, sheets, and higher-order structures as rings and gauzes (Weirich et al. 2008, Beise and Trimble 2011). Subsequent studies identified five Septin orthologs in *Drosophila* – Sep1, Sep2, Sep4, Sep5 and Peanut (Pnut) –, with the canonical hexamer only comprising Sep1, Sep2 and Pnut (orthologs of

mammalian SETP2, SEPT6 and SEPT7, respectively) and arranged as follows: Sep1-Sep2-Pnut-Pnut-Sep2-Sep1 (Field et al. 1996, Mendonca et al. 2019). In *C. elegans* (Maddox et al. 2005), *Drosophila* (Oegema et al. 2000, Hickson and O'Farrell 2008b) and mammalian (Liu et al. 2012) cells, as well as in *Drosophila* spermatocytes and mouse fibroblasts (Goldbach et al. 2010), Septins are recruited to the cleavage furrow by Anillin. In fly spermatocytes, it was shown that the expression of *Drosophila* E-cadherin suppresses the cytokinesis defects observed upon Anillin depletion, by recruiting catenins to the cleavage furrow and stabilizing F-actin at the cell equator (Goldbach et al. 2010). These results suggest that the Anillin-Septins and Cadherin-Catenins complexes can serve as alternative anchors of the contractile ring to the plasma membrane, by physically coupling F-actin and Myosin II to the cleavage furrow, and driving cytokinesis completion. On the contrary, the localization of Anillin is not affected by Septins depletion in the *Drosophila* pupal notum, although the levels of Anillin within the contractile ring are significantly reduced (Founounou et al. 2013). The direct interaction between a human heteroligomer of SEPT2, SEPT6 and SEPT7 and *Xenopus* Anillin was first demonstrated *in vitro* (Kinoshita et al. 2002), and it was subsequently shown that Anillin-mediated recruitment of Septins requires the C-terminal third of Anillin, which encompasses the PH domain and the adjacent terminal part of the AHD (Maddox et al. 2005, D'Avino et al. 2008, Hickson and O'Farrell 2008b). In line with this, the expression of an Anillin mutant lacking the PH domain failed to recruit Septins to the plasma membrane of the intercellular bridge (ICB) during abscission in HeLa cells (Liu et al. 2012). A follow-up study recently showed that the PH domain of Anillin is able to directly interact with octameric septin structures enriched in SEPT11 but depleted of SEPT9; nevertheless, SEPT9-enriched structures are also required for ICB elongation and maturation (Panagiotou et al. 2022). In this case, Anillin was shown to indirectly interact with Septins via the adaptor protein CIN85 (a vertebrate homologue of the *Drosophila* Cindr), which simultaneously binds the N-terminal region of Anillin and SEPT9, allowing ICB elongation and subsequent maturation. Since Septins can also bind F-actin (Mavrakis et al. 2014) and Myosin II (Joo et al. 2007), which also bind Anillin, a recent study addressed to what extent the actomyosin-dependent interactions contributed to the Anillin-dependent localization of Septins during cytokinesis in both HeLa and *Drosophila* S2 cells (Carim and Hickson 2023). In line with what has been reported so far, it was shown that Septins recruitment to the contractile ring occurs solely via the C-terminal AHD-PH region of Anillin. Expression of an Anillin construct lacking this region (Anillin-ΔN) failed to properly localize Septins to the cleavage furrow, although it retained the ability to scaffold actomyosin and to allow the formation of a partially functional contractile ring (Carim and Hickson 2023).

Since the AHD of Anillin also harbors a well-mapped RhoA-binding region, the same authors further dissected the involvement of RhoA in regulating Anillin ability to recruit

Septins to the cleavage furrow (Carim and Hickson 2023). Mutations within this region abrogating the RhoA-Anillin binding, either alone or in combination, disrupted the ability of both full-length Anillin and Anillin-ΔN to recruit Septins to the cleavage furrow during cytokinesis. This recruitment is independent of F-actin, not only supporting a role for RhoA in the Anillin-mediated recruitment of Septins, but also corroborating previous observations of Anillin and/or Septin-based structures that are formed at the beginning of cytokinesis upon inhibition of F-actin polymerization (Oegema et al. 2000, Hickson and O'Farrell 2008b, D'Avino et al. 2008, Lebedev et al. 2023). Furthermore, mutations in the Anillin-RhoA binding also slowed contractile ring constriction and destabilized ring attachments to the plasma membrane, invariably leading to cytokinesis failure (Carim and Hickson 2023). This suggests that the Anillin-RhoA binding is a pre-requisite for the Anillin-mediated recruitment of Septins to the cleavage furrow, for timely contractile ring closure and, ultimately, for cytokinesis fidelity. Moreover, the equatorial accumulation of RhoA seems to depend on Anillin in *Drosophila* spermatocytes, as it is dramatically reduced upon Anillin depletion (Goldbach et al. 2010). On the contrary, over-expressing Anillin in human lung cancer cells increases active RhoA (Suzuki et al. 2005), a phenotype that has been observed in different human tumors (Sahai and Marshall 2002). Aberrant RhoA activation promotes the migration of these neoplastic cells through the reorganization of the actin cytoskeleton, contributing to its invasive and metastatic potential (Suzuki et al. 2005). Remarkably, the Anillin-RhoA binding at the cell equator seems to be transient, to ensure a cyclical resetting of cortical RhoA. Beyond its ability to bind RhoA, Anillin also locally concentrates PIP_2 from the plasma membrane, which then retains active RhoA in its free state at the membrane, after it has disengaged from Anillin (Budnar et al. 2019). This cycling allows the dissociation kinetics of active RhoA to be repeatedly reset, increasing the dwell time for RhoA to promote its downstream signaling and, thus, ensure an efficient cell division. Noteworthy, being RhoA upstream of Anillin in the regulation of cytokinesis – through multiple interactions with pivotal cytokinetic proteins –, its depletion in different systems completely blocks contractile ring assembly and subsequent ingression (Drechsel et al. 1997, Prokopenko et al. 1999, Jantsch-Plunger et al. 2000, Yuce et al. 2005), whilst Anillin depletion results in a less severe phenotype, in which contractile ring form and partially ingresses, despite doing so in an oscillating manner (Straight et al. 2005, Piekny and Glotzer 2008, Hickson and O'Farrell 2008b, Kechad et al. 2012).

Further supporting the idea that Anillin regulates the localization and stabilization of RhoA, it was shown that Anillin also interacts with Ect2, a RhoA activator (Frenette et al. 2012). A mutation in the PH domain of Ect2, which interacts with the AHD of Anillin, not only decreases its binding to Anillin, but concomitantly impairs the association of Ect2 with the plasma membrane and the generation of active RhoA by Ect2, suggesting a double role for

Anillin in ensuring the efficient production and stable localization of active RhoA at the cleavage furrow (Frenette et al. 2012). A recent study by Husser and colleagues (Husser et al. 2022) elegantly characterized the cytokinetic diversity in five different human cell lines, using endogenously-tagged versions of Anillin, Ect2 and RhoA. Besides assessing the spatiotemporal distribution of these cytokinetic regulators in several cell lines, the authors also reported that differences in Anillin localization correlate with distinct ring closure kinetics, showing an association between the breadth of Anillin and the rate of ring closure.

Altogether, these results place Anillin as a hub for the cytokinetic machinery, whose binding partners – probably not all known yet – reflect the myriad roles of this protein in ensuring a successful cytokinesis amongst animal cells (Figure 1.2).

2. The epithelial tissue

The word *epithelia* dates back to 1703, when it was introduced by the Dutch botanist and anatomist Frederik Ruysch (1638-1731) in the third volume of a series of twelve anatomical books, collective designated *Thesaurus Anatomicus*, published between 1701 and 1728. *Epithelia* derives from the Greek *epi-* = upon/on top of + *thēlē* = papillae, and was used to refer the type of tissue Ruysch found when dissecting the lip of a cadaver, which he observed to be different from the skin (epi*dermis*) and described instead as "a different substratum covered with sensitive (nervous) papillae, which are finely injected red". Nevertheless, it was only about 150 years later that the epithelial tissue became at the center stage of the anatomy/medicine field. In 1837, just around the same time the Cell Theory was being proposed by Schwann and Schleiden, Jacob Henle (1809-1885), a German anatomist, carefully described the microscopic appearance of the surface layer of the intestine in his habilitation thesis to lecture at the University of Berlin, *Symbolae ad Anatomiam Villorium Intestinalium, Imprimis Eorum Epithelii et Vasorum Lacteorum* ("*Contribution to intestinal villi anatomy with special reference to the epithelium and lymph or lacteal vessels*") (Henle 1837). In this thesis, Henle reported that the internal surface of the intestinal tract was lined by a thin layer of densely packed cells with specific morphological features, devoid of blood vessels and rich in nerve endings. Building on these findings, he noted that these cells could also be found in the uppermost layers of the different organs that comprise the digestive tract, concluding that all mucous membranes, rather than being encircled by "coagulated mucus", as it was assumed by then, are covered by layers of cells with similar morphological and functional features – the epithelial cells. Henle's work even led him to classify these layers of epithelial cells into three different types – squamous, columnar and ciliated columnar, each of which were characteristic of a segment of the intestinal tract (Henle 1837). Although nowadays this classification is more detailed, based on cell shape, the number of cell layers and the presence or absence of specialized structures like cilia and microvilli, the accuracy of Henle's original classification is remarkable and still partially present (Figure 1.3). Henle later extended his research to the whole human body, discovering that epithelial cells line the surface of the different body organs, as well as of body cavities, composing the epithelial tissue (Kinne-Saffran and Kinne 1994).

From Henle's first drawings of epithelial cells to our current cutting-edge microscopy to live imaging them, countless studies on the architecture and function of the epithelial tissue have been published. In this section, I will focus on the interactions that epithelial

cells establish with each other and with the extracellular matrix to ensure the cohesiveness of the epithelial tissue and, ultimately, its architecture and proper function.

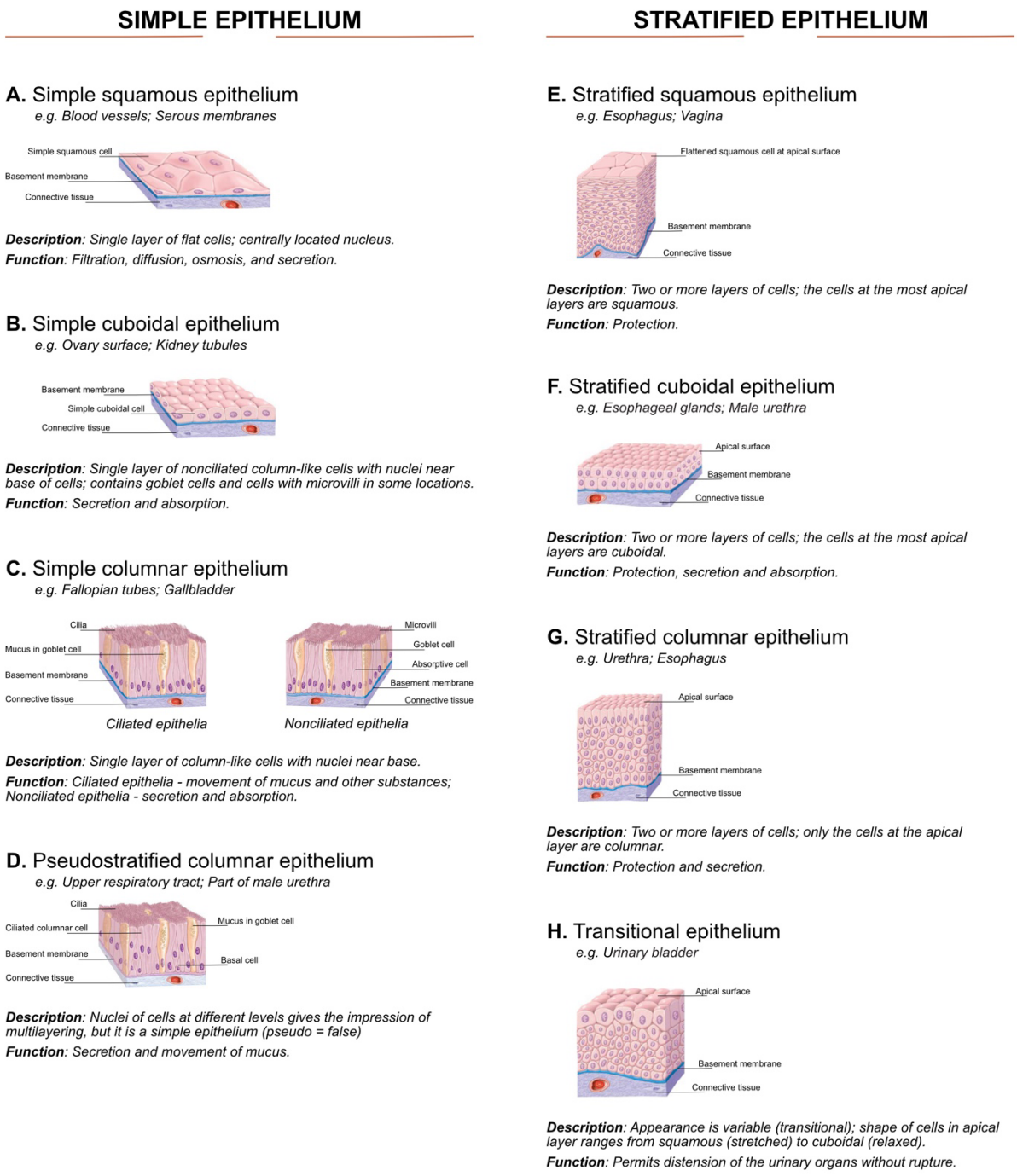


Figure 1. 3. Current classification of epithelial tissues. Epithelial tissues are mainly classified according to the cell shape and the arrangement of layers, and further specified according to the presence or absence of specialized structures, such as microvilli and cilia. Epithelia can be classified as *squamous*, *cuboidal* or *columnar* regarding cell shape and *simple*, *pseudostratified* or *stratified*, regarding the arrangement of layers. The combination of these two criteria originates the 8 types of epithelial tissues depicted in the figure (adapted from (Tortora 2017)).

2.1. Overview on epithelial organization

The epithelial tissue is essential for organizing multicellularity, functioning as a dynamic barrier that separates the interior and exterior of organs and, thus, allowing the coexistence of distinct microenvironments within the same organism (Miller et al. 2013a, Herszterg et al. 2014, Thieleke-Matos et al. 2017). Contrarily to most cell types, which display transient cell surface domains, one particularity of epithelial cells is their ability to establish stable molecular and functional asymmetries along its apical-basal axis, a feature termed intrinsic polarity, which is crucial for the proper function of epithelial tissues (Giepmans and van Ijzendoorn 2009, Miller et al. 2013a, St Johnston and Ahringer 2010). The asymmetric distribution of lipids, proteins and cytoskeletal components along the plasma membrane divide epithelial cells into two major domains – apical and basal-lateral. These domains can be further regionalized into four topological and functionally distinct subdomains – apical, junctional, lateral and basal. Usually, the apical domain of epithelial cells faces the luminal spaces of internal organs or the exterior of the epithelia and the lateral domain faces the neighboring cells, whilst the junctional and the basal domains mechanically couple epithelial cells with each other and with the extracellular matrix, respectively (Figure 1.4) (Rodriguez-Boulan and Macara 2014, Thieleke-Matos et al. 2017, Tepass et al. 2001). I will now focus on the specialized structures that mediate cell-cell and cell-matrix interactions, describing their composition and contribution to epithelial organization and function.

2.1.1. Cell-cell adhesion

The junctional domain of epithelial cells harbors specialized intercellular junctions, that hold the cells together – providing structural stability to the epithelial tissue and allowing cells to sense forces transmitted along it –, and also form a semipermeable barrier for the diffusion of solutes through the intercellular space (Giepmans and van Ijzendoorn 2009, Miller et al. 2013a, Thieleke-Matos et al. 2017). An electron microscopy study in epithelial cells from different mammalian organs originally identified three morphologically distinct intercellular junctions, aligned from the apical to the basal domain – Tight Junctions (or Zonula Occludens), Intermediate Junctions (or Zonula Adherens, most commonly named Adherens Junctions) and Desmosomes (or Macula Adherens) (Farquhar and Palade 1963). Since then, several studies have been characterizing these adhesive structures in the

epithelial tissue of both vertebrates (Hatte et al. 2018, Higashi et al. 2016) and invertebrates (McMahon et al. 2001, St Johnston and Ahringer 2010, Lecuit and Yap 2015) (Figure 1.4). Due to the scope of this thesis, which uses *Drosophila* epithelial tissues as a model, I will mainly focus on Occluding Junctions (Tight/Septate Junctions) and Adherens Junctions, as *Drosophila* epithelial cells do not harbor Desmosomes (Tepass et al. 2001).

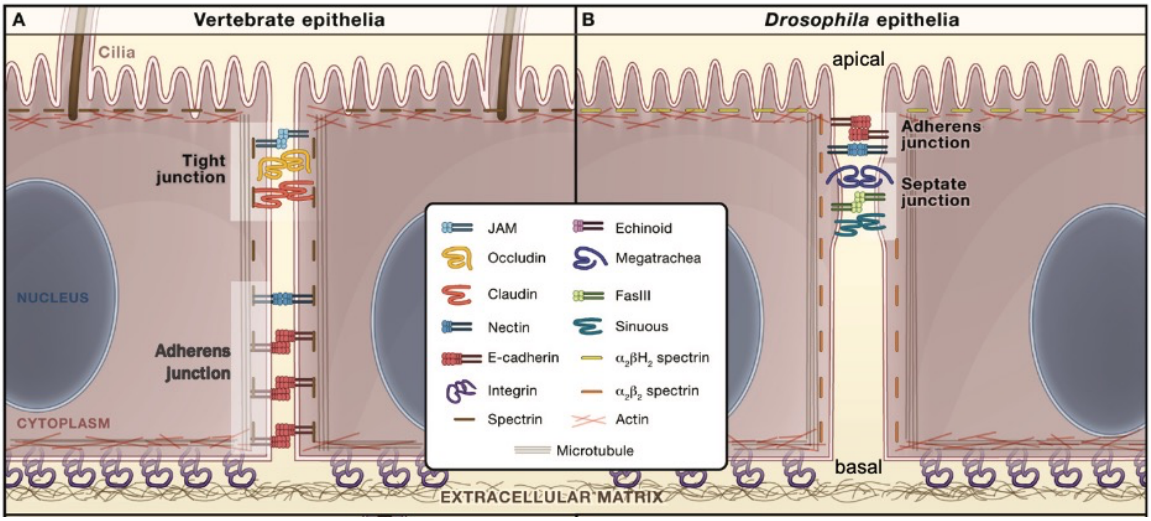


Figure 1. 4. Overview on the positioning of intercellular junctions in vertebrate (A) and *Drosophila* (B) epithelia. Both vertebrates and *Drosophila* present a set of cell-cell junctions that ensure adhesion (Adherens Junctions) and act as a semi-permeable barrier for paracellular diffusion (Tight or Septate Junctions). Noteworthy, the positioning of these junctions in the apical-basal axis of epithelial cells is reversed between vertebrates and flies – while in vertebrates Adherens Junctions are more spread along the lateral domain, below Tight Junctions, in *Drosophila* they are restricted to the apical-lateral interface, above Septate Junctions, the functional homolog of vertebrate Tight Junctions (adapted from (St Johnston and Ahringer 2010)).

Tight Junctions (TJs) are characterized by a dense branching network of transmembrane proteins, forming a particularly tight apposition on adjoining membranes, to seal the intercellular gap between two cells (Farquhar and Palade 1963). These junctions act as a semipermeable barrier that restricts paracellular transport, preventing the leakage of the different organ contents to the bloodstream or to the surrounding tissues (Giepmans and van Ijzendoorn 2009, Hatte et al. 2018). In vertebrates, TJs are positioned at the boundary between the apical and lateral domains, above the Adherens Junctions (AJs) (Hatte et al. 2018, Higashi et al. 2016), whereas the positioning of its functional homologue in flies, named Septate Junctions (SJs), is usually inverted (Figure 1.4) (Lecuit and Yap 2015). In contrast, in the *C. elegans* epithelia, TJs and AJs form a single apical junctional complex (McMahon et al. 2001).

TJs are composed by a set of homophilic transmembrane proteins anchored to the actin cytoskeleton through a junctional plaque (St Johnston and Ahringer 2010, Zihni et al.

2016). In vertebrates, the main transmembrane proteins present in TJs are Claudins, Occludins, Tricellulin and Junctional Adhesion Molecules (JAMs), which are all clustered together via a junctional plaque that interacts with both the cytoplasmic domains of these proteins and with the actin cytoskeleton. The junctional plaque is composed by adaptor proteins that contain multiple protein-protein interaction motifs, such as Zonula Occludens-1, -2 and -3 (ZO-1, ZO-2 and ZO-3, respectively), MAGUK (membrane-associated guanylate kinase-like homology) proteins, which have similar domains to ZO proteins but in a different arrangement, and multi-PDZ-containing proteins, as MUPP1 and PATJ (St Johnston and Ahringer 2010, Zihni et al. 2016). TJs assembly and stabilization seems to be facilitated by AJs in different epithelial contexts, as AJs formation precedes the assembly of TJs (Fleming et al. 1994, Vasioukhin et al. 2001, Maiers et al. 2013). A role for E-cadherin, a core component of AJs, in the formation of TJs was described both *in vitro* (Gumbiner et al. 1988) and *in vivo*, during early mouse development (Fleming et al. 2000). Similarly, E-cadherin depletion in intestinal (Schneider et al. 2010, Bondow et al. 2012) and mammary (Shigetomi et al. 2018) epithelial cells and keratinocytes (Tunggal et al. 2005, Tinkle et al. 2008, Michels et al. 2009, Rubsam et al. 2017) impaired TJs barrier function, although in some simple epithelia, such as the liver and thyroid gland, it did not seem to affect intercellular junctions (reviewed in (Boggetti and Niessen 2012)). Nevertheless, this regulation might be bi-directional in some extent, as TJs were shown to negatively regulate the tensile forces applied to AJs in vertebrate epithelium, so AJs do not become excessively strengthened and prevent successful cytokinesis completion (Hatte et al. 2018).

The occluding barrier present in the invertebrate epithelia is ensured by Septate Junctions (SJs), which are functionally similar to the TJs found in vertebrates (Figure 1.4) (Alhadyian et al. 2021, Rice et al. 2021, De et al. 2022). *Drosophila* SJs are composed by a myriad of proteins, with at least 33 genes required for its formation and maintenance (reviewed in (Rice et al. 2021). These genes encode proteins from different families, that can be grouped into three categories according to their localization and the level of requirement for SJs assembly and maintenance – core proteins, resident proteins and assembly proteins (Alhadyian et al. 2021, Rice et al. 2021, De et al. 2022). Core SJ proteins localize at the junctions and are required for its formation and maintenance. These include proteins from the Claudins family, as Megatrachea, Kune-Kune and Sinuous, cell-adhesion molecules, namely Neurexin-IV, Lachesin, Contactin and Neuroglian, and cytoplasmic proteins, as Varicose and Coracle. SJs resident proteins also localize to the mature junction, but are not required for its assembly and maintenance, such as the polarity proteins Discs-large and Scribble and the cell-adhesion molecules Fasciclin 2 and Fasciclin 3. Finally, assembly proteins are required for the formation and maintenance of SJs, although they do

not permanently localize at SJs. Assembly proteins include members of the Leukocyte antigen 6 family of proteins and also proteins involved in membrane vesicle trafficking, as Rab5 and Rab11 (Rice et al. 2021, De et al. 2022). SJs biogenesis initiates at stage 12 of *Drosophila* embryogenesis, when core SJs proteins start to localize along the lateral membrane. During stages 14-15, these proteins become restricted to an apico-lateral region, below AJs, via Rab5 and Rab11-mediated endocytosis and recycling (Tiklova et al. 2010). By the end of stage 15, SJs become tightly apposed, displaying a ladder-like septa appearance by stage 17 (Tepass and Hartenstein 1994). Remarkably, in the *Drosophila* follicular epithelium, core SJs proteins localize to the lateral membrane of follicle cells during early-to-mid oogenesis and, from stage 11 onwards, become progressively enriched basally to AJs (at the presumptive SJs) (Alhadyian et al. 2021). Depletion of core SJs components drives egg chamber elongation defects and the formation of abnormal dorsal appendages, suggesting additional roles for these proteins in morphogenesis during egg development.

Contrarily to TJs, which are closely apposed, AJs display an intercellular space of over ~200 Å in-between two parallel cell membranes (Farquhar and Palade 1963). Deep-etch electron microscopy further revealed that the intercellular space of the AJs is occupied by rod-shaped proteins bridging the membranes of neighboring epithelial cells (Hirokawa and Heuser 1981, Miyaguchi 2000). In the polarized epithelia of flies and vertebrates, AJs typically form an encircling adhesion belt around its apex – the Zonula Adherens – that completely encloses cells along with the actin cytoskeleton. AJs can assume different morphologies in non-epithelial cells or in epithelial tissues with a different topology. For instance, in keratinocytes in stratified epithelium, immature epithelial cells, neurons or fibroblasts, AJs usually appear as scattered puncta (termed Punctum Adherens), whereas at tricellular junctions (points within epithelial tissues that bridge three or more cells) it assembles at the vertex, forming tricellular AJs (Bosveld and Bellaiche 2020, Yonemura 2011). These junctions are highly dynamic and undergo extensive remodeling to accommodate morphogenetic processes during development, such as tissue folding or bending, which often require cell division, and also throughout adult life, as epithelial tissues are highly proliferative (Campas et al. 2024). Structurally, AJs are composed by two adhesive complexes – the classical Cadherin/Catenins and the Nectin/Afadin complexes (Figure 1.5).

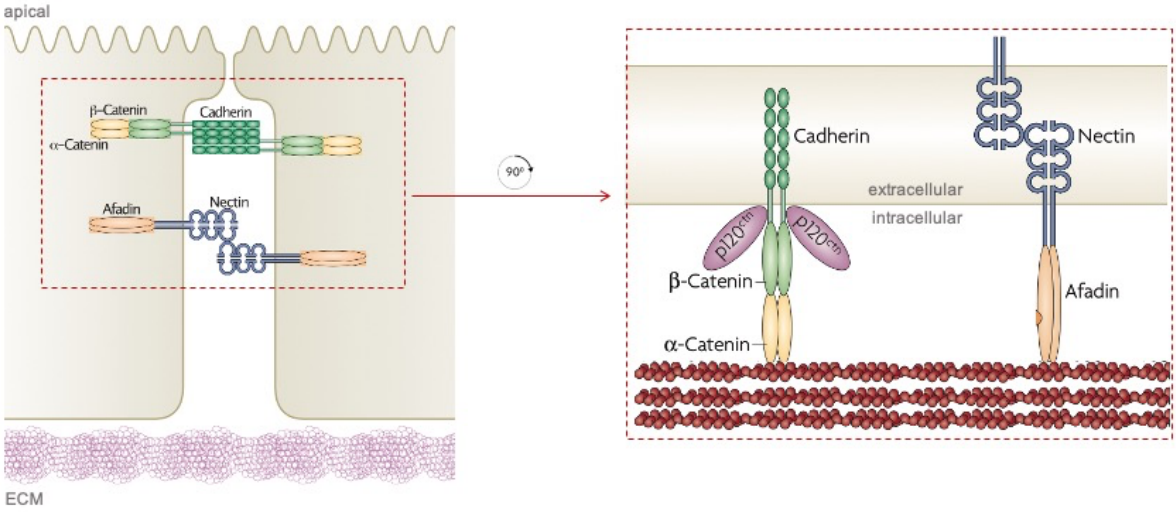


Figure 1. 5. Adherens Junctions (AJs) distribution and molecular composition. AJs are composed by two major adhesive complexes – Cadherin/Catenins and Nectin/Afadin. The Cadherin/Catenins complex involves a stoichiometric interaction between a cadherin (dark green) molecule (usually E-cadherin in epithelial tissues), and three catenins – α- (yellow), β- (light green) and p120-catenin (lilac). While the extracellular domain of cadherins establish homophilic interactions with other cadherins in apposed cells, the intracellular domain binds β- and p120-catenin, that indirectly modulate the actin cytoskeleton. The Nectin/Afadin complex reinforces cell-cell contacts at the AJs through both homophilic and heterophilic Nectin (grey) dimers of adjacent cells. Intracellularly, Nectin binds to Afadin (orange), an actin-binding and actin-crosslinker protein, reinforcing the connection between AJs and the actomyosin cytoskeleton (adapted from (Takai et al. 2008)).

The Cadherin/Catenins complex

Classical cadherins are single-pass transmembrane glycoproteins that mediate calcium-dependent homophilic cell-cell adhesion (Figure 1.5). The two most extensively studied cadherins, E-(epithelial, *Dm* Shotgun or DE-Cad) and N-(neural) cadherins, present almost mutually exclusive expression patterns – while E-cadherin is primarily expressed in epithelial cells, N-cadherin is more prevalent in non-epithelial tissues, being found in a variety of cells, namely neural, muscle and endothelial (Radice 2013, Loh et al. 2019). The structural difference between these two cadherins refers to the cytoplasmic tail, as E-cadherins binds a shorter isoform of the intracellular ligand p120-catenin, whilst N-cadherin binds the longer isoform (Loh et al. 2019).

Cadherins present an extracellular domain that display repetitive regions, termed extracellular cadherin (EC) repeats, harboring calcium-binding sequences that switch cadherins' adhesive function on and off through conformational changes (Pokutta et al. 1994, Overduin et al. 1995). These calcium-sensitive sequences are highly conserved amongst cadherins, although its number is variable – while E- and N-cadherin presents five EC domains in vertebrates (termed classical cadherins) (Radice 2013), *Drosophila* E-cadherin has seven (Oda et al. 1994) and N-cadherin remarkably harbors 17 EC domains

(Iwai et al. 1997). Noteworthy, fly cadherins contain additional domains between the EC repeats and the transmembrane domain, such as laminin globular-like and EGF-like domains, which turn these cadherins much larger than the vertebrate ones (Meng and Takeichi 2009). Through the calcium-binding regions of the EC domains, both E- and N-cadherins preferentially establish homophilic interactions at the AJs with cadherins from apposed cells (Figure 1.5), although they are also able to establish heterophilic interactions (Zuppinger et al. 2000, Meng and Takeichi 2009, Loh et al. 2019). The most commonly accepted model for the homophilic interactions between classical cadherins on adjacent cells involves a *trans*-binding that occurs between six highly conserved N-terminal residues on the EC1 domain of apposing cadherin molecules. The “adhesion arm” from one cadherin mutually exchanges a Tryptophan residue with a conserved hydrophobic “acceptor pocket” on the opposing cadherin, forming a strand-swap dimer (Leckband and de Rooij 2014). Although mutations in the EC1 domain of E-cadherin abolish cell-cell adhesion (Berx et al. 1998), the fact that non-classical and invertebrate cadherins lack the signature sequence for strand-swap dimer formation has been arguing in favor of a more intricated mechanism of homophilic cadherin interactions, involving additional domains. In line with this, an alternative cadherin-binding interface was identified in the non-classical T-cadherin, which is mostly expressed in endothelial cells and that lacks the Tryptophan residue essential for strand-swap dimer formation (Ciatto et al. 2010). Despite this, T-cadherin was shown to retain the binding properties reported for classical cadherins (Ranscht and Dours-Zimmermann 1991), acquiring an antiparallel and crossed configuration, termed X-dimer, which involves extensive cadherin-cadherin contacts through the EC1-EC2 domains, near calcium-binding sites, that do not swap (Ciatto et al. 2010). It was subsequently demonstrated that strand-swapping mutants of classical cadherins can also transiently form X-dimers, suggesting a two-step adhesive binding in classical cadherins, in which an intermediate binding X-dimer conformation facilitates the formation of final strand-swap dimers (Harrison et al. 2010). Of note, cadherins can also establish lateral *cis*-binding interactions, which occurs between proteins on the same cell membrane, to increase the strength of intercellular adhesion at the AJs (Leckband and de Rooij 2014).

The cytoplasmic domain is also highly conserved amongst classical cadherins and mediates key structural and signaling functions required for adhesion, through stoichiometric binding to three different catenins – α-, β- and p120-catenins (Figure 1.5) (Niessen et al. 2011, Leckband and de Rooij 2014, Rubsam et al. 2018). The distal region of the cadherins’ cytoplasmic tail directly binds β-catenin (*Dm* Armadillo, and its homologous γ- catenin/Plakoglobin, a component of Desmosomes), which seems to be crucial for the adhesive function of cadherins (Ozawa et al. 1990, Huelsken et al. 2000). On one hand, β-catenin was shown to protect the cadherin cytoplasmic domain from proteolysis *in vitro*

(Huber et al. 2001). On the other hand, β-catenin was shown to prematurely associate with cadherins in the endoplasmic reticulum, where it is required to escort E-cadherin for proper plasma membrane targeting (Chen et al. 1999). Once the E-cadherin/β-catenin complex reaches the membrane of epithelial cells, β-catenin then exerts its most important function at AJs level – the recruitment of α-catenin, an actin-binding protein (Rimm et al. 1995, Aberle et al. 1996, Bajpai et al. 2008). α-catenin links the E-cadherin/β-catenin complex to the underlying actomyosin cytoskeleton, through its ability to bind F-actin. Despite previous *in vitro* (Yamada et al. 2005, Drees et al. 2005, Miller et al. 2013b) and *in vivo* (Borghi et al. 2012) studies suggested that α-catenin could either bind the E-cadherin/β-catenin complex or F-actin, depending on its dimerization state, a subsequent study reconciled these data, demonstrating that α-catenin undergoes a conformational change under actomyosin-generated tension, that allows it to simultaneously bind the E-cadherin/β-catenin complex and actin filaments (Buckley et al. 2014). In addition, α-catenin binds several actin-binding proteins and actin regulators, namely Vinculin, Afadin, Formin-1, ZO-1 and α-actinin, thus providing further pathways for the modulation of the actomyosin cytoskeleton at AJs, but also additional support for cadherin-based adhesion, as some of its binding partners also bind cadherins (Imamura et al. 2005, Gumbiner 2005, Leckband and de Rooij 2014, Takeichi 2014). The physical connection of E-cadherin to the actin cytoskeleton is crucial for AJs to not only mechanically couple epithelial cells together, but also to distribute the mechanical stresses that drive cell rearrangements along the epithelial tissue, by modulating the actomyosin cytoskeleton (Lecuit and Yap 2015).

Actin dynamics is also indirectly regulated at the AJs, via interaction of the juxtamembrane region of the cytoplasmic tail of E-cadherin with p120-catenin (Shibamoto et al. 1995). Although p120-catenin does not directly bind actin, increasing evidence suggests that it modulates actin dynamics, through direct and indirect binding to RhoA. First, p120-catenin directly binds RhoA in *Drosophila* (Magie et al. 2002), although it seems dispensable for adult viability in flies (Pacquelet et al. 2003, Fox et al. 2005), in contrast to vertebrates, where p120-catenin ablation is embryonic lethal (Yu et al. 2016). Second, the interaction of p120-catenin with cadherins indirectly regulates RhoA activation/inhibition, affecting the organization of actin-based stress fibers (Anastasiadis et al. 2000, Thoreson et al. 2000). A subsequent study dissected the mechanism underpinning this p120-catenin-mediated RhoA activation/inhibition, showing that, besides binding E-cadherin, p120-catenin can also interact with a RhoGAP (p190RhoGAP) at the AJs, in a mutually exclusive manner (Wildenberg et al. 2006). The binding of p120-catenin to p190RhoGAP coordinates Rac1/RhoA antagonism, inducing Rac1-mediated inhibition of RhoA activity and, thus, modulating actin organization. In agreement, ablating the Rho-suppressing activity mediated by p120-catenin disrupts the integrity of the apical surface of the epithelia, due to

excessive actomyosin contractility along the lateral membrane of epithelial cells (Yu et al. 2016). By regulating the actin cytoskeleton, as well as by promoting cadherin clustering (Yap et al. 1998), p120-catenin is able to convert weak into strong intercellular adhesion at the AJs level (Thoreson et al. 2000). In line with a positive role in stabilizing AJs-mediated adhesion, p120-catenin binding to E-cadherin physically masks a highly conserved motif that targets its endocytosis (Xiao et al. 2005, Miyashita and Ozawa 2007, Nanes et al. 2012), as shown by the rapid internalization and degradation of the cadherin complex upon p120-catenin ablation, both *in vitro* (Davis et al. 2003, Xiao et al. 2003) and *in vivo* (Davis and Reynolds 2006, Smalley-Freed et al. 2010, Marciano et al. 2011, Kurley et al. 2012). Noteworthy, by modulating actomyosin contractility, p120-catenin plays a dual role in epithelial cells – as a regulator of cell-cell adhesion, in a manner dependent on its association with cadherins (Shibamoto et al. 1995), and as a regulator of epithelial cytokinesis, in a cadherin-independent manner (van de Ven et al. 2016). p120-catenin becomes enriched at the equatorial cortex of epithelial cells during cytokinesis, where it binds RhoA, regulating its spatiotemporal activity and also its cycling, through concomitant binding to the centralspindlin component MKLP1. Loss of p120-catenin induces continuous cytokinesis defects, due to oscillatory furrows that ultimately give rise to chromosomally unstable multinucleated cancer cells, in both mouse and human cancer models (van de Ven et al. 2016). Additionally, p120-catenin is also able to mediate the connection of cadherins to microtubules, both through the interaction with microtubule-binding proteins, as Pleckstrin homology domain-containing A7 (PLEKHA7) (Meng et al. 2008) and CLIP-associated protein 2 (CLASP2) (Shahbazi et al. 2013), and also through direct binding to Kinesin heavy chain (a motor protein that moves along microtubules) (Chen et al. 2003), mediating the connection between cadherins and microtubules.

Summing up, the Cadherin/Catenins complex mediates cell-cell adhesion through homophilic interactions between cadherins of apposed cells, whose stabilization at the cell surface is ensured by the cytoplasmic interactants β- and p120-catenins (Figure 1.5). Furthermore, this complex associates with both the actin cytoskeleton (through α-catenin), modulating its dynamics (via α- and p120-catenins), and with the microtubule network (through p120-catenin), thus providing a crucial hub for the epithelial tissue to respond to different extracellular stimuli. In fact, increasing attention has been given to the role of AJs in the tissue-level response to these stimuli, both during morphogenesis and homeostasis. The breakthroughs of the last years in super-resolution, expansion and advanced electron microscopy, as well as the development of junction- and cytoskeleton-related biosensors and optogenetic probes, have allowed a deeper understanding both on the junctional architecture and dynamics of AJs as on tissue mechanics, connecting them (nicely reviewed in (Janssen and Huveneers 2024)). By regulating cell-cell adhesion, being coupled to

signaling pathways and displaying mechanosensitivity, AJs can modulate the mechanical properties of cell-cell interactions which, collectively, translate into tissue-level changes (Campas et al. 2024).

The Nectin/Afadin complex

The second adhesive complex found at AJs is the Nectin/Afadin complex (Figure 1.5). The Nectin sub-family belongs to the Immunoglobulin (Ig)-superfamily, and is composed by four calcium-independent transmembrane adhesion molecules (Nectin-1 to Nectin-4) (Takai et al. 2008). Nectins contain an extracellular domain composed by three N-terminal Ig-like loops – the first one of a variable-type Ig, followed by two of a constant-type Ig – that preferentially establish heterophilic interactions, rather than homophilic ones, with other Nectins (Samanta et al. 2012). The cytoplasmic domain of Nectins binds the PDZ-domain of Afadin/AF6 (*Dm* Canoe) (Figure 1.5), an actin-binding and actin-crosslinker protein (Mandai et al. 1997, Takahashi et al. 1999), thus providing an alternative connection between AJs and the underlying actomyosin cytoskeleton. Therefore, similar to the Cadherin/Catenins complex, the Nectin/Afadin complex not only mediates cell-cell adhesion, but also modulates actin cytoskeleton dynamics. Beyond its central role on the Nectin-based complex, Afadin also interacts with other adhesive complexes, namely AJs, through binding to α-catenin (Tachibana et al. 2000) and p120-catenin (via PLEKHA7) (Meng et al. 2008), and TJs, by interacting with ZO-1 and JAMs (Takai et al. 2008, Mandai et al. 2013), providing additional stability to these adhesion complexes. In fact, in mammalian cells, the Nectin/Afadin complex was shown to be required to initiate AJs-mediated cell-cell adhesion (Tachibana et al. 2000).

In *Drosophila*, Echinoid, which also belongs to the Ig-superfamily, acts as the functional homologue of Nectins, localizing at the AJs and interacting with Canoe, the fly ortholog of Afadin (Wei et al. 2005). Similar to Nectins, Echinoid establishes homophilic interactions, although it is also able to bind heterotypically to Neuroglian, a cell-adhesion molecule of SJs (Islam et al. 2003). As its human counterpart, Canoe also interacts with several proteins rather than Echinoid, namely E-cadherin (Wei et al. 2005), the small GTPase Rap1 (Boettner et al. 2003) and F-actin (Sawyer et al. 2009), contributing to stabilize the connection of AJs to the actin cytoskeleton. In line with this, upon Canoe ablation during gastrulation in *Drosophila* embryos, the actomyosin network of mesodermal cells is still able to apically constrict, but it cannot induce the subsequent cell-shape changes, as the cytoskeleton disengages from AJs (Sawyer et al. 2009). Similarly, disruption of Canoe function during dorsal closure of *Drosophila* embryos halts this

morphogenetic process (Takahashi et al. 1998, Boettner et al. 2003), suggesting a pivotal role for the Echinoid/Canoe complex in attaching AJs to the underlying actin cytoskeleton during morphogenesis. Additionally, Canoe is essential for the establishment of apical-basal polarity in the *Drosophila* embryo, guiding the initial positioning of AJs at the apical domain (Choi et al. 2013). These different studies in *Drosophila* have been underpinning Rap1 as the key regulator of Canoe in several morphogenetic processes. Accordingly, abolishing the two Ras-association (RA) domains present at the N-terminal region of Canoe, to which Rap1 binds to, disrupts these functional roles of Canoe (Perez-Vale et al. 2021). A recent follow-up study showed that, even if Rap1 binds with similar affinity to RA1 and RA2 domains, they play different roles during morphogenesis (McParland et al. 2024). While RA1 is essential for several processes during embryonic development, such as mesoderm invagination, germband extension and dorsal closure, RA2 is dispensable. Additionally, replacing the missing RA1 with a second copy of RA2 does not rescues Canoe function, suggesting that the number of RA domains is not the only variable that accounts for protein function (McParland et al. 2024). Overall, whilst RA2 seems to be dispensable for AJs stabilization, RA1 ensures Canoe enrichment at AJs under higher tension, as tricellular junctions, to reinforce them and to allow cells to physically respond to modulations on the actin dynamics. In fact, Canoe is particularly relevant for epithelial cells to retain their columnar architecture during processes that require intensive AJs remodeling, such as cell division and cell intercalation. *Drosophila* embryos lacking both Canoe and Polychaetoid, the fly homologue of ZO-1 known to physically and genetically interact with Canoe, present junctional failure and widespread loss of epithelial integrity (Manning et al. 2019). This is consistent with previous studies in cultured mammalian cells (Choi et al. 2016), suggesting a synergistic role for Canoe and Polychaetoid in ensuring junctional integrity during morphogenesis, by maintaining the linkage of AJs to the actin cytoskeleton. Finally, Canoe also provides an alternative anchorage of the adhesion machinery to the microtubule network, through its interaction with PLEKHA7 (Kurita et al. 2013), a microtubule-binding protein that also binds p120-catenin.

Altogether, the Nectin/Afadin complex not only mediates intercellular adhesion, but also regulates the connection of the adhesion machinery with the underlying actin and microtubule networks. Of note, Nectin/Afadin complexes are predominant in epithelial cells, fibroblasts and synaptic junctions in neurons, either acting autonomously or in concert with other adhesion complexes (Takai et al. 2008, Mandai et al. 2013).

Atypical cadherins

A loose definition for cadherins refers to any protein that contains an extracellular cadherin (EC) domain, turning this group of proteins into a superfamily (Shapiro and Weis, 2009). Cadherins can be divided into classical cadherins, which are linked to the actin cystoskeleton via catenins (described in the section *The Cadherin/Catenins complex*), and non-classical cadherins, which lack the catenin-binding domain and present a divergent cytoplasmic tail (Fung et al. 2008, Oda and Takeichi 2011). Non-classical cadherins can be further categorized into sub-families, according to their domain composition, namely desmosomal cadherins, protocadherins and also atypical cadherins, such as Fat-like cadherins, protein kinase cadherins, seven-pass transmembrane cadherins and Calsyntenin cadherins (Tepass et al. 2000, Hill et al. 2001, Fung et al. 2008). In *Drosophila*, 17 cadherin genes encode for representatives of all main cadherin families, with exception of protocadherins and demosomal cadherins (Figure 1.6) (Fung et al. 2008), as flies lack this type of adhesive structures. Notably, *Drosophila* also displays seven other cadherins that do not fall into any particular sub-family, leaving open the identification of the domain composition of their cytoplasmic region – Cad74A, Cad86C, Cad87A, Cad88C, Cad89D, Cad96Cb and Cad99C.

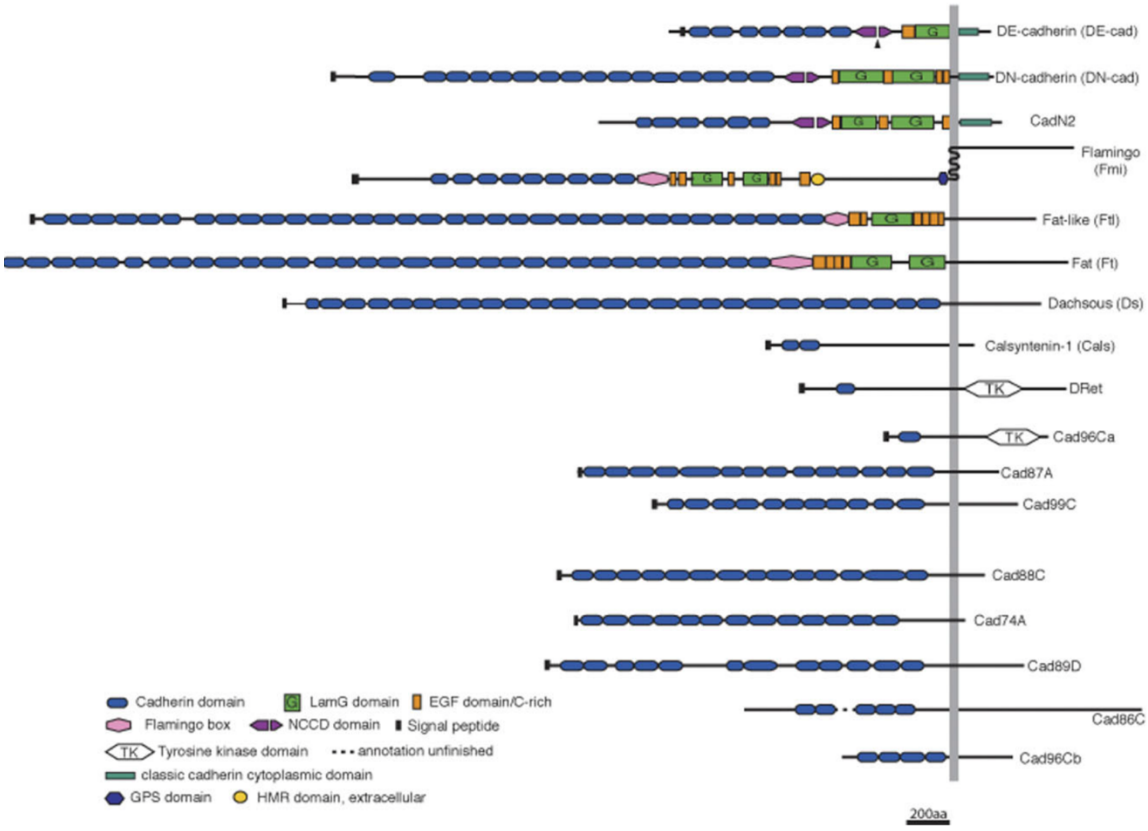


Figure 1. 6. The *Drosophila* cadherin superfamily. Complete genome sequencing in *Drosophila* identified 17 cadherin genes, which encode for classical (DE-cadherin, DN-cadherin and CadN2) and non-classical cadherins. Structurally, classical cadherins display a catenin-binding domain in the cytosolic tail, whilst non-classical cadherins lack this domain. Non-classical cadherins can be further subdivided according to their domains into Fat-like cadherins (Fat, Fat-like and Dachsous), seven-pass transmembrane cadherins (Fmi), Calsyntenin cadherins and protein kinase cadherins (DRet and Cad96Ca). The remaining seven cadherins (Cad74A, Cad86C, Cad87A, Cad88C, Cad89D, Cad96Cb and Cad99C) are not categorized into any of the four sub-families (adapted from (Fung et al. 2008)).

Similar to classical cadherins, non-classical cadherins establish homophilic and heterophilic interactions with other cadherins (Oda and Takeichi 2011). Several atypical cadherins have been shown to play adhesion-independent roles, aside their involvement in cell-cell adhesion (Kumar et al. 2020). For instance, Fat-like cadherins are major regulators of planar cell polarity in *Drosophila* (Saburi and McNeill 2005, Cetera et al. 2014, Barlan et al. 2017), where they also regulate axon terminal organization (Vien et al, 2024) and epithelial cell size dynamics during dorsal thorax morphogenesis (Kumar et al. 2020). Furthermore, Calsyntenin organizes microtubule polarity during axon arbor development in vertebrates (Lee et al. 2017) and Cad99C has been shown to regulate the length of microvilli on the apical surface of *Drosophila* ovarian follicle cells (D'Alterio et al. 2005, Schlichting et al. 2006). Interestingly, based on BLAST analysis, Cad99C was identified as the fly homolog of the vertebrate Protocadherin 15 (D'Alterio et al. 2005, Schlichting et al. 2006), which

localizes to the hair cells of vertebrates inner-ear (Choudhary et al. 2020), despite the absence of a defined protocadherins sub-family in *Drosophila* (Fung et al. 2008).

Despite variations in the overall junctional architecture between vertebrates and invertebrates, cell-cell adhesion is essential to the evolution of multicellularity. Remarkably, the machinery that ensures intercellular adhesion allows the compartmentalization of tissues into organs and the co-existence of different microenvironments, while being flexible enough so different morphogenetic processes can occur.

2.1.2. Cell-matrix interactions

Interactions between the cell cytoskeleton and the extracellular matrix (ECM) are mainly mediated by integrins, transmembrane receptors that fulfil vital biological roles through multiple signaling pathways. Besides supporting cell anchorage and, thus, contributing for tissue architecture, integrins also act as bidirectional signaling platforms between the inside and the outside of cells, either driving the reorganization of the underlying cytoskeleton or of the ECM, both during physiological and pathological processes (Kechagia et al. 2019, Tvaroska et al. 2023, Kanchanawong and Calderwood 2023).

Integrins heterodimerize through non-convalent interactions between an α- and a β-subunit (Hynes 2002), forming an extracellular headpiece with a cytosolic tail extending from each subunit. To date, 18 α- and 8 β-subunits have been identified in mammals, theoretically allowing 144 different heterodimeric configurations. Nevertheless, only 24 integrin complexes have been reported so far (Kechagia et al. 2019, Tvaroska et al. 2023), suggesting a dimerization preference between the two subunits. In *Drosophila*, the integrin family is much shorter, comprising 5 α- (αPS1 to 5) and 2 β-subunits (βPS/*myospheroid* and βv) (Brown et al. 2000). While βv expression seems to be restricted to the endodermal cells of the gut, βPS has a broader expression pattern, being the only β-subunit expressed in the follicular epithelium of the fly ovary (Delon and Brown 2009, Devenport and Brown 2004). Although mutating the βPS subunit does not affect spindle orientation in follicle cells (Bergstralh et al. 2013), it disrupts the monolayered follicular epithelium at the terminal regions of the egg chambers (Delon and Brown 2009, Lovegrove et al. 2019). Interestingly, βPS seems to be required for proper organization of the follicular epithelium only during early oogenesis, as depleting this subunit from stage 3/4 of oogenesis onwards does not affect the morphology of the fly epithelium (Lovegrove et al. 2019). Additionally, βPS has

been showing to play a role on the organization of actin stress fibers at the basal side of follicle cells, although different studies using the same model report conflicting results. On one hand, Delon and Brown showed that mutant clones for βPS display ~33% more actin bundles than its adjacent wild-type counterparts, which are displaced towards the periphery and do not present the typical parallel arrangement (Delon and Brown 2009). This suggests that integrins are not required for the assembly of actin stress fibers, but rather to limit the number of fibers and to properly orient them. This contrasts with early studies in cultured cells, implying a direct role for integrins in the formation of basal stress fibers (Couchman and Rees 1979), and with studies in the *Drosophila* follicular epithelium showing a reduction on the levels of basal actin and myosin when integrins are impaired (He et al. 2010, Qin et al. 2017). In agreement, integrins disruption in the follicular epithelium leads to a reduction of the stress fibers and to a reorganization of the actomyosin network into a new type of membrane protrusions (Santa-Cruz Mateos et al. 2020). Additionally, laser ablation experiments showed that membrane tension increases at the interface between two integrin mutant follicle cells, resulting in a defective expansion of the basal surface of follicle cells (Santa-Cruz Mateos et al. 2020). Altogether, this unveils a role for integrins in controlling both the architecture and the mechanical properties of basal actomyosin networks, which ultimately regulate epithelial cell shape.

Although integrins dimerization occurs early in the endoplasmic reticulum, these heterodimers are transported onto the cell surface in a bent and inactive conformation that is not able to bind ligands and, therefore, cannot transduce signals (Figure 1.7) (Kechagia et al. 2019, Tvaroska et al. 2023, Kanchanawong and Calderwood 2023). Instead, a conformational change *in loco* drives integrins activation through the binding of adaptor proteins to the cytosolic tail of integrin β-subunit, such as Talin (Calderwood et al. 1999, Calderwood et al. 2002, Tadokoro et al. 2003) and Kindlin (Moser et al. 2008, Montanez et al. 2008, Harburger et al. 2009), that further recruits additional signaling and cytoskeletal proteins, an event termed *inside-out* signaling. This binding induces the separation of the cytosolic tails of the α- and β-subunits, triggering a conformational change in the extracellular headpiece, which goes from a low-affinity bent conformation to a high-affinity extended conformation, capable to bind extracellular ligands and, thus, initiating cell-ECM adhesion (Figure 1.7) (Kechagia et al. 2019, Kadry and Calderwood 2020, Pang et al. 2023, Tvaroska et al. 2023).

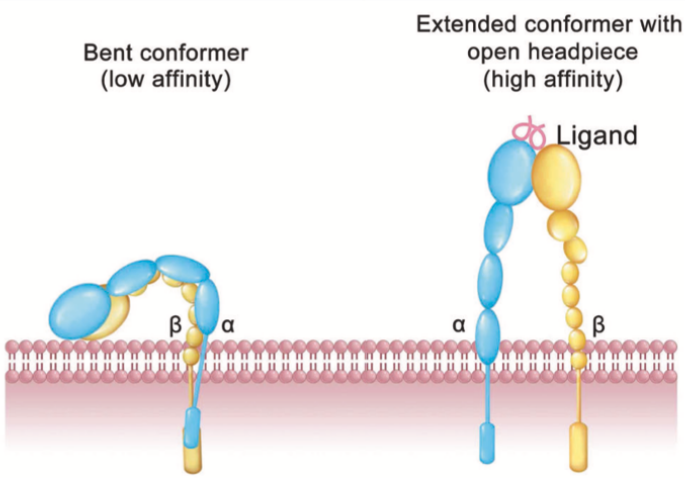


Figure 1. 7. A conformational change on integrins drives extracellular ligand binding. Integrins dimerize in the endoplasmic reticulum and reach the cell surface in a bent closed conformation, which does not allow ligand binding. The interaction of intracellular adaptor proteins (as Talin and Kindlin) with the cytosolic tails of integrins drives a conformational change to an extended open status, increasing the ligand-binding affinity – an event termed inside-out signaling (adapted from (Pang et al. 2023)).

Following conformational activation, integrin heterodimers engage with extracellular ligands that drive full integrin activation and clustering into adhesion complexes. The formation of initially small integrin clusters triggers the recruitment of additional adaptor, scaffold and signaling proteins, as well as protein kinases, phosphatases and GTPases – a cascade referred to as *outside-in* signaling. As integrin clusters expand and mature, a complex network of cell-matrix attachments is formed, generally termed as the integrin adhesome (Kechagia et al. 2019, Kadry and Calderwood 2020, Tvaroska et al. 2023, Kanchanawong and Calderwood 2023), that reinforces and stabilizes the adhesion of epithelial cells to the ECM. In addition, integrins act as mechanosensing platforms that not only sense ECM rigidity, but also respond to extracellular mechanic stimuli and transduce them into biochemical signals to the interior of the cell (mechanotransduction) (Kechagia et al. 2019, Kanchanawong and Calderwood 2023). By sensing the mechanical forces exerted extracellularly, integrins activate multiple signaling cascades to respond to those stimuli, while inducing actomyosin cytoskeleton rearrangements via actin-binding proteins present at the integrin adhesome, to ultimately modulate events as cell proliferation, survival/apoptosis, polarity, motility, differentiation and shape (Legate et al. 2009, Pang et al. 2023).

The most common and best described integrin-based adhesions are focal adhesions; in addition, non-canonical integrin adhesions that lack focal adhesion proteins have also been reported, as reticular adhesions (also termed clathrin plaques due to their enrichment in clathrin) (Lock et al. 2018, Lock et al. 2019) and retraction fibers (Jiang et al. 2019, d'Alessandro et al. 2021). Recently, it was shown that canonical and non-canonical

adhesions can reciprocally interconvert, through the selective exchange of components on a common integrin backbone, suggesting that non-canonical adhesions may actually act as points of origin for the assembly of canonical focal adhesions (Lukas et al. 2024). In line with this, focal adhesions present within stress fibers were shown to change their molecular composition during *Drosophila* oocyte maturation (Delon and Brown 2009), reinforcing the idea that the structure of the integrin adhesome is highly dynamic and dictates distinct roles of integrins during morphogenesis. Beyond integrin-based adhesion, cell-ECM contacts are also reinforced by the non-integrin receptor Dystroglycan, whose structure and biological roles I will describe in detail in the next section.

2.1.3. The extracellular matrix

Beneath the basal domain of epithelial cells, lies a basement membrane that is critical for the development of epithelial tissues and organs – the extracellular matrix (ECM). Electron microscopy studies show that the ECM is a thin sheet, with approximately 100 nm thickness (Isabella and Horne-Badovinac 2015). This matrix is typically composed of two web-like meshworks of Laminin and Collagen IV, that are highly interconnected by the proteoglycan Perlecan and the glycoprotein Nidogen (Isabella and Horne-Badovinac 2015, Topfer et al. 2022). *De novo* assembly of the ECM starts with the deposition of Laminins, followed by Collagen IV, suggesting that this is a hierarchical process (Matsubayashi et al. 2017). Besides these four core components, a number of accessory proteins have been found to provide additional support to the ECM, as Hemicentin, although they are not strictly required for its assembly or organization, lending structural and functional diversity to this matrix (Zhang et al. 2022).

Although the *Drosophila* genome comprises a much simpler set of genes encoding the core components of the ECM, these are well conserved between flies and vertebrates (Figure 1.8). In *Drosophila*, four genes (wing blister, Laminin A, Laminin B1 and Laminin B2) encode two distinct laminin trimers, in contrast to the 16 trimers found in humans (Isabella and Horne-Badovinac 2015, Topfer et al. 2022). Laminins are a large family of glycoproteins that self-assemble into heterotrimers in a cross-shaped structure, composed of one α-, one β- and one γ-chains. In *Drosophila*, only one β- and one γ-chains exist, which can be assembled together either with the α-chain wing blister or Laminin A. Laminins can interact with the epithelial tissue both via integrins and Dystroglycan (Isabella and Horne-Badovinac 2015, Topfer et al. 2022). Collagen IV is composed by two α1-chains and one α2-chain, forming a triple helical structure through covalent interactions that are strengthened through

their terminal domains (Wu and Ge 2019). Two genes encode the two α-chains of Collagen in *Drosophila*, which form a single trimer, while humans display 3 different Collagen trimers (Isabella and Horne-Badovinac 2015). Along with Laminins, Collagen IV contributes for the structural backbone of the ECM. Nidogen and Perlecan are encoded by single genes in *Drosophila*, Ndg and trol (terribly reduced optic lobes), respectively (Hynes and Zhao 2000, Topfer et al. 2022). While these proteins are not essential for ECM assembly, they are required for the maintenance of ECM integrity in some tissues (Dai et al. 2018). Perlecan has been reported as the ECM ligand with more affinity for Dystroglycan in non-muscle cells, binding it with five times more affinity than the other known ECM-binding partner of Dystroglycan, Laminin (Talts et al. 1999). The more reduced diversity of ECM components turns *Drosophila* into a powerful system to dissect their functions, overcoming the redundancy that is common in vertebrates.

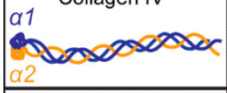
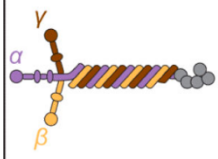
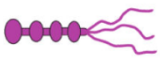
Protein	*Drosophila* genes	Human homologs
Collagen IV 	α1: *Collagen gene at 25C (Cg25C)*	*COL4A1, COL4A3, COL4A5*
	α2: *viking (vkg)*	*COL4A2, COL4A4, COL4A6*
Laminin 	α1,2: *wing blister (wb)*	*LAMA1, LAMA2*
	α3,5: *Laminin A (LanA)*	*LAMA3, LAMA4, LAMA5*
	β: *Laminin B1 (LanB1)*	*LAMB1, LAMB2, LAMB3, LAMB4*
	γ: *Laminin B2 (LanB2)*	*LAMC1, LAMC2, LAMC3*
Perlecan 	*terribly reduced optic lobes (trol)*	*HSPG2*
Nidogen	*Nidogen/entactin (Ndg)*	*NID1, NID2*

Figure 1. 8. The core ECM components are conserved across flies and humans. Despite the *Drosophila* genome is much simpler than the human one regarding the major ECM proteins, these proteins are well conserved between flies and vertebrates (adapted from (Isabella and Horne-Badovinac 2015)).

Due to its mechanical properties, the ECM has been primarily established as a physical scaffold, that allows tissues to maintain its shape and integrity by resisting deformation forces (Isabella and Horne-Badovinac 2015). Several *in vitro* studies using different cell lines in culture have shown that the modulation of the physical properties of the ECM regulate dynamic cellular activities, such as cell migration, contractile behavior and adhesive interactions (nicely reviewed in (Charras and Sahai 2014)). *In vivo*, the ECM undergoes extensive remodeling to assist and regulate morphogenesis. One such example occurs in the *Drosophila* egg chambers, where alterations on the ECM structure drive changes in the activity of follicle cells, to allow egg chamber elongation (Cetera et al. 2014,

Isabella and Horne-Badovinac 2016, Cerqueira Campos et al. 2020). At stage 5 of oogenesis, linear fibrils of Collagen IV, Laminin and Perlecan are deposited and incorporated onto the pre-existing ECM (Haigo and Bilder 2011, Cetera et al. 2014). Until then, the ECM presents no obvious structure, but the perpendicular alignment of the deposited fibrils in relation to the anterior-posterior axis of the egg chambers polarizes the ECM. Concomitantly, linear F-actin bundles in the basal cortex of each follicle cell, which are mechanically linked to the ECM via integrin-mediated adhesion, align parallelly to the ECM fibrils (Gutzeit 1990). By acting in coordinated action, the ECM fibrils and the actin bundles form a molecular corset that physically constrains egg chamber growth in relation to its apical-basal axis, promoting its anterior-posterior elongation. Interestingly, a more recent study showed that Dystroglycan and its intracellular binding partner, Dystrophin, are required to orient the deposition of the ECM fibrils from stages 4-8 of oogenesis. These fibrils are maintained until later stages of oogenesis, acting as a long-term polarity cue for the subsequent planar orientation of the actin fibers, unveiling a role for Dystroglycan and Dystrophin on egg chamber elongation (Cerqueira Campos et al. 2020). This phenomenon also depends on a dramatic rotational movement of the egg chambers, which is powered by the follicle cells that migrate along the ECM, allowing the polarized deposition of the ECM fibrils. Accordingly, disruption of this rotational follicle movement abolishes the polarized deposition of the ECM fibrils and the alignment of the actin bundles, preventing egg chamber elongation (Haigo and Bilder 2011, Cetera et al. 2014).

2.2. The Dystrophin-Associated Protein Complex – structure and functions

In 1987, two apparently unrelated proteins were identified in different biochemical contexts – Dystrophin (Dys) and Dystroglycan (Dg). The first one was identified in the muscle system of both patients with muscular dystrophy and *mdx* mice (which have a point mutation ablating Dys expression), as the primary biochemical defect in Duchenne muscular dystrophy (DMD) (Hoffman et al. 1987), a type of muscular dystrophies characterized by progressive muscle degeneration (Gao and McNally 2015). The second one was reported as a novel 120 kDa laminin-binding protein in the plasma membrane of different cell types (rodent neural hybrid cells, embryonic chicken brain and mouse fibroblasts), and originally named *Cranin* due to its localization (brain) (Smalheiser and Schwartz 1987). In 1990, through biochemical assays in the muscle tissue, Ervasti and colleagues described the same protein as part of an oligomeric Dystrophin complex composed of four glycoproteins (Ervasti et al. 1990). The authors observed that protein levels were reduced for one of them – the heaviest one – in the muscle tissue from DMD patients and *mdx* mice, suggesting for the first time a role for this Dystrophin-associated complex in the molecular pathogenesis of muscular dystrophies (Ervasti et al. 1990). Two years later, Ibraghimov-Beskrovnaya and co-workers established the primary sequence of two of the four glycoproteins identified by Ervasti *et al.*, which were encoded by a single mRNA and were the result of post-translational modifications in a 97 kDa precursor protein – one transmembrane (43 kDa) and the other one extracellular (156 kDa), the last one acting as a receptor for extracellular matrix laminin. For the first time, the name *Dystroglycan* was proposed – an agglutination between *Dystrophin* and *glycosylation* – and remained ever since (Ibraghimov-Beskrovnaya et al. 1992). Since its discovery, Dystroglycan has been detected in a different range of tissues, both during development and in adulthood, suggesting a role for this protein beyond the muscle tissue.

Several additional proteins have been identified as part of the currently called Dystrophin-Associated Protein Complex (DAPC) or Dystrophin-glycoprotein complex (DGC) since its pioneering discovery, some of which are also implicated in other genetic disorders associated with muscle defects (Kanagawa 2021, Vainzof et al. 2021). Besides Dystrophin and Dystroglycan, the DAPC also comprises Sarcoglycans, Sarcospan, Dystrobrevin and Syntrophins (Figure 1.9) (Ervasti and Sonnemann 2008, Constantin 2014, Gao and McNally 2015). While Dystroglycan, Sarcoglycans and Sarcospan are transmembrane proteins, Dystrophin, Syntrophins and Dystrobrevin are cytosolic proteins. Dystrophin is able to directly bind Dystroglycan (Suzuki et al. 1992), Syntrophins (Suzuki et

al. 1995) and Dystrobrevin (Sadoulet-Puccio et al. 1997), placing it at the core of the complex, while also directly interacting with the actin cytoskeleton (Way et al. 1992). Additionally, each protein of the DAPC interacts with other components of the complex, to further hold the DAPC together, and with cytoskeleton, ECM and/or signaling proteins. In a nutshell, the DAPC links the actin cytoskeleton to the ECM, establishing an additional connection to integrins between the inside and the outside of the cell. Furthermore, the DAPC has also been shown to provide mechanical robustness to muscle cells, maintaining their biochemical integrity, as the vast majority of the studies performed so far have been using the skeletal muscle as a model (Ervasti and Sonnemann 2008, Gao and McNally 2015, Mirouse 2023). Nonetheless, this complex is not restricted to the muscle, being expressed in a wide range of tissues, as the epithelia (Durbeej and Campbell 1999). The dissection of the functional roles of the DAPC beyond the muscle tissue will therefore bring important insights for a broader understanding of this complex. In line with this, during the course of my PhD, I have identified the DAPC as a new regulator of cytokinesis in the *Drosophila* follicular epithelium. I will therefore detail its molecular composition and functional relevance in this section.

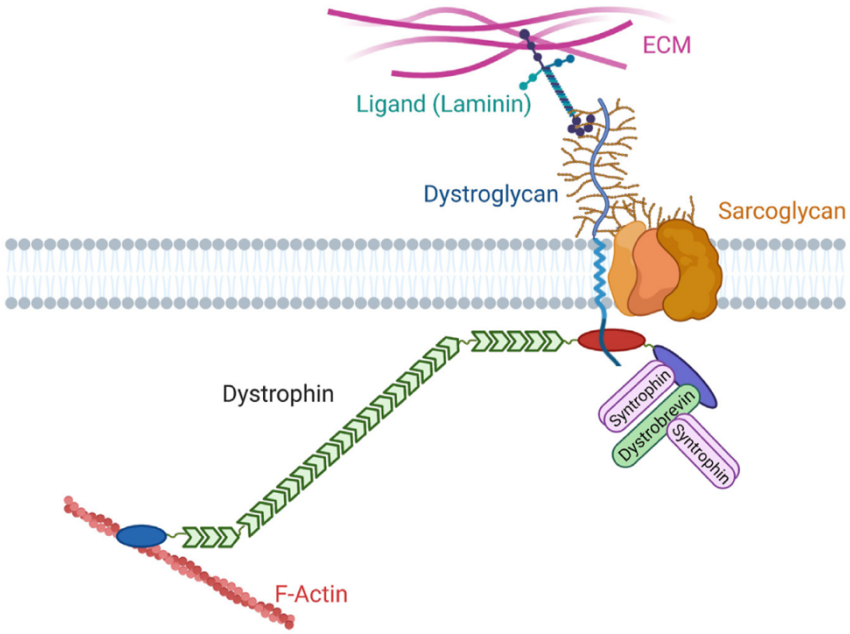


Figure 1. 9. Canonical organization of the Dystrophin-Associated Protein Complex (DAPC). The DAPC is composed by four major modules – Dystrophin, Dystroglycan, Sarcoglycans/Sarcospan and Dystrobrevin/Syntrophins, which connect the extracellular matrix (ECM) to the intracellular actin cytoskeleton. Extracellular Dystroglycan (blue) is highly glycosylated (brown), and interacts with ECM proteins containing laminin globular domains, as Laminin. Transmembrane Dystroglycan interacts with the C-terminal domain of Dystrophin (red), which, in turn, links filamentous actin (blue). The Sarcoglycans/Sarcospan complex localize near Dystroglycan, reinforcing the interaction between its extracellular and transmembrane subunits. Dystrophin also harbors binding domains for Syntrophins and Dystrobrevin (purple) (adapted from (Mirouse 2023)).

2.2.1. Composition of the DAPC

Reflecting its complexity, a number of studies have suggested that, besides its pivotal protein – Dystrophin –, the DAPC can be biochemically sub-divided into three sub-complexes: the Dystroglycan, the Sarcoglycan/Sarcospan and the Dystrobrevin/Syntrophin complexes (Ervasti and Sonnemann 2008). I will thus describe each of these complexes, preceded by an overview on the Dystrophin structure.

Dystrophin structure overview

The *Dystrophin* gene (*DMD*) is the largest known human gene, located on the X chromosome. It covers almost 2400 kb, roughly 0.1% of the whole genome (Koenig et al. 1987). Although its *Drosophila* orthologue, also known as *Dystrophin-like protein* (*DLP*), is about 20 times smaller, spanning approximately 130 kb, it is also one of the largest genes in the fly genome and, curiously, also occupies about 0.1% of the whole genome, as its human counterpart (Neuman et al. 2001, van der Plas et al. 2007). Both genes encode at least six known isoforms, expressed predominantly in the muscle and nervous system, and all of them bear a conserved C-terminal region, which is involved in the interaction of Dys with Dystroglycan and with the Dystrobrevin/Syntrophins complex (Neuman et al. 2001, Neuman et al. 2005, van der Plas et al. 2007, Mirouse 2023). In the full-length Dys isoforms reported so far in the fly – DLP1/Dys-RF, DLP2/Dys-RA, DLP3/Dys-RG, Dys-RC and Dys-RH –, transcription is initiated upstream of most coding exons. The shorter Dys isoforms – Dp205/Dys-RD, Dp117/Dys-RE and the central nervous system-specific Dp186/Dys-RB – are transcribed from downstream promoters, and display a variable number of spectrin repeats upstream of the C-terminal region (Neuman et al. 2001, Neuman et al. 2005, van der Plas et al. 2007). Dystrophin transcripts present tissue-specific expression patterns in both flies and mammals. A recent ribosome-affinity purification study showed that only one full-length (Dys-RH) and one truncated (Dys-RE) isoform are expressed in the *Drosophila* follicular epithelium (Vachias et al. 2023).

The canonical long isoform of human Dys expressed in the muscle is a 427 kDa protein, divided into four main domains based on sequence homology (Figure 1.10) (Koenig et al. 1988). The N-terminal part comprises and actin-binding domain (ABD) composed of two calponin homology (CH) modules (CH1 and CH2), common to several proteins binding filamentous actin, each of which composed of ~100 amino acids arranged in a core of four α-helices and two shorter helices, folding into a globular domain (Korenbaum and Rivero

2002, Yin et al. 2020). The ABD is involved in the direct binding of Dystrophin to the actin network (Way et al. 1992), which seems to induce a conformational opening of the ABD (Fealey et al. 2018). The CH1 module, but not the CH2, has the intrinsic ability to bind F-actin, although this occurs with much higher affinity when CH2 binds in tandem with CH1 (Stradal et al. 1998).

Adjacent to this ABD, there is a long rod-domain composed of 24 spectrin repeats (SRs), each of which formed by ~110 amino acid motifs organized in triple α-helices that fold on each other, into small ~5 nm rods (Koenig et al. 1988, Broderick and Winder 2005, Liem 2016). The enrichment of some SRs in basic amino acids, which display higher affinity to establish electrostatic interactions with the acidic actin filaments (Amann et al. 1998), suggests that these regions may act as secondary ABDs (Figure 1.10). In fact, *in vitro* biochemical assays in mammalian muscle extracts identified the SR11-SR17 of Dys rod-domain as an alternative ABD (ABD2) (Rybakova et al. 1996). Subsequent studies proposed that this ABD2 collaborates with the canonical ABD, mediating a strong binding of a single Dystrophin molecule with a portion of F-actin with more than 20 monomers (Rybakova and Ervasti 1997). Nevertheless, it remains unclear if this secondary ABD may also be present in *Drosophila*, since SR12-SR17 are poorly conserved in evolution and are enriched in acidic amino acids in all invertebrate dystrophins. Strikingly, aside from SR1-SR2, SR11 is the most evolutionarily conserved SR, suggesting a relevant role in Dys structure and function (Mirouse 2023). Dystrophin's rod-domain is also able to bind microtubules with high affinity through SR20-SR23 (Prins et al. 2009, Belanto et al. 2014), and an *in vitro* assay showed that Dys pauses microtubule polymerization, suggesting a role for Dys in organizing the microtubule lattice in the skeletal muscle (Belanto et al. 2014). Furthermore, impairing the Dys-microtubules interaction results in a disordered microtubule lattice in muscle cells and has been shown to increase reactive oxygen species (ROS) signaling in *mdx* mice, which exacerbates the pathophysiological phenotype of DMD (Khairallah et al. 2012). Dystrophin-induced microtubule lattice disorganization further contributes to the loss of muscle force induced by eccentric contraction in *mdx* mice, pinpointing a role for Dys in conferring muscle protection after repeated contractions (Nelson et al. 2018). In agreement, expression of a nearly full-length Dystrophin in the *mdx* background rescues microtubule lattice organization (Belanto et al. 2014). *In vitro* studies have showed that the rod-domain can also bind membrane phospholipids, through tryptophan residues present in SR2 (Le Rumeur et al. 2003). Although it remains unknown how the interaction with membrane phospholipids shapes Dys function, it has been speculated that this interaction possibly facilitates the targeting of Dys onto the sarcolemma, the plasma membrane of muscle cells, possibly modifying the physical properties of the lipid bilayer of the sarcolemma and/or associated proteins. Subsequent studies showed that the

Dys-phospholipids interaction extends to SR1-SR3, which are sufficient for Dys recruitment onto the sarcolemma in both skeletal and cardiac muscle (Legardinier et al. 2008, Zhao et al. 2016), and also identified an additional membrane-binding domain at SR10-12 in the skeletal muscle (Zhao et al. 2016).

The 24 SRs of the Dys rod-domain are intercalated by four proline-rich spacers, termed hinges, due to the elasticity that they provide to the large Dys protein (Koenig and Kunkel 1990). In particular, the fourth hinge is at the end of the rod-domain, after SR24, and comprises a WW domain, consisting in two conserved tryptophans, which is involved in protein-protein interactions with proline-rich molecules (Ilsley et al. 2002). The WW domain is followed by a C-terminal cysteine-rich domain spanning two EF-hand motifs and a zinc-finger (ZZ) domain. The region from the WW to the ZZ domains is necessary and sufficient to bind the C-terminal region of transmembrane Dystroglycan (Figure 1.10) (Suzuki et al. 1994, Jung et al. 1995, Chung and Campanelli 1999, Rentschler et al. 1999, Ishikawa-Sakurai et al. 2004). Each EF motif is composed by two α-helices linked by a short loop, which undergoes a conformational change upon calcium-dependent binding (Koenig et al. 1988), even if subsequent contradictory *in vitro* studies leave unanswered if EF-mediated Dys-Dg binding is calcium-sensitive or not (Milner et al. 1992, Ervasti and Campbell 1993, Chung and Campanelli 1999). The ZZ domain contains two small protein motifs displaying conserved cysteine residues, that fold into finger-like protrusions in the presence of divalent metal cations, as Zn^{2+} (Ponting et al. 1996). *In vitro* studies with recombinant human Dystrophin revealed that this domain is able to bind the first EF motif of Dys C-terminal, to reinforce its binding to Dg (Ishikawa-Sakurai et al. 2004). Finally, the C-terminal (CT) domain of Dys is composed by two polypeptides folded into coiled coils, which are commonly involved in protein-protein interactions (Figure 1.10) (Blake et al. 1995). These protein domain display binding sites for Dystrobrevin and Syntrophins (Figure 1.10), which also take part of the DAPC, providing additional stabilization to the complex (Suzuki et al. 1995, Sadoulet-Puccio et al. 1997). The CT domain also harbors a membrane-binding domain that contributes for the recruitment of DAPC components onto the sarcolemma in the skeletal muscle (Zhao et al. 2016).

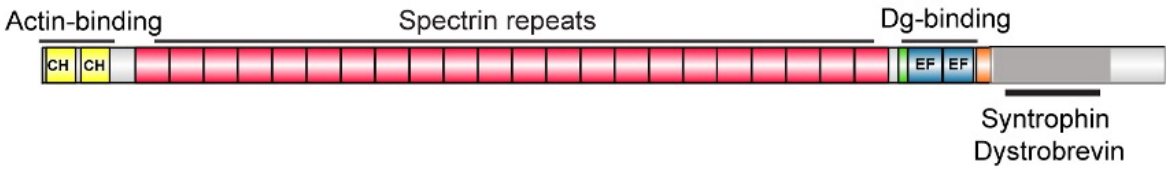


Figure 1. 10. Schematic representation of full-length Dystrophin domains in *Drosophila* (Dys-RH). Dystrophin harbors an N-terminal actin-binding domain (ABD), composed of two calponin homology (CH) regions (yellow), which are followed by a rod-domain comprised of 24 spectrin repeats (red). Some spectrin repeats have been suggested to act as secondary ABDs. Adjacent to the rod-domain, Dystrophin harbors a Dystroglycan-binding domain, consisting in a WW domain (green) followed by two EF-hand motifs (blue) and a zinc-finger (ZZ) domain (orange). The coiled-coil C-terminal domain displays binding sites for Dystrobrevin and Syntrophins (grey), additional components of the DAPC.

Dystroglycan

Built on the initial findings reported by Ibraghimov-Beskrovnaya and colleagues (Ibraghimov-Beskrovnaya et al. 1992, Ibraghimov-Beskrovnaya et al. 1993), it is currently known that Dg is encoded by a single gene (*DAG1*), whose product is a precursor protein that undergoes post-translational proteolytic cleavage. This produces two tightly, although non-covalently, associated subunits – α- and β-Dystroglycan. After proteolytic cleavage, the extracellular α-Dg subunit also undergoes extensive *O*- and *N*- glycosylation in its mucin-like region, prior to being sorted to its extracellular location. This seems to be crucial for the interaction of α-Dg with its extracellular binding partners, particularly for the binding affinity to Laminin, and the subsequent stabilization of the connection between the ECM and the intracellular actin cytoskeleton (Ervasti and Campbell 1993, Kanagawa et al. 2004, Barresi et al. 2004). In fact, mutations in several genes encoding glycosyltransferases involved in the *O*-mannosylation of α-Dg, which overall disrupt the interaction between α-Dg and Laminin, have been described in a sub-group of muscular dystrophies, globally called Secondary Dystroglycanopathies (Muntoni et al. 2008, Bozzi et al. 2009). Due to the hypoglycosylation of α-Dg in this sub-group of disorders, the glycoepitope of α-Dg has been widely used as a diagnostic tool in Dystroglycanopathies, which range from mild congenital muscular dystrophy to severe brain and eye anomalies, suggesting a role for Dg beyond the muscle tissue (Bozzi et al. 2009). Besides Laminin, the glycoepitope of α-Dg mediates its binding to other ECM components containing laminin G domains, as Agrin and Perlecan in the muscle (Barresi and Campbell 2006) and Neurexin in the brain (Sugita et al. 2001). In fact, biochemical assays using recombinant protein fragments showed that Perlecan is a stronger extracellular ligand of α-Dg, binding with five-times more affinity than Laminin (Talts

et al. 1999), and a recent *in vivo* study in *C. elegans* showed that Perlecan is required for proper Dg localization at the surface of muscle cells (Peysson et al. 2024).

The C-terminal domain of α-Dg binds to the unstructured N-terminal extracellular domain of β-Dystroglycan (Di Stasio et al. 1999, Boffi et al. 2001). On the other hand, the 121-residue C-terminal cytoplasmic domain of β-Dg directly binds the WW, EF and ZZ modules of Dystrophin or Utrophin (a Dystrophin homologue expressed in non-muscular tissues), which is anchored to the actin cytoskeleton (Suzuki et al. 1992, Jung et al. 1995, Rentschler et al. 1999, Chung and Campanelli 1999, Ishikawa-Sakurai et al. 2004). In addition, the cytosolic tail of β-Dg is also known to bind a variety of signaling and structural proteins. On such example is Growth factor Receptor-Bound protein 2 (Grb2) (Spence et al. 2004), an adapter protein from the ERK-MAP kinase cascade involved in signal transduction and cytoskeletal organization (Yang et al. 1995). Another β-Dg-binding partner is Caveolin-3 (Sotgia et al. 2000), the main structural component of muscle caveolae, which are plasma membrane invaginations involved in signal transduction. Strikingly, Caveolin-3 is highly expressed in DMD patients and transgenic mice overexpressing this protein display a DMD-like phenotype, suggesting that a competitive downregulation of Dys might occur in the muscle, due to the excessive binding of Caveolin-3 to β-Dg (Ehmsen et al. 2002).

The Sarcoglycan/Sarcospan complex

All the Sarcoglycan isoforms are glycoproteins presenting long extracellular domains, but rather short cytoplasmic ones (Roberds et al. 1993, Lim et al. 1995, Noguchi et al. 1995), being α-Sarcoglycan an exception, with its N-terminus oriented extracellularly (Roberds et al. 1993). The most prevalent form of the Sarcoglycan sub-complex is composed of four single-pass transmembrane isoforms of Sarcoglycan, in stoichiometric amounts – α, β, γ and δ, each of which encoded by a different gene (Ervasti and Sonnemann 2008) – and by Sarcospan (Crosbie et al. 1999). The four Sarcoglycan subunits are tightly assembled and translocated as a complex within the muscle myofiber, being its expression mostly restricted to the skeletal muscle (Chan et al. 1998). In fact, mutating any of the individual subunit abolishes the localization of the unmutated Sarcoglycans from the sarcolemma (Ehmsen et al. 2002, Gao and McNally 2015). Beyond the four canonical Sarcoglycan subunits described as a Dystrophin sub-complex, two additional mammalian Sarcoglycan isoforms have been identified – ε and ζ. While ε-Sarcoglycan is more homologous to α-Sarcoglycan in both gene sequence and protein structure, compensating its loss in the skeletal muscle (Imamura et al. 2005), it also takes part on another complex present in the muscle, aside from the DAPC (Durbeej et al. 2000). ζ-Sarcoglycan is similar

to γ- and δ-Sarcoglycan in sequence homology and protein structure (Wheeler et al. 2002), and can even compensate for γ-Sarcoglycan loss in the Sarcoglycan sub-complex of the DAPC (Shiga et al. 2006). Three Sarcoglycan orthologs have been identified in *Drosophila* and *C. elegans* – one similar to mammalian α-/ε-Sarcoglycan, another one to β-Sarcoglycan and the last one to γ-/δ-/ζ-Sarcoglycan (Gao and McNally 2015). The Sarcoglycan sub-complex plays a structural role in stabilizing the DAPC in the skeletal muscle, via two mechanisms identified so far. On one hand, Sarcoglycans are tightly associated with the Dystroglycan sub-complex (Rafael et al. 1996, Chan et al. 1998), reinforcing the interaction between α- and β-Dg. On the other hand, Sarcoglycans have been shown to directly interact Dystrobrevin (Yoshida et al. 2000), which heterodimerizes with Dystrophin (Sadoulet-Puccio et al. 1997), further holding the complex together.

The other component of this sub-complex is Sarcospan, a 25 kDa protein encompassing four transmembrane-spanning segments. This protein is tightly associated with Sarcoglycans, and belongs to the tetraspanin family of proteins, known to interact with integrins (Crosbie et al. 1997). Sarcospan-null mice present a mild myopathy which, in synergy with the loss of the α7-integrin subunit, becomes exacerbated and resembles the DMD phenotype (Marshall et al. 2012), paving the way on the importance of the Sarcoglycan-α7-integrin interaction for the pathogenesis of muscular dystrophies. It has been proposed that Sarcospan can form homo-oligomers that enhance protein-protein interactions, clustering different subunits of the DAPC, to facilitate its anchorage to the sarcolemma (Miller et al. 2007).

The Dystrobrevin/Syntrophin complex

Besides Dystroglycan and the Sarcoglycan/Sarcospan complex, Dystrophin also directly interacts with Dystrobrevins and Syntrophins, two families of cytoplasmic proteins that are expressed in a tissue-specific manner (Ervasti and Sonnemann 2008). Alternative splicing of two Dystrobrevin genes produces multiple isoforms of two highly homologous proteins, expressed in a variety of tissues – α- and β-Dystrobrevin (Blake 2002). While β-Dystrobrevin is only found in non-muscle tissues, namely in the brain, where it interacts with two truncated Dystrophin isoforms (Blake et al. 1998), α-Dystrobrevin is expressed in a wide array of tissues, particularly in the skeletal muscle, where α-Dystrobrevin-1 localizes to the neuromuscular junction and α-Dystrobrevin-2 is uniformly distributed throughout the sarcolemma (Peters et al. 1998, Nawrotzki et al. 1998). α-Dystrobrevin shares significant homology with the C-terminal domain of Dystrophin (Wagner et al. 1993, Sadoulet-Puccio

et al. 1996). It interacts with Dystrophin via the coiled-coil motifs present in the C-terminal region of both proteins (Sadoulet-Puccio et al. 1997, Peters et al. 1998). This interaction seems to be crucial for α-Dystrobrevin stabilization at the sarcolemma, since *mdx* mice display reduced levels of muscle α-Dystrobrevin (Blake 2002). In the DAPC, α-Dystrobrevin was also shown to interact with the Sarcoglycan complex (Yoshida et al. 2000), and to directly bind to Syntrophins (Newey et al. 2000).

Syntrophins are a family of membrane-associated adapter proteins, with five known isoforms – α-1, β-1, β-2, γ-1 and γ-2 (Okumura et al. 2008). Each Syntrophin isoform presents a unique tissue expression and sub-cellular localization (Bhat et al. 2013). Syntrophins share common modular domains, consisting in a Syntrophin-unique (SU) domain (transversal to this family of proteins), a PDZ domain and two pleckstrin homology (PH) domains. The SU is a highly conserved C-terminal domain with 57 amino acids that, along with the PHs, interacts with the C-terminal domain of Dystrophin (Suzuki et al. 1995), and probably also contains binding sites for different proteins of the Dystrophin family (Gao and McNally 2015). The PH domains consist in modules of ~100 amino acids commonly found in a wide range of signaling proteins (Gao and McNally 2015). Within each first PH domain is a PDZ domain that facilitates homo- and hetero-dimerization with other proteins also containing a PDZ domain. This suggests that Syntrophins function as adaptor proteins involved in the recruitment of signaling proteins to the plasma membrane (Piluso et al. 2000), namely neural nitric oxide synthase (nNOS), the enzyme responsible for the production of nitric oxide in the muscle (Hillier et al. 1999). Additionally, the SU and the second PH domain harbor actin-binding sites (Iwata et al. 1998, Iwata et al. 2004), placing Syntrophins as a linker between their different binding partners and the actin cytoskeleton.

2.2.2. Functions of the DAPC

The DAPC, in particular Dystrophin, is closely associated with muscular dystrophies and, therefore, has been extensively characterized in the pathological context. In fact, a deletion spanning one or multiple Dys exons is responsible for 60-70% of all DMD cases and 80-85% of the Becker muscular dystrophy (BMD) cases, a less severe form of the disease (Flanigan et al. 2009, Tuffery-Giraud et al. 2009). Consequently, DAPC functions have been studied in the muscle system in particular, where it provides mechanoprotection and structural stability to the sarcolemma, act as a hub for cell signaling molecules, regulates ion channels and, possibly, is involved in mechanosensing/transduction (Wilson et al. 2022, Mirouse 2023).

Dystrophin plays the leading role regarding DAPC functions. Part of this role relies on the longest Dys domain, the rod-domain, which has the elastic ability to extend and unfold, stretching up to 800 nm (Bhasin et al. 2005, Le et al. 2018). This mechanical property points the rod-domain as a flexible spacer between the N-terminal and the C-terminal domains of the protein, suggesting that Dystrophin may act as a shock absorber during muscle contraction/stretch, thereby providing mechanical resistance and stabilizing the sarcolemma (Ervasti 2007, Mirouse 2023). In line with this, several studies describe sarcolemmal fragility/rupture (Mokri and Engel 1975, Weller et al. 1990, Matsuda et al. 1995), costamere disorganization (Porter et al. 1992, Ehmer et al. 1997, Williams and Bloch 1999), muscle weakness (Carlson and Makiejus 1990, Cox et al. 1993) and necrosis (Torres and Duchen 1987) in the skeletal muscle of patients with DMD or in animal models of the disease. Expression of full-length Dystrophin in *mdx* mice rescues the majority of these defects (Weller et al. 1990, Cox et al. 1993, Clarke et al. 1993), supporting a role for Dys in stabilizing the sarcolemma under the mechanical forces resultant from muscle contraction.

The molecular mechanisms underlying the protective role of Dys at the sarcolemma remain elusive. On one hand, *mdx* mice expressing the Dp71 isoform (the human orthologue of the fly Dp186/Dys-RB), which lacks both the canonical ABD and the rod-domain, show that Dp71 localizes to the sarcolemma and is able to restore the expression and localization of the other DAPC components, but does not revert the dystrophic phenotype observed in control *mdx* mice (without endogenous Dys and Dp71) (Cox et al. 1994, Greenberg et al. 1994). Conversely, another study in *mdx* mice expressing Dys isoforms with a deletion in the ABD presented a very mild dystrophy variant, suggesting that the canonical ABD is not essential for Dys function (Corrado et al. 1996) and further supporting the notion of secondary actin-binding domains in Dystrophin. Another study in *mdx* mice expressing different deletions in the cysteine-rich domain, which harbors the binding site for β-Dystroglycan, showed a severe dystrophic phenotype, indicating that this domain is necessary for Dystrophin function (Rafael et al. 1996). Nevertheless, in light of the results from Cox *et al.* and Greenberg *et al.*, it suggests that the cysteine-rich domain, although necessary, is not sufficient for Dystrophin function. Abolishing the Syntrophins or Dystrobrevin binding sites in Dystrophin does not affect the localization of these proteins nor affects muscle function in *mdx* mice (Rafael et al. 1994, Rafael et al. 1996). As Syntrophins and Dystrobrevin directly bind each other in addition to Dystrophin (Newey et al. 2000), abolishing one of the two binding sites on Dys might not sever the link between Dystrophin, Syntrophins and Dystrobrevin. Surprisingly, *mdx* mice expressing a Dys variant lacking both Syntrophins and Dystrobrevin binding sites also displayed normal muscle function (Crawford et al. 2000), suggesting that other proteins of the DAPC beside Dystrophin might present binding sites for Syntrophins and Dystrobrevin, compensating the

disruption of their interaction with Dystrophin, or, alternatively, that this interaction is not essential for proper muscle function.

The full-length Dys isoform seems to also be crucial in the cardiac muscle, since *mdx* mice develop DMD-related cardiomyopathy, an impairment of the cardiac function tightly associated with muscular dystrophies (Masubuchi et al. 2013). In the same study, the authors compared *mdx* mice, which lack the full-length Dys isoform (Dp427) but express the truncated Dp71 isoform (the human orthologue of the fly Dp186/Dys-RB), with DMD-null mice, in which all Dys isoforms are abolished. Strikingly, nNOS levels in the cardiac muscle of DMD-null mice were lower than in *mdx* mice, suggesting that Dp71 may play a role in the regulation of this enzyme (Masubuchi et al. 2013). nNOS, which interacts with Syntrophins from the DAPC, is responsible for the production of nitric oxide (NO) in the muscle tissue. NO increases the local blood flow to accompany the high metabolic load of muscular contraction, and its production is thought to be impaired in DMD patients, which show abnormal blood vessel constriction (Ehmsen et al. 2002).

Additionally, the localization of the DAPC at costameres – sub-membranous lattice structures involved in lateral force transmission from the sarcomere to the sarcolemma and to the ECM (Peter et al. 2011) – suggests a role for DAPC in mechanotransduction in the muscle. This idea is further supported by the ability of spectrin repeats to fold or unfold (Bhasin et al. 2005), and by the co-localization of the DAPC with integrins at costameres (Elsherif et al. 2008), such as with the transmembrane complex Integrin-Talin-Vinculin (Anastasi et al. 2003). Integrins are known to act as mechanotransducers, converting mechanical stimuli across the ECM into a biochemical response, through its interaction with the intracellular actin network (Wilson et al. 2022). In the cardiac and skeletal muscle in particular, β1 integrins (which dimerize with the α7 subunit), along with the DAPC, comprise two major linkers between the intracellular cytoskeleton and the ECM (Elsherif et al. 2008). In fact, *mdx* mice have been shown to develop dystrophic phenotypes in the absence of either α7-integrin or Dystrophin, and present accelerated myopathy and premature lethality when both components are abolished concomitantly (Mayer et al. 1997, Elsherif et al. 2008). Furthermore, α7-integrin levels become increased in DMD patients and *mdx* mice in the absence of Dystrophin, as a compensatory mechanism (Hodges et al. 1997). Altogether, these studies support a cross-talk between DAPC and integrins and further strengthens the concept of DAPC as a mechanotransducer. A role for the DAPC as a mechanosensor in the NO production pathway has also been suggested in mouse cardyomyocites. In these cells, the DAPC promotes the mechanical activation of nNOS, by regulating the AMP-activated protein kinase (AMPK) activity (Garbincius and Michele 2015), suggesting an active role for this complex in mechanotransduction in muscle cells.

Beyond the muscle tissue, it has been shown that the truncated isoform Dp71 plays a canonical function within the central nervous system, as a regulator of the water-channel Aquaporin-4 (AQP4) and the potassium-channel Kir4.1. Dp71 disruption has been shown to induce alterations in the permeability of the blood-brain barrier in adult dystrophic *mdx* mice (Nico et al. 2004). Furthermore, since these channels localize to retinal Muller glial cells, where Dp71 is the only Dystrophin isoform expressed, DMD patients with mutations that abolish Dp71 production present an abnormal electroretinogram (Naidoo and Anthony 2020). The role of Dp71 in nNOS regulation in the muscle and water/potassium channel regulation in the brain pinpoints a rod-domain-independent function for Dystrophin, as Dp71 lacks both the ABD and the SRs, only displaying the C-terminal domains.

Contrarily to Dystrophin, reports of mutations in the *DAG1* gene (which encodes Dystroglycan) as a cause of muscular dystrophies are scarce, most likely related to the vital role of Dg during embryonic development (Williamson et al. 1997). Homozygous Dg-mutant mice are embryonic lethal due to disruption of the Reichert's membrane, an extra-embryonic basement membrane, which also disrupts the localization of Laminin and Collagen IV (Williamson et al. 1997). Several mutations in different proteins involved in the *O*-mannosylation of α-Dg have been identified, comprising a sub-group of muscular diseases termed Secondary Dystroglycanopathies. In general, these mutations impair the multiple interactions of α-Dg with its extracellular binding partners, particularly laminin, destabilizing the connection of the actin cytoskeleton to the ECM (Muntoni et al. 2008, Bozzi et al. 2009). However, only three mutations in the *DAG1* gene itself have been reported so far. In 2011, a missense mutation (T192M) was identified in a patient with limb-girdle muscular dystrophy, a less aggressive form of the disease, which affected a highly conserved residue in the N-terminal of α-Dg, impairing its *O*-mannosylation (Hara et al. 2011). Two years later, another missense mutation in *DAG1* (C669F) was reported in two siblings, who presented a severe muscle-eye-brain disease with additional brain malformations. This mutation is predicted to affect the secondary structure of β-Dg by substituting a helix by a β-strand, and, consequently, disrupt the α-/β-Dg binding interface (Geis et al. 2013). Through whole-exome sequencing, two novel *DAG1* mutations were identified in a patient with mild muscular dystrophy, affecting the N-terminal region of α-Dg and leading to its hypoglycosylation (Dong et al. 2015), which destabilizes the interaction of the actin network with the ECM.

Beyond the muscle system, Dystroglycan has also been shown to play a crucial role in the neural tissue. A role for Dg in organizing axon guidance cue distribution was identified in mice, both by directly binding and regulating the distribution of the axon cue signaling

molecule Slit, and by being glycosylated by two distinct enzymes, which is crucial for neuronal migration (Wright et al. 2012).

As aforementioned, and despite the vast knowledge that emerged from studies in the skeletal muscle, the DAPC is not limited to the muscle tissue. In fact, since its first *in vitro* characterization in epithelial cells (Durbeej and Campbell 1999), increasing evidence has been implicating DAPC components in a variety of epithelial-derived tumors. Dystroglycan alterations have been reported in different epithelial tumors, namely prostate, breast (Henry et al. 2001a, Muschler et al. 2002, Sgambato et al. 2003) and colon (Sgambato et al. 2003). Overall, Dg presents a reduced expression in these types of cancer, correlating with tumor progression and worst prognosis. Furthermore, abolishing β-Dg in a myoblast cell line led to centrosome amplification, a hallmark of cancer (Martinez-Vieyra et al. 2013). The same study reported a role for β-Dg in a nuclear import pathway, probably acting as a scaffolding protein involved in nuclear organization (Martinez-Vieyra et al. 2013). Additionally, several studies point to a correlation between *DMD* gene mutations and/or alterations in Dystrophin with the pathogenesis of a variety of tumors, including melanomas, lymphomas, leukaemia's, brain tumors, sarcomas and various carcinomas (Jones et al. 2021). In particular, the ratio of short *versus* long Dys isoforms seems to be imbalanced in these tumors, with the full-length Dys isoform absent and the truncated Dp71 isoform present in the majority of cancer cells. Characterizing the DAPC in other tissues beyond the muscle, as the epithelial tissue, will thus provide valuable insights, not only to further understand pathological contexts associated with alterations in this complex, as muscular dystrophies and cancer, but also regarding its role in physiological contexts. In line with this, a number of studies have been dissecting the role of the DAPC during development in different epithelial tissues, which I will now describe.

The DAPC during development

The DAPC has been implicated in several morphogenetic processes in various model organisms, such as ovarian follicle elongation (Cerqueira Campos et al. 2020), adult neck formation (Villedieu et al. 2023) and retina development (Walther et al. 2024) in *Drosophila*, and notochord (Buisson et al. 2014) and pronephros (a rudimentary, non-functional kidney) development (Bello et al. 2008) in the *Xenopus* embryo. Although these processes are different in many aspects, they seem to share some features regarding the contribution of DAPC for their success (Figure 1.11). Overall, the DAPC seems to play a

role in tissue morphogenesis via two interconnected mechanisms. On one hand, it is a pivotal player for proper basement membrane (BM) organization (Henry and Campbell 1998, Satz et al. 2008, Haigo and Bilder 2011, Buisson et al. 2014, Walther et al. 2024), a vital structure for the generation of the mechanical force required to the majority of the morphogenetic processes aforementioned. On the other hand, the DAPC is able to modulate the organization of F-actin fibers at the basal side of epithelial cells, influencing tissue shape (Cerqueira Campos et al. 2020, Villedieu et al. 2023).

The BM underlies epithelial cells, providing mechanical support and influencing cell shape through its mechanical properties (Topfer et al. 2022). Impairment of Dg function, through defective α-Dg glycosylation, was shown to alter BM structure in mice muscle cells, leading to the formation of a less compact BM that reduced the physiological function of the muscle and predisposed it to dystrophy (Goddeeris et al. 2013). A similar requirement of Dg for proper BM assembly was observed in the mouse retina and brain (Henry and Campbell 1998, Satz et al. 2008), suggesting a transversal role for the DAPC in morphogenetic processes across different tissues. Furthermore, as previously referred, mice in which Dg was knocked out are embryonic lethal due to defects in the Reichert's membrane, one of the first BMs to be produced in the mouse embryo (Williamson et al. 1997).

A variety of studies also imply a role for Dg in BM organization and dynamics in other vertebrate and invertebrate contexts. Both during notochord (Buisson et al. 2014) and pronephros (Bello et al. 2008) development in the *Xenopus* embryo, Dg was shown to play a key role in Laminin assembly, anchoring the cells to the BM and helping to maintain the shape of both notochordal and pronephric cells. Additionally, a study in the *Drosophila* testis suggests a relationship between these two proteins during fly spermatogenesis (Tseng et al. 2022). In this model, germline stem cells (GSCs) compete for space and resources, and the absence of the transcriptional repressor Chinmo confers a competitive advantage to GSCs. Chinmo-depleted GSCs were shown to secrete Perlecan to the BM, creating a *de novo* ECM around the niche to evict non-depleted GSCs, in a Dg-dependent manner. Abolishing Dg from Chinmo-depleted GSCs decreased their niche occupancy, pointing a role for Dg in this competition mechanism of GSCs. Furthermore, the *de novo* ECM surrounding the GSCs niche also displayed Laminin, whilst other ECM proteins were expressed at low levels (Tseng et al. 2022), supporting a role for the DAPC in BM organization. A similar role was observed in the *Drosophila* retina, where the DAPC is required for the proper BM patterning, by accumulating Laminin at specific locations across the basal surface of retinal cells (Walther et al. 2024). This localized accumulation of DAPC-Laminin is also crucial to direct integrin adhesion, which in turn induces cell shape remodeling during morphogenesis of the fly neuroepithelia. The DAPC is also specifically required for BM assembly in female flies (Isabella and Horne-Badovinac 2015, Cerqueira

Campos et al. 2020). During *Drosophila* oogenesis, both Dg and Dys were shown to modulated BM fibril deposition, which act as a persistent cue for egg chamber elongation (Cerqueira Campos et al. 2020).

Beyond its role in BM assembly and organization, the DAPC has also been shown to modulate the organization of F-actin fibers at the basal side of epithelial cells, although this role cannot be completely dissociated from the previous one. Specifically, the DAPC is required for proper orientation of the F-actin fibers during follicle elongation of the *Drosophila* ovary (Cerqueira Campos et al. 2020) and during fly neck formation (Villedieu et al. 2023). In the first model, the DAPC was shown to orient F-actin stress fibers in a manner that relies on the preceding DAPC-dependent BM fibril deposition, playing a dual role in organizing the basal domain of epithelial ovarian cells that is crucial for its elongation (Cerqueira-Campos et al, 2020). Disrupting Dg or Dys function also impairs basal actomyosin organization during *Drosophila* neck formation, slowing down neck folding (Villedieu et al, 2023). The DAPC has also been implied in cytoskeleton reorganization during notochord development in *Xenopus* embryos, through targeting myosin to notochordal cells that creates an adhesome composed of Laminin-Dg-Myosin (Buisson et al. 2014). Overall, these studies suggest a role for the DAPC as an organizer of the extracellular basement membrane and of the intracellular cytoskeleton during several morphogenetic processes in different organisms (Figure 1.11).

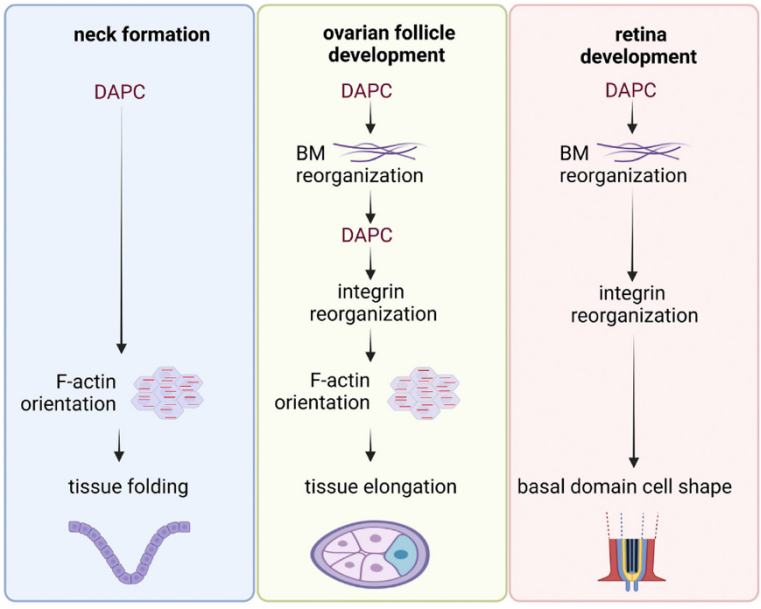


Figure 1. 11. The DAPC is involved in different morphogenetic processes. The DAPC seems to play a dual role in tissue morphogenesis, by organizing the basement membrane underlying epithelial cells and/or by modulating the orientation of the actin cytoskeleton. These processes are common to several morphogenetic processes in *Drosophila*, such as neck formation, ovarian follicle elongation and retina development (adapted from (Mirouse 2023)).

Early studies suggested that the DAPC also plays a role in the polarization of the oocyte and epithelial cells of the *Drosophila* ovary (Deng et al. 2003). Specifically, it was found that disrupting Dg function in the follicular epithelium results in the extension of apical markers of polarity to the basal side of the cells and loss of lateral markers. On the contrary, Dg over-expression reduces the apical localization of the same markers, implying a cell-autonomous role for the DAPC in the maintenance of the apicobasal polarity of epithelial cells (Deng et al. 2003). Furthermore, the organization of Laminin in Dg-mutant cells and of cortical F-actin in the neighboring cells of the Dg-mutant ones was also disrupted, suggesting a non-cell-autonomous role for the DAPC in basal actin organization and planar polarity of the follicular epithelium. This is consistent with a role for Dg in the organization of extracellular Laminin architecture in mouse embryonic stem cells (Henry et al. 2001b), emphasizing the importance of DAPC on BM organization. Moreover, it was shown that proper Dg localization (but not integrin localization) in these cells depends on extracellular Perlecan, and that abolishing Perlecan causes polarity defects similar to those of Dg-mutant cells (Schneider et al. 2006). Dg interaction with Perlecan does not require its mucin-like domain, suggesting that this domain might regulate the binding affinity of Dg with its extracellular interactants and implying a function for Dg that does not rely on its mucin region. Finally, Dg was also shown to take part on another type of polarity in the *Drosophila* ovary – the anteroposterior polarization of the oocyte. In Dg-mutant germline clones, early polarity markers failed to localize to the posterior part of the oocyte (Deng et al. 2003), suggesting a role for Dg in anteroposterior polarity. Overall, these studies pinpoint the DAPC as an organizer of cellular polarity, although the contribution of each specific component still needs to be further dissected. In addition, the recent studies using different allelic combinations of Dg and Dystrophin did not provide evidence for the dramatic effects on epithelial organization that would be expected if the DAPC had a central role in epithelial cell polarity, making its cell polarity functions controversial.

3. The challenges of epithelial cytokinesis

The mechanisms underlying cytokinesis previously described in Section 1.1. have been mostly dissected in individual cells, both *in vitro* (in cells growing in culture) and *in vivo*, during early embryonic stages (in one-cell embryos) (Green et al. 2012, D'Avino et al. 2015). While these models are crucial to understand how the cytokinetic machinery operates, providing the range of knowledge that I described in the previous Sections, cytokinesis in a multicellular context entails several challenges aside the cytokinetic process itself. One of these challenges is the establishment of a novel set of adhesive contacts between the newly formed daughter cells following cytokinesis, in order to preserve tissue integrity and epithelial polarity.

In the last decade, a wealth of knowledge on epithelial cytokinesis in invertebrate (Guillot and Lecuit 2013, Herszterg et al. 2013, Founounou et al. 2013, Morais-de-Sa and Sunkel 2013, Bourdages et al. 2014, Pinheiro et al. 2017, di Pietro et al. 2023) and vertebrate (Wang et al. 2014, Hatte et al. 2014, Higashi et al. 2016, Taneja et al. 2016, Hatte et al. 2018, Uroz et al. 2019, Landino et al. 2023) models has emerged. Aside from deciphering how the adhesive contacts with neighboring cells are remodeled and/or synthesized *de novo* upon division, these studies highlighted one common cytokinetic feature to different animal models – the prevalent asymmetric constriction of the contractile ring, which ultimately positions the midbody at the apical side of epithelial cells. In this section, I will start by discussing the mechanisms that ensure these two features of epithelial cytokinesis (asymmetric ring constriction and adhesion remodeling), followed by an overview on how the adhesion contacts impact the efficiency of cytokinesis in the epithelial context.

3.1. Mechanisms controlling furrow asymmetry and junctions remodeling during epithelial cytokinesis

3.1.1. Asymmetric furrowing during epithelial cytokinesis

In single-cell systems, the absence of cell-cell contacts typically leads to the symmetric constriction of the contractile ring. This is also observed during epithelial orthogonal divisions – when the cell divides perpendicular to the plane of the epithelium –, as the contractile ring does not face the resisting forces exerted by intercellular adhesion (Founounou et al. 2013). However, in most monolayered epithelial tissues, cell division occurs parallel to the plane of the epithelium (termed planar divisions), with the contractile ring directly contacting the AJs. Interestingly, planar divisions in most epithelial contexts display a unilateral (or asymmetric) ingression of the cleavage furrow, in which the contractile ring constricts from the basal to the apical domain of epithelial cells (Herszterg et al. 2014, Thieleke-Matos et al. 2017).

Asymmetric ring constriction has been observed *in vitro*, in cultured monolayers of Madin-Darby Canine Kidney (MDCK) cells (Reinsch and Karsenti 1994) and in immortalized mouse hepatocytes with induced expression of Tight Junctions (TJs) proteins (Kojima et al. 2001). Additionally, a number of studies also reported this feature of epithelial cytokinesis *in vivo*. In vertebrates, asymmetric furrowing has been described in the mouse small intestine (Jinguji and Ishikawa 1992, Fleming et al. 2007), mouse (Kosodo et al. 2004) and zebrafish (Das et al. 2003) neuroepithelia and in chicken (Firmino et al. 2016), *Xenopus* (Higashi et al. 2016, Hatte et al. 2018, Hatte et al. 2021) and mouse (Paim and FitzHarris 2022) embryos. Similarly, asymmetric furrowing has been observed in different epithelial tissues of invertebrates, namely in the *Drosophila* pupal notum (Founounou et al. 2013, Herszterg et al. 2013), embryonic ectoderm (Guillot and Lecuit 2013) and follicular epithelium (Morais-de-Sa and Sunkel 2013), and in *C. elegans* embryos (Maddox et al. 2007) and vulval epithelial precursor cells (Bourdages et al. 2014). These studies shed light on the mechanisms that ensure asymmetric furrowing during epithelial cytokinesis, which mainly differ from each other in the distribution of proteins within the contractile ring and on the external forces exerted onto this structure, mainly by the AJs.

Asymmetric furrowing through heterogeneous distribution of ring components

In some systems, asymmetric furrowing seems to be ensured by the unequal distribution of structural and scaffold proteins along the contractile ring. In the *C. elegans* early embryo, asymmetric furrowing is promoted by intrinsic anisotropy of the contractile ring. The stochastic accumulation of Anillin at one side of the ring promotes the subsequent recruitment of Septins and the coalescence of myosin and F-actin at the same side, which constricts first and the most (Maddox et al. 2007). Depletion of Anillin or Septins in this system disrupts the polarized distribution of myosin within the contractile ring and the asymmetry of furrowing. Of note, this is observed even in the first zygotic division of *C. elegans*, which, by occurring in a single-cell context, is the only one devoid of potential cell-cell adhesion interference, suggesting that Anillin and Septins directly act as regulators of asymmetric ring constriction in this context. The absence of known polarized cues at the equator of the *C. elegans* zygote has hindered how the random symmetry breaking event occurs in the contractile ring. Mathematical modeling has proposed that a feedback mechanism involving membrane curvature-dependent alignment and contractility of actomyosin filaments is responsible for the stochastic furrow asymmetry in this system. Local actomyosin contractility, potentiated by the alignment of membrane-associated filaments parallel to the division plane, would gradually propagate around the ring, promoting its asymmetric constriction (Dorn et al. 2016). An alternative model was subsequently proposed, based on radially asymmetric cortical inflows that drive the alignment of actin filaments circumferentially around the ring, leading to its asymmetric closure (Menon et al. 2017). Further observations added to this model the notion that asymmetric outflows (around the contractile ring) of microdomains composed by Anillin and Septins would also account for the asymmetry of furrowing, which starts in a region of the ring offering least resistance (Carim et al. 2020). Additionally, differences in the upstream regulators of cytokinesis might also affect the kinetics of the contractile ring. While impairing RhoA activation, which is required for subsequent Anillin recruitment and activation, does not alter the asymmetry of ring constriction in *C. elegans* embryos (Tse et al. 2012), disruption of RhoA upon *Chlamydia* infection in HeLa cells affects the bidirectionality of cleavage furrow ingression (Sun et al. 2011). In line with this, the upstream regulator of RhoA, Ect2, displays a divergent localization in HeLa cells (restricted to the equatorial cortex) (Yuce et al. 2005) and *C. elegans* embryos (spread throughout the zygote cortex) (Gomez-Cavazos et al. 2020), suggesting that the regulation of asymmetric cytokinesis is needs to be addressed in the complex and broad environment where it occurs.

Heterogeneous distribution of structural components within the contractile ring is also observed in the *Drosophila* pupal notum (Figure 1.12) (Founounou et al. 2013, Herszterg et al. 2013). However, in contrast to the random distribution of Anillin and Septins in the contractile ring of *C. elegans* embryos, the accumulation of these proteins in the notum occurs preferentially at the basal side of the ring. Consequently, this side of the ring displays a higher rate of constriction, thus driving basal-to-apical furrowing in this epithelial context (Founounou et al. 2013, Herszterg et al. 2013). Noteworthy, depletion of Anillin or Septins in the fly notum does not make the furrowing symmetric, suggesting that, in this particular context, furrow asymmetry is regulated by other cytokinetic component(s). Accordingly, while the heterogeneous distribution of Anillin and Septins drives the polarization of Myosin and F-actin within the contractile ring in the *C. elegans* embryo (Maddox et al. 2007), the same does not occur in the *Drosophila* pupal notum. Although a reduction in Myosin accumulation in cells neighboring *anillin* or *peanut* dividing cells has been reported in this epithelial context (Pinheiro et al. 2017), the total amount of Myosin within the contractile ring seems to remain unaffected in *peanut* mutant cells, despite a reduction of about 40% in the levels of Anillin along the contractile ring (Founounou et al. 2013). Altogether, this suggests an Anillin-independent mechanism to ensure asymmetric ring constriction in the *Drosophila* pupal notum. Alternatively, it remains to be uncovered if cell-cell and cell-matrix adhesion play an additional role in this particular type of asymmetric cytokinesis, where the contractile ring *per se* displays an intrinsic asymmetry. Thus, to identify a possible regulator of asymmetric cytokinesis in the *Drosophila* pupal notum, it would be interesting to deplete the different adhesion proteins present in this type of epithelial tissue and assess if furrow asymmetry becomes disrupted.

Asymmetric furrowing despite homogeneous distribution of ring components – a role for cell-cell and cell-matrix adhesion on asymmetric cytokinesis

In contrast to the aforementioned studies, in some epithelial contexts, the distribution of Anillin and Septins along the contractile ring does not follow a particular gradient that could explain its asymmetric constriction. In the *Drosophila* embryonic ectoderm (Guillot and Lecuit 2013) and follicular epithelium (Morais-de-Sa and Sunkel 2013), most contractile ring components are spread homogeneously throughout the ring (Figure 1.12), suggesting that intrinsic polarization of ring proteins is dispensable for asymmetric furrowing in these contexts, which must be ensured by extrinsic factors. In

agreement, laser nanodissection of the mechanical properties of the contractile ring in the embryonic epithelia showed that the intrinsic tension exerted by the ring apically and basally is undistinguishable, despite its asymmetric constriction (Guillot and Lecuit 2013). This further corroborates a role for an extrinsic regulation of furrow directionality, that lifts a uniformly contracting ring upwards.

Interestingly, in both epithelial contexts, the apical side of the contractile ring remains in close contact with AJs throughout constriction (Figure 1.12). Thus, the bias in furrow ingression observed in these tissues could arise if the contractile ring, while constricting uniformly, was being positioned upwards by a mechanical anchorage to the junctions. In fact, disruption of α-catenin, β-catenin or E-cadherin functions abolished the asymmetry of ring constriction in these systems (Guillot and Lecuit 2013, Morais-de-Sa and Sunkel 2013), supporting a vital role for these proteins in polarizing the constriction of an otherwise nearly symmetric ring. Furthermore, additional laser ablation experiments in fly embryos depleted of α-catenin or E-cadherin showed that, despite its uniform intrinsic contractility, the ring is deformed laterally as it constricts due to the tension coming from its anchoring to AJs, reinforcing the idea that polarized ingression in these contexts largely relies on the forces exerted by the AJs on the contractile ring (Guillot and Lecuit 2013).

In line with the aforementioned hypothesis, induced polarization of AJs in otherwise non-polarized *Drosophila* S2 cells, through the expression of a construct where the cytoplasmic tail of E-cadherin was fused to the extracellular domain of Echinoid, was sufficient to direct furrow constriction and midbody positioning towards the sites of E-cadherin enrichment (Morais-de-Sa and Sunkel 2013). This suggests that AJs are sufficient to orient ring constriction in the absence of other polarization cues, and that the tension exerted by these junctions might be sufficient to promote asymmetric constriction. Similarly, in the simple epithelium of *C. elegans* formed by vulval precursor cells, contractile ring constriction is asymmetric and polarized towards the apical domain, in a ring displaying a homogeneous Myosin distribution in both apical and basal domains (Bourdages et al. 2014). In contrast with what happens in the *C. elegans* embryo (Maddox et al. 2007), interfering with Anillin function does not alter the asymmetry of furrowing in these cells, uncovering different Anillin requirements for cytokinesis in epithelial versus early embryonic tissues in *C. elegans.* This suggests that, similar to the *Drosophila* epithelia (Founounou et al. 2013, Herszterg et al. 2013, Morais-de-Sa and Sunkel 2013, Guillot and Lecuit 2013), furrow asymmetry in *C. elegans* vulval precursor cells occurs independently of Anillin. Remarkably, asymmetric constriction was maintained in cells depleted of E-cadherin (Bourdages et al. 2014), suggesting that, in this particular context, a different extrinsic mechanism must ensure furrow asymmetry, rather than the anchoring of the contractile ring to the AJs.

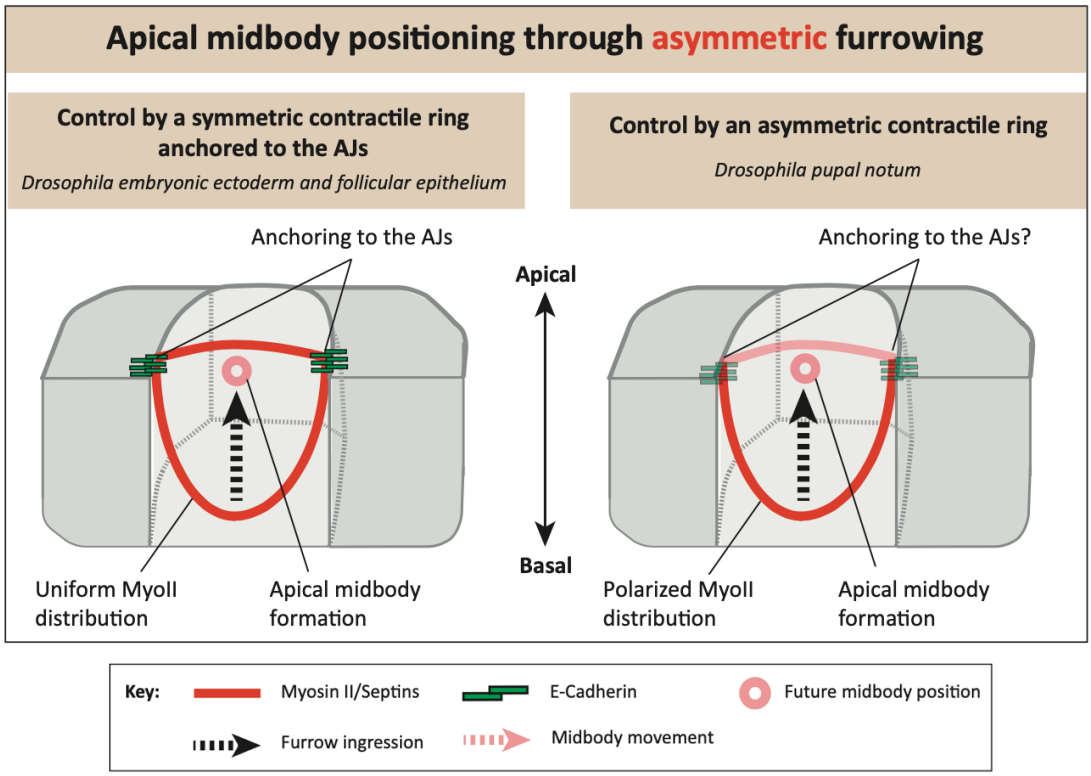


Figure 1. 12. Two mechanisms control furrow asymmetry in *Drosophila* epithelia. Apically-directed constriction of the contractile ring is ensured either by a nearly symmetric ring anchored to apical Adherens Junctions (left) or by the heterogeneous distribution of structural components within the contractile ring (right) (adapted from (Herszterg et al. 2014)).

Aside the contribution of AJs to asymmetric epithelial cytokinesis, it is likely that other adhesion molecules might also shape the directionality of ring constriction. For instance, the presence of integrins that directly connect the basal domain of epithelial cells to the actin cytoskeleton, hints that there might be an interplay between the extracellular matrix (ECM) and the contractile ring, acting in coordination to the one observed with the AJs. In line with this, integrins present at the basal side of cultured HeLa cells, which cluster into Focal Adhesions, were shown to control furrow asymmetry in these cells (Taneja et al. 2016). In particular, the extent of adhesion of these cells to the underlying substrate via a population of mitotic Focal Adhesions composed by Paxillin, Focal Adhesion Kinase and Vinculin, tightly correlated with furrow asymmetry. Enhancing adhesion of HeLa cells to the substrate caused a higher resistance of an otherwise nearly concentric ring to ingress, resulting in apical-to-basally directed ring constriction. On the contrary, decreasing the adhesive strength of these cells to the substrate resulted in less resistive force at the bottom of the cell, inducing symmetric ring constriction (Taneja et al. 2016). Additionally, increasing adhesion to the substrate in monolayered epithelial cells, that usually display basal-to-apical ring constriction, directed less ingression of the contractile ring from the basal domain

(Taneja et al. 2016), suggesting that adhesion to the ECM acts in coordination with cell-cell adhesion to shape furrow ingression in epithelial monolayers.

Challenging this model, a study using human RPE1 cells reported that the majority of Focal Adhesions components that are present during interphase, like Talin, Zyxin and Paxillin, are lost upon mitotic entry, with the exception of β1-integrin (Dix et al. 2018). Although the relevance of cell-substrate adhesion to furrow (a)symmetry was not addressed in these cells, β1-integrin-mediated adhesion to the substrate was shown to guide migration of dividing cells with a compromised actomyosin ring, thus enabling successful cytokinesis. In line with the loss of Focal Adhesions upon mitotic entry, it was recently shown that Kindlins (cytoplasmic adaptor proteins that, along with Talin, are involved in the inside-out activation of integrins, allowing them to bind extracellular interactors (described in Section 2.1.2)) are degraded upon entry in mitosis in mammalian cell culture (Chen et al. 2022). Cdk1-CyclinB1-mediated phosphorylation of Kindlin induces its ubiquitination and subsequent degradation, which is crucial for Focal Adhesions disassembly and mitotic rounding and, thus, for cells to progress accurately through mitosis. Based on these findings, it would be interesting to assess if Talin is also degraded at mitotic entry, which would explain its disappearance during mitosis observed in RPE1 cells (Dix et al. 2018). As Kindlins directly bind actomyosin (Bledzka et al. 2016), it is possible that its degradation acts as a safety mechanism to limit the strength of basal adhesion applied to the contractile ring, so it can effectively constrict. In line with this hypothesis, it was recently reported that integrins in mitotic cells in culture are still able to engage adhesion with the ECM in a Kindlin- and Talin-dependent manner (Huber et al. 2023). However, both Kindlins and Talin cannot bind the actomyosin cytoskeleton, decreasing the overall adhesion of the cell to the ECM. It is thus possible that distinct mechanisms mediate cell adhesion to the ECM, whose molecular composition and dynamics of assembly/distribution varies according to cell- or even tissue-context. Despite this, a balance between cell-cell and cell-matrix adhesion must contribute for the polarized ingression of the cleavage furrow in the intricated context of epithelial cytokinesis.

Remarkably, regardless of the (a)symmetry of furrowing, epithelial cytokinesis seems to conserve a crucial feature – the systematic positioning of the midbody at the apical part of the daughter cells interface (Herszterg et al. 2014, Thieleke-Matos et al. 2017). In line with this – and despite asymmetric furrowing being the prevalent mode of cytokinesis in most epithelial tissues –, it was shown that symmetric ring constriction in a few epithelial contexts still ends up with the apically positioning of the midbody. In the *Drosophila* pupal wing, the contractile ring constricts symmetrically and presents a homogeneous distribution of Myosin along the ring. However, as the dividing cell becomes partially extruded from the

epithelium during division, the midbody is positioned off-centered, towards the apical domain of the tissue (Figure 1.13) (Herszterg et al. 2013). Interestingly, an inverse correlation between apical extrusion and furrow asymmetry has been reported in the *Drosophila* follicular epithelium, showing that the more a cell extrudes from the epithelial monolayer during division, the more symmetric is ring constriction (Morais-de-Sa and Sunkel 2013). Additionally, even though furrowing occasionally occurs in a symmetric fashion in this system, positioning the midbody more centrally, an AJs-dependent upward lifting ensures that the midbody is ultimately relocated to the apical domain of the tissue (Figure 1.13). Altogether, this suggests that epithelial cells evolved self-regulatory mechanisms to ensure an apical midbody positioning at the end of cytokinesis. This position enables the midbody to act as a spatial determinant in different *Drosophila* epithelial tissues, to ensure the propagation of apical-basal polarity and the preservation of epithelial architecture during proliferation (as discussed in the following sub-section) (Herszterg et al. 2013, Morais-de-Sa and Sunkel 2013).

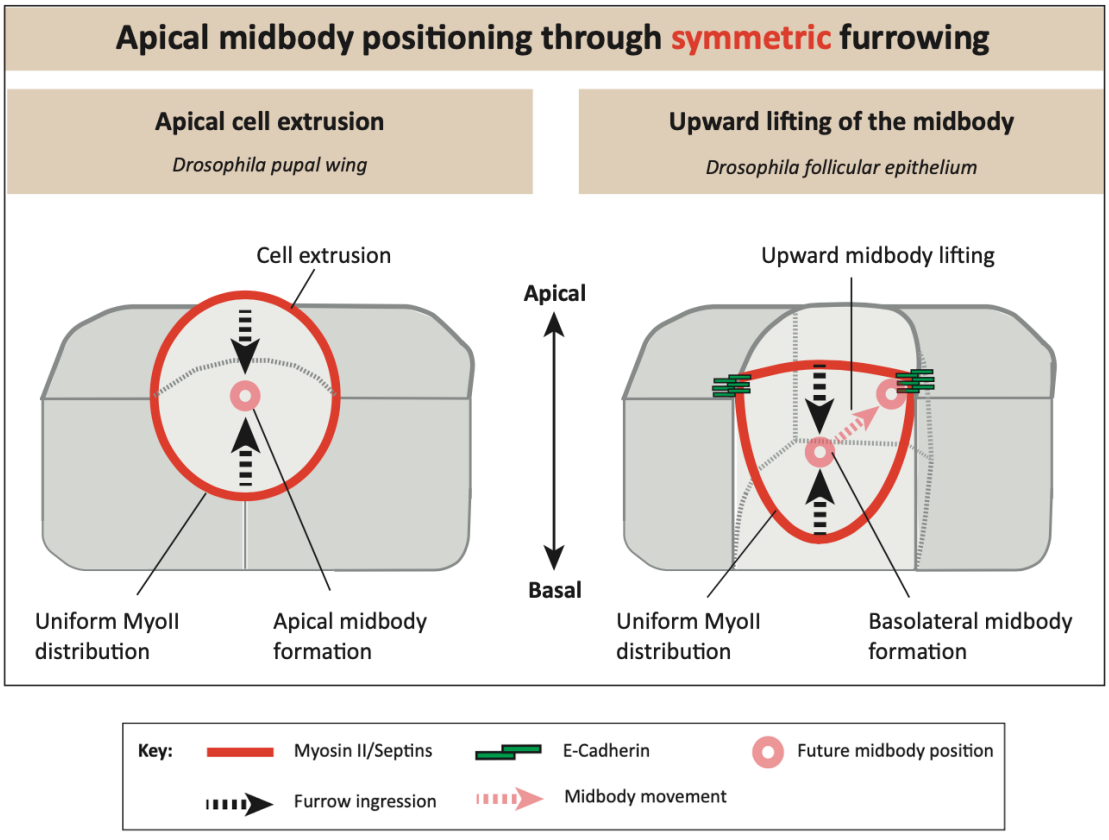


Figure 1. 13. Two mechanisms ensure apical midbody positioning through symmetric furrowing in *Drosophila* epithelia. In the *Drosophila* pupal wing (left) and follicular epithelium (right), the contractile ring presents a uniform Myosin distribution and can constrict in a symmetric fashion. Despite this, the midbody is ultimately positioned at the apical domain of the epithelium, either through partial apical cell extrusion of the dividing cell (left) or through an AJs-dependent upward lifting, at the end of cytokinesis (adapted from (Herszterg et al. 2014)).

3.1.2. Remodeling of intercellular junctions during epithelial cytokinesis and *de novo* junction formation

Epithelial cohesiveness is a crucial feature of epithelial tissues, being essential for its barrier function. Remarkably, although epithelial cells undergo extensive reshaping during division, the integrity of the tissue is maintained, largely due to the adhesive junctions present at the apical-basal axis of these cells. Cell-cell junctions were initially thought to be stable structures that were maintained throughout cell division (Jinguji and Ishikawa 1992, Baker and Garrod 1993, Reinsch and Karsenti 1994). Fluorescence recovery after photobleaching (FRAP) experiments in cultured MDCK cells (Yamada et al. 2005) and in *Drosophila* embryos (Huang et al. 2011) subsequently showed that, in fact, AJs are highly dynamic structures during epithelial cytokinesis, despite the maintenance of tissue architecture. These observations paved the way for understanding the remodeling of intercellular contacts during epithelial cell division, in order to accommodate contractile ring constriction and, later, to properly establish *de novo* cell-cell contacts between the newly formed daughter cells.

In vivo clonal analysis has shown that, in the majority of *Drosophila* epithelia, daughter cells remain close neighbors after division by forming cohesive groups of clones, indicating that adhesion between these cells is sufficient to prevent their dissociation (Baena-Lopez et al. 2005, Farhadifar et al. 2007, Ragkousi and Gibson 2014). Despite this, a reduction in the E-cadherin levels at the interface between the cleavage furrow of the dividing cell and its neighbors has been observed in time-lapse imaging of cytokinesis in different *Drosophila* epithelia (Founounou et al. 2013, Herszterg et al. 2013, Guillot and Lecuit 2013, Morais-de-Sa and Sunkel 2013). This decrease drives a local and transient reduction of adhesion at the AJs and, consequently, an uncoupling between the dividing and the neighbouring cells at the furrow zone, termed adhesion disengagement, which allows the formation of the new interface between the nascent daughter cells (Figure 1.14) (Founounou et al. 2013, Guillot and Lecuit 2013).

Local adhesion disengagement seems to require a balance between opposite mechanical forces occurring during cell division – in one direction, active pulling forces exerted by the contractile ring on the AJs, to which it is anchored, and, in the opposite direction, the reactive forces exerted by the homophilic interactions of E-cadherin with neighbouring cells, to maintain epithelial shape (Herszterg et al. 2014, Ragkousi and Gibson 2014). Therefore, in order to efficiently complete cytokinesis, the contractile ring needs to produce enough force to bypass the opposite tension resulting from the adhesive contacts with the neighbors. In line with this, reducing contractile ring constriction in the *Drosophila*

embryonic ectoderm (Guillot and Lecuit 2013) and pupal notum (Founounou et al. 2013, Herszterg et al. 2013, Pinheiro et al. 2017), through the depletion of Anillin or Septins, prevents or delays adhesion disengagement and subsequent *de novo* junction formation, suggesting that these proteins contribute to produce the necessary force for the ring to constrict and overcome the opposite tension from intercellular adhesion. Alternatively, as E-cadherin dilution at the edge of the contractile ring is minimal upon Anillin or Septins disruption (Founounou et al. 2013, Guillot and Lecuit 2013), these proteins might act as regulators of the anchorage of the contractile ring to AJs, ensuring adhesion remodeling and subsequent ring disengagement to allow an efficient cytokinesis. In agreement with this, adhesion disengagement is also delayed when the edge of the cleavage furrow faces a vertex of a neighboring cell, where the opposing tensile forces exerted onto the contractile ring are likely higher (Founounou et al. 2013) or when adhesion strength at the AJs is reinforced through E-cadherin is over-expressed (Guillot and Lecuit 2013). In the embryonic epithelium, it is proposed that extrinsic tension exerted by the neighbouring cells needs to be present so the contractile ring can transiently disengage. Accordingly, ablating the tension exerted by the neighbors strikingly delayed the detachment of the contractile ring from the cell junctions in the dividing cell (Guillot and Lecuit 2013).

In the *Drosophila* pupal notum, the membrane of the neighbouring cells becomes stretched and is pulled towards the dividing cell as the contractile ring constricts, resulting in a local E-cadherin dilution. In response, the neighbouring cells accumulate Myosin at the base of the co-ingressing membrane, providing the cortical tension required for membrane juxtaposition with the dividing cell and ensuring maintenance of cohesion between these cells (Figure 1.14) (Founounou et al. 2013, Herszterg et al. 2013, Pinheiro et al. 2017). Accordingly, disruption of Myosin accumulation in the neighbors impairs membrane juxtaposition and shortens the length of the new interface formed between the daughter cells (Herszterg et al. 2013), suggesting that the non-autonomous Myosin enrichment promotes a primordial adhesive contact between the future daughter cells that sets the geometry of the emerging interface. Myosin accumulation was subsequently shown to be driven by a mechanosensing mechanism that coordinates the remodeling of AJs with actomyosin dynamics (Pinheiro et al. 2017). The pulling forces exerted by the contractile ring in the dividing cell promote junction elongation and local E-cadherin dilution, which further induces self-organized actomyosin flows that lead to the accumulation of Myosin at the base of neighbouring cells. While this seminal work highlighted the importance of AJs mechanosensitivity during epithelial cytokinesis, a recent study has further dissected how mechanosensing is transduced in a biochemical response in neighbouring cells (di Pietro et al. 2023). In particular, it was shown that the local enrichment of the RhoGEF Cysts in neighbouring cells contributes for this mechanosensing response. Cysts accumulation

triggers the activation of Rho-dependent actomyosin contractility, promoting cell membrane juxtaposition to allow *de novo* junction formation (di Pietro et al. 2023).

As in the *Drosophila* pupal notum, epithelial cohesiveness is also maintained throughout cytokinesis in vertebrates (Figure 1.14), as shown by the close apposition between the dividing and neighbouring cells through electron microscopy of the mouse small intestine (Jinguji and Ishikawa 1992). Remarkably, in contrast to what has been described for *Drosophila*, the constriction of the contractile ring in vertebrate epithelia triggers adhesion strengthening at the cleavage furrow, rather than junction dilution. FRAP experiments in the *Xenopus* embryo suggest that TJs are under stable tension throughout cytokinesis, to preserve the barrier function for paracellular transport of the epithelium during cell division. Additionally, the contractile ring was shown to remain in close contact with the AJs until the end of cytokinesis, without displaying adhesion disengagement (Higashi et al. 2016).

The maintenance of the apical junctional complex during cytokinesis in the *Xenopus* embryo seems to rely on a balance between two mechanisms. On one hand, Vinculin recruitment to the cleavage furrow seems to stabilize E-cadherin levels at the AJs, reducing its turnover. Disruption of Vinculin recruitment induces a transient reduction of AJs at the points of contact with the contractile ring, driving its acceleration (Higashi et al. 2016). On the other hand, TJs negatively regulate the mechanical tension applied to the AJs during epithelial cytokinesis. Depletion of the core TJs proteins ZO-1 and GEF-H1 increase the tensile forces applied to AJs, enhancing its resistance to ring furrowing and ultimately driving cytokinesis failure (Hatte et al. 2018).

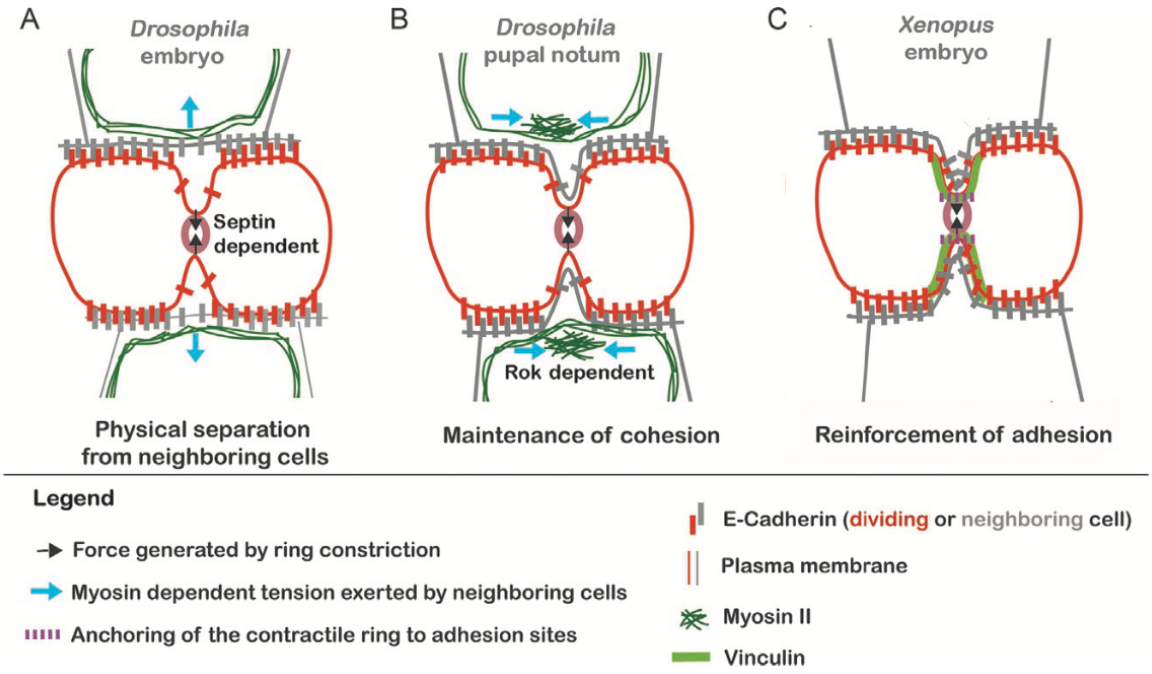


Figure 1. 14. Regulation of tissue cohesiveness and AJs remodeling between a dividing cell and its neighbors during cytokinesis in *Drosophila* and *Xenopus* epithelia. Schematic representation of a dividing and neighbouring cells in an epithelial tissue. In the embryonic ectoderm (A), the pulling forces exerted by the contractile ring overcomes the restraining strength of E-cadherin-based junctions, termed adhesion disengagement, driving the formation of a gap between the dividing and the neighbouring cells. In contrast, cell-cell cohesiveness is maintained in the pupal notum (B), where the membrane of the neighbouring cells remains in tight juxtaposition with the membrane of the dividing cell, due to tension generated by a Rok-dependent accumulation of Myosin at the base of the neighbouring cells. Similarly, cell-cell contacts are also maintained throughout constriction in the *Xenopus* embryonic epithelia (C), where these contacts are even reinforced by the recruitment of Vinculin to the cleavage furrow (adapted form (Thieleke-Matos et al. 2017)).

Despite the differences in AJs remodeling between vertebrate and invertebrate epithelia, a striking similarity was recently reported – the accumulation of Myosin at the neighbouring cells. In the *Xenopus* embryo, neighbouring cells accumulate actomyosin arrays adjacent to the cytokinetic furrow, generating restraining tension in response to the pulling forces of contractile ring (Landino et al. 2023). This tension needs to be balanced in order to drive a faithful cytokinesis, as increased neighbour cell contractility on both sides of the furrow bypasses the pulling forces of the contractile ring, leading to cytokinesis failure (Landino et al. 2023). Therefore, despite suggesting that the role of Myosin accumulation in cytokinesis might be tissue-dependent, this observation reinforces an active role for the non-dividing neighbouring cells in the modulation of epithelial cytokinesis, and further pinpoints that this non-autonomous regulation is evolutionarily conserved.

In the majority of epithelial tissues, ring constriction is followed by the formation of a new junctional interface between the nascent daughter cells. The mechanisms underlying

de novo junction formation have been mostly dissected in the *Drosophila* pupal notum (Herszterg et al. 2013, Pinheiro et al. 2017, di Pietro et al. 2023), where neighbour-mediated tension and apical midbody positioning guide AJs establishment and ensure the maintenance of epithelial geometry. In this tissue, a mechanosensing response from the neighbors to the pulling forces exerted by the contractile ring in the dividing cell ensures a tight juxtaposition between both cell membranes. Although this initially sets the topology of the new daughter-daughter interface (Herszterg et al. 2013, Pinheiro et al. 2017), neighbouring cell membranes must then be removed to enable the formation of a new daughter-daughter AJ interface. The apical positioning of the midbody becomes particularly relevant at this stage, as it drives a wave of actin polymerization, regulated by Arp2/3 and Rac, that ensures the retraction of neighbouring cell membranes to modulate the length of *de novo* junction and the dynamics of cell-cell arrangements (Herszterg et al. 2013, Morais-de-Sa and Sunkel 2013). Recently, it was shown that the localization of active Rac at the newly formed interface upon midbody formation is determined by RhoGEF4 (di Pietro et al. 2023). However, how RhoGEF4 enrichment is associated with the midbody remains unclear.

In contrast, in some epithelial contexts, the neighbouring cells are not withdrawn and remain interposed in-between the daughter cells. One such example occurs during gastrulation in the *Xenopus* embryo (Higashi et al. 2016). The stabilization of the apical junctional complex throughout cytokinesis may contribute to these intercalations. After cell division, a transient interface is established between the neighbouring cells, which is subsequently rearranged so that only a minor part of the neighbors remains apposed (Higashi et al. 2016). Cell intercalation has also been described during gastrulation of the chick embryo (Firmino et al. 2016). Remarkably, depending on the developmental stage, the new interface can be established either in-between the two daughter cells or in-between neighbouring cells, with the latter being more frequent in the latest stages of gastrulation. Additionally, cell intercalation has been shown to be dependent on the accumulation of low levels of actomyosin in the neighbouring cells in this epithelium (Firmino et al. 2016). Thus, although in the chick embryo the mechanosensing response of the neighbouring cells contrasts with the one described for the *Drosophila* notum (Herszterg et al. 2013, Pinheiro et al. 2017), where it promotes membrane cohesiveness, both show that the neighbouring cells respond during cytokinesis to facilitate specific tissue topologies, underscoring that the interplay between the dividing cell and the surrounding environment is essential for cytokinesis in the epithelia.

Altogether, these studies gather critical insights in both vertebrates and invertebrates into the mechanisms that ensure asymmetric furrowing and junction

remodeling during epithelial cytokinesis, two remarkable features of this intricated process. Overall, these studies pinpoint that, in highly proliferative tissues, the crosstalk between the dividing cell and the surrounding environment is crucial to cope the maintenance of the general tissue architecture with the extensive cell shape changes that occur during cell division.

3.2. The impact of adhesion on the efficiency of epithelial cytokinesis

A crucial aspect when studying cytokinesis is an epithelial context is the presence of a tightly arranged neighbouring environment, that needs to be maintained despite cell proliferation. As described in the previous section, epithelial architecture is ensured by cell-cell and cell-matrix complexes that ensure the connection between epithelial cells and with the underlying matrix, and whose contribution for the efficiency of epithelial cytokinesis cannot be overlooked. To understand how cells in a multicellular context cope adhesion with cytokinesis, several *in vivo* studies have been addressing this process in different animal models and, within the same model, in different tissues. Noteworthy, the majority of these studies have focused on several aspects influencing the dynamics of cytokinesis, such as the remodeling of adhesive contacts (Founounou et al. 2013, Herszterg et al. 2013, Higashi et al. 2016, Firmino et al. 2016, Pinheiro et al. 2017), the *de novo* junction formation following cytokinesis (di Pietro et al. 2023) or the impact of adhesion on the (a)symmetry of ring closure (Guillot and Lecuit 2013, Morais-de-Sa and Sunkel 2013). Nevertheless, studies reporting the impact of the adhesive contacts that the dividing cell establishes with its neighbors and with the underlying ECM on the completion of cytokinesis remain scarce.

In the planar divisions of the *Drosophila* pupal notum epithelium, the presence of Adherens Junctions (AJs) seems to promote cytokinesis failure, as Septin mutant cells have to overcome the force exerted by the surrounding AJs to divide (Founounou et al. 2013). In contrast, during orthogonal divisions, Septin mutant cells are able to efficiently complete cytokinesis, as the counteracting adhesion force exerted by AJs is absent. In agreement, abolishing AJs during the planar divisions of the notum allow cells to divide successfully, suggesting that AJs-mediated apical adhesion causes an opposing tension that resists furrowing, hindering cytokinesis efficiency (Founounou et al. 2013). A similar effect for intercellular adhesion on cytokinesis completion was observed in vertebrates. In the *Xenopus* embryonic gastrula, two Tight Junctions (TJs)-associated proteins were shown to negatively regulate the mechanical tension that is applied to AJs during cytokinesis, ensuring cytokinesis efficiency (Hatte et al. 2018). Depletion of Zonula Occludens-1 (ZO-1) or Guanine nucleotide Exchange Factor-H1 (GEF-H1) increased the tensile forces applied to AJs, strengthening the resistive forces of these junctions to cytokinesis completion. This led to cytokinesis failure, and also impaired the geometry and the speed of constriction of the contractile ring. Expression of ZO-1 or GEF-H1 in the respective knocked-down embryos rescued these defects (Hatte et al. 2018). Similarly, alterations in the tension at AJs level impaired cytokinesis completion in Madin Darby Canine Kidney (MDCK) cells

(Wang et al. 2023). In this study, α-catenin mechanosensitive mutants, which enhance the binding of this catenin to the actin cortex and the overall epithelial monolayer strength, were sufficient to drive cytokinesis failure in a monolayer of MDCK cells. Strikingly, cytokinesis fidelity was not compromised in MDCK-single cells, suggesting that α-catenin-induced cytokinesis failure is mediated by AJs in a manner that depends on neighboring cell-cell adhesion (Wang et al. 2023). A more direct contribution of neighboring cells on the non-autonomous regulation of epithelial cytokinesis was recently shown in *Xenopus* embryos (Landino et al. 2023). In this system, non-dividing neighboring cells accumulate contractility factors and assemble actomyosin arrays adjacent to the cytokinetic furrow. When hyper-contractility is induced, either through over-expression of an F-actin cross-linker in neighbor cells or through optogenetic activation in only one neighbor, furrow ingression is restrained and the dividing cell fails cytokinesis, indicating an active role for non-dividing neighbors in the efficiency of epithelial cytokinesis (Landino et al. 2023). Altogether, these studies suggest that AJs-based apical adhesion generally oppose the force exerted by the contractile ring, challenging cytokinesis efficiency in the epithelial context.

In contrast with the aforementioned studies, a positive role for AJs on cytokinesis completion was reported in both vertebrates and invertebrates (Goldbach et al. 2010). In *Drosophila* spermatocytes, abolishing Anillin function induces multinucleation, which is rescued upon over-expression of E-cadherin. E-cadherin is able to recruit α- and β-catenin to the cleavage furrow, strengthening AJs and stabilizing the contractile ring beyond its canonical anchorage to the plasma membrane through Anillin and Septins. This compensatory mechanism via E-cadherin over-expression was also observed in mouse fibroblasts, another non-epithelial tissue (Goldbach et al. 2010), suggesting that AJs might oppose or foster cytokinesis efficiency in a tissue-specific manner.

Although the majority of the studies reported so far focused on the impact of adhesion exerted at the apical level, increasing evidence indicates that the interaction of the dividing cell with the underlying extracellular matrix also shapes cytokinesis efficiency. In the Zebrafish epicardium, reinforcement of focal adhesions at the basal domain prevents contractile ring closure and subsequently leads to cytokinesis failure (Uroz et al. 2019). Studies in different epithelial cell lines have been suggesting that cell-ECM adhesion requires a fine-tuned regulation for a successful cytokinesis. In Chinese Hamster Ovary (CHO) cells, decreasing the activity of β1-integrin prevents the formation of a bipolar spindle, which ultimately drives cytokinesis failure (Reverte et al. 2006). Accordingly, maintenance of active β1-integrin puncta with the substrate in mitotic human retinal epithelial cells (RPE1) is crucial for an efficient cytokinesis (Dix et al. 2018). Through these integrin-substrate contacts that are initially formed during the mitotic rounding, dividing RPE1 cells are able to move away from one another in the absence of a visible contractile

ring and successfully complete cytokinesis, whilst cells plated in non-adherent substrates fail cytokinesis. A similar observation was also reported in non-epithelial cells in culture (Sambandamoorthy et al. 2015). Human dermal fibroblasts undergo successful cytokinesis when plated on hard substrates, but not on soft ones. Strikingly, cell-substrate adhesion on these fibroblasts seems to also be dependent on β1-integrin, as depleting this integrin leads to cytokinesis failure. Noteworthy, the compliance of the substrate *per se* may influence the efficiency of cytokinesis, as reinforcing cell-substrate adhesion in mouse mammary epithelial cells plated on soft substrates, through an autoclustering mutant of β1-integrin, is not sufficient to drive a successful cytokinesis (Rabie et al. 2021). Finally, a role for matricellular proteins on non-epithelial cytokinesis efficiency has also been reported, in both vertebrates and invertebrates (Xu and Vogel 2011). These proteins, as Hemicentin, are not directly required for basement membrane assembly or organization, but provide additional support to it (Zhang et al. 2022). In both *C. elegans* germline and preimplantation mouse embryos, Hemicentin depletion led to cleavage furrow retraction prior to completion and subsequent cytokinetic failure, suggesting a role for this protein on stabilizing the cytokinetic furrow (Xu and Vogel 2011).

Altogether, these findings progressively support the notion that cell division is not strictly a cell-autonomous process, as both the dividing cell and its neighbors contribute for this process. Therefore, understanding the mechanisms underlying the cytokinetic apparatus is crucial, but the contribution of the machinery that regulates the adhesive contacts that the dividing cell establishes with its neighbors and with the extracellular matrix, which ensure tissue integrity, have also to be taken into account in a multicellular context.

Chapter II



Crafting the roadmap

Main goals & Outline of approach

Every day is a new day. It is better to be lucky. But I would rather be exact.
Then when luck comes you are ready.
— Ernest Hemingway, *The Old Man and the Sea*

Epithelial function relies on the correct distribution of cell-cell and cell-matrix adhesion complexes along its apical-basal axis, which defines overall organ shape and ensures the packed architecture of this type of tissue (St Johnston and Ahringer 2010, Miller et al. 2013a). Cytokinesis therefore arises as a challenging event for epithelial architecture, as the dividing cell has to accommodate dramatic shape changes to allow division to occur, while remodeling its connection to the adjacent cells and to the underlying extracellular matrix. Although countless studies in single-cell systems have thoroughly dissected the molecular mechanisms that regulate cytokinesis (nicely reviewed on (Verma et al. 2019) and (Ozugergin and Piekny 2022)), it is anticipated that the tissue context imposes specific requirements for the cytokinetic apparatus.

A number of studies in different animal models and in a variety of epithelial tissues have been tackling the question of how cytokinesis occurs in a multicellular context, and provided important insights to understand several aspects of epithelial cytokinesis *in vivo* (described in detail in Chapter I) (Founounou et al. 2013, Herszterg et al. 2013, Guillot and Lecuit 2013, Morais-de-Sa and Sunkel 2013, Higashi et al. 2016, Firmino et al. 2016, Pinheiro et al. 2017, Hatte et al. 2018, di Pietro et al. 2023, Landino et al. 2023). The general emerging picture is that, rather than being a cell autonomous process, epithelial cytokinesis is based in a multicellular coordination between the intrinsic cytokinetic apparatus of the dividing cell and the adhesive contacts that are established with its neighbors and with the extracellular matrix, to maintain tissue architecture and function. In particular, studies in the *Drosophila* pupal notum (Founounou et al. 2013), *Xenopus* embryo (Hatte et al. 2018) and cultured MDCK cells (Wang et al. 2023) highlight that intercellular adhesion at the apical domain of the epithelia, ensured by the Adherens Junctions (AJs), generally exerts an opposite tension to the pulling forces of the contractile ring, posing a challenge for cytokinesis to occur and impairing its fidelity. Interestingly, an opposite effect of AJs on cytokinesis efficiency was reported in the *Drosophila* spermatocytes and mouse fibroblasts (Goldbach et al. 2010), where AJs-based adhesion seems to foster cytokinesis, hinting that adhesion might differently modulate cytokinesis in a tissue-specific fashion. Additionally, a number of proteins distributed at the lateral domain of the epithelia also mechanically couples neighbouring cells with each other, assembling Tight/Septate Junctions. Nevertheless, and although these proteins might also account to promote or hinder proper

cytokinesis completion, their contribution for the efficiency of epithelial cytokinesis has remained unexplored. Similarly, only one *in vivo* study has addressed the effect of the basal contacts with the extracellular matrix in cytokinesis efficiency (Uroz et al. 2019). This study, in combination with others performed in a variety of cultured cells to assess the impact of the modulation of substratum stiffness in cytokinesis (Reverte et al. 2006, Sambandamoorthy et al. 2015, Dix et al. 2018, Rabie et al. 2021), suggests that a tight regulation of basal cell adhesion to the underlying matrix needs to be ensured for a faithful cytokinesis. Nevertheless, the question of how cell-matrix adhesion affects cytokinesis in an epithelial context still remains poorly addressed.

To understand how epithelial cells accomodate their adhesion to the neighbors and to the underlying matrix during cytokinesis, this work primarily aimed to *dissect the impact of the major regulators of cell-cell and cell-matrix interactions on epithelial cytokinesis efficiency* (Chapter II). To this end, I performed a modifier RNAi screen to test the role of 24 transmembrane adhesion proteins/ECM components present in the *Drosophila* follicular epithelium (Figure 2.1). The follicular epithelium has been widely used as a model to study different cellular processes *in vivo*, such as morphogenesis and cell division (Bastock and St Johnston 2008). It consists of a polarized monolayer of epithelial cells that encloses the germline of the *Drosophila* ovaries, protecting it. Each ovary contains 16-20 ovarioles, each composed by a string of egg chambers in different developmental stages. Individual egg chambers contain 16 germ cells that are encapsulated by the follicular epithelium, and develop throughout 14 stages of oogenesis, giving rise to mature eggs (Bastock and St Johnston 2008, Bilder and Haigo 2012). The follicular epithelium develops in close coordination with the germline, to support its growth (Wu et al. 2008). During stages 2-6 of oogenesis, follicle cells proliferate through constant rounds of cell division. However, from stage 6 onwards, these cells cease their proliferation and switch to endoreplication cycles (Calvi et al. 1998), making the follicular epithelium a particularly interesting model to interfere with epithelial cytokinesis during the proliferative stages of oogenesis and assess the presence of multinucleated cells when follicle cells are no longer dividing. Another remarkable feature of this model is that *Drosophila* egg chambers can be cultured *ex vivo* for at least 4 hours (Morais-de-Sa and Sunkel 2013, Cetera et al. 2014), allowing real-time imaging of cell division, either along its planar axis (imaging an egg chamber at the surface) or along its apical-basal axis (imaging an egg chamber at the midsagittal plane). Additionally, for the purpose of this work, the myriad of RNAi lines that are available in different *Drosophila* stock centers was also particularly valuable, prompting us to test the effect of depleting different adhesion proteins on cytokinesis efficiency.

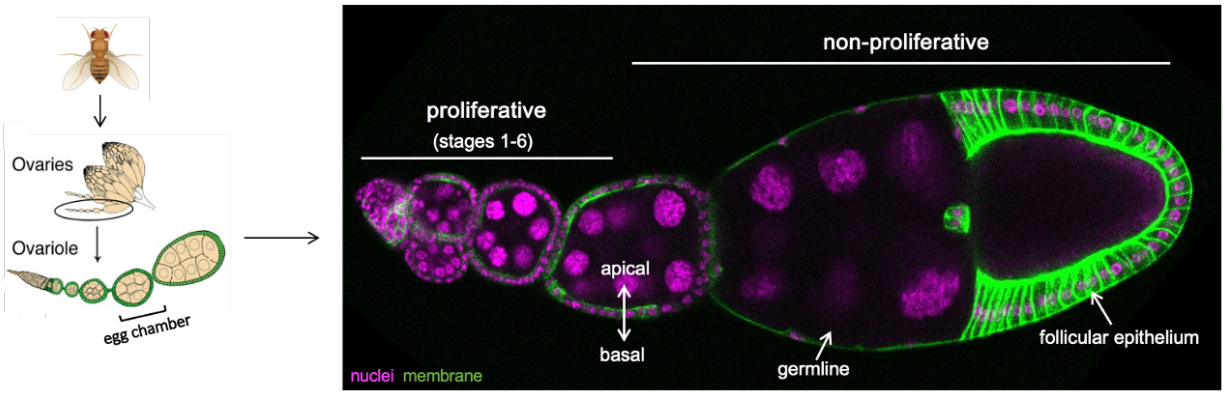


Figure 2. 1. The *Drosophila* follicular epithelium. A *Drosophila* female possesses a pair of ovaries, each of which are composed by 16-20 strings of individual egg chambers in different developmental stages. In each egg chamber, the follicular epithelium (green) envelops the growing germline. The apical side of the follicular epithelium faces the germline, while its basal side contacts the extracellular matrix.

The modifier RNAi screen allowed me to identify major regulators of epithelial cytokinesis efficiency in the *Drosophila* follicular epithelium. I have then focused on Dystroglycan (Dg), a non-integrin basal receptor, as we found that, in contrast to integrins, depletion of Dg increased epithelial cytokinesis defects. I aimed to *dissect the localization and function of Dg during epithelial cytokinesis*, by combining fly genetics, analysis of fixed ovaries and live imaging of both *Drosophila* ovarioles and S2 cells. Using these approaches, I found that Dg acts together with Dystrophin (Dys), a cytoskeleton-binding protein, to ensure epithelial cytokinesis efficiency in two distinct moments. At the beginning of cytokinesis, their function is required to ensure normal ring constriction rate, whereas they also facilitate the maintenance of daughter-cell separation post-constriction. Altogether, this work uncovers an unanticipated role for the Dys-Dg complex on cytokinesis efficiency, and paves the way to understand the molecular mechanisms that coordinate the remodeling of cell-matrix interactions to secure efficient cytokinesis completion in epithelial tissues.

Chapter III



Insights unveiled

Experimental work

The Dystrophin-Dystroglycan complex ensures cytokinesis efficiency in *Drosophila* epithelia

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The manuscript here included was published in EMBO Reports, in January 2025 (volume 26, issue 2, pages 307-328). Together with Eurico Morais de Sá, I designed the project and experiments. I performed most experiments and analyzed most data. Catarina Lopes contributed to finish the RNAi modifier screen for the Extracellular matrix proteins (Figure 3.1G), imaged Dys:sfGFP with Tubulin:RFP (Figure 3.3E) and the tagged Dys constructs in Supplementary Figure 3.5A-C, F, G, and performed the Western Blot in Figure 3.4A. Catarina Lopes also validated the RNAi lines featured in Supplementary Figure 3.S2 and Supplementary Figure 3.S3A, and measured the aspect ratio of basal adhesion proteins (Supplementary Figure 3.3B). Carolina Ramos imaged the S2 cells featured in Supplementary Figure 3.4A and B. Mariana Osswald coded the macro for FIJI for the quantification of the multinucleation defects from the RNAi modifier screen. Hervé Alégot and Graziella Richard (Mirouse lab) generated transgenic lines encoding mutant and GFP-tagged Dys short and long versions. Floris Bosveld (Bellaiche lab) did the experiments in the notum (Supplementary Figure 3.S4C and D). Eurico Morais de Sá collected the images in Figure 3.3C, Figure 3.4B, Supplementary Figure 3.S5D and E. I prepared the figures together with my supervisor and I wrote the figure legends, the Material & Methods section and the first draft of the Introduction. Eurico Morais de Sá wrote the remaining manuscript, which I helped to review.

It is one thing to mortify curiosity, another to conquer it.
— Robert Louis Stevenson, Strange Case of Dr Jekyll and Mr Hyde

The Dystrophin-Dystroglycan complex ensures cytokinesis efficiency in *Drosophila* epithelia

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Abstract

Cytokinesis physically separates daughter cells at the end of cell division. This step is particularly challenging for epithelial cells, which are connected to their neighbors and to the extracellular matrix by transmembrane protein complexes. To systematically evaluate the impact of the cell adhesion machinery on epithelial cytokinesis efficiency, we performed an RNAi-based modifier screen in the Drosophila follicular epithelium. Strikingly, this unveiled adhesion molecules and transmembrane receptors that facilitate cytokinesis completion. Among these is Dystroglycan, which connects the extracellular matrix to the cytoskeleton via Dystrophin. Live imaging revealed that Dystrophin and Dystrogly- can become enriched in the ingressing membrane, below the cytokinetic ring, during and after ring constriction. Using multiple alleles, including Dystrophin isoform-specific mutants, we show that Dystrophin/Dystroglycan localization is linked with unantici- pated roles in regulating cytokinetic ring contraction and in pre- venting membrane regression during the abscission period. Altogether, we provide evidence that, rather than opposing cyto- kinesis completion, the machinery involved in cell–cell and cell–matrix interactions has also evolved functions to ensure cytokinesis efficiency in epithelial tissues.

Keywords

Cell adhesion / Cytokinesis / Dystrophin / Epithelial tissues / Extracellular matrix

Introduction

Cytokinesis, the physical separation of daughter cells, relies on a dramatic remodeling of the cytoskeleton. It begins with the assembly of an actomyosin ring at the cell equator, which drives membrane ingression, and is completed by the abscission of a small intercellular bridge, or midbody, formed at the end of ring constriction. Cytokinesis has long fascinated researchers, leading to the identification of conserved structural components and regulators (Pollard and O'Shaughnessy 2019). These include formins, which drive F-actin polymerization, non-muscle Myosin II, which drives actomyosin contraction, and anillin and septins, which tether the ring to the plasma membrane. Additionally, cytokinetic ring contraction must be adapted to different animal cell types (Cabernard et al. 2010, Jordan et al. 2016, Davies et al. 2018, Ozugergin and Piekny 2022, Paim and FitzHarris 2022). A particularly interesting case is that of epithelia, which must maintain tissue cohesion and the epithelial barrier throughout cell division. Here, dividing cells face challenges posed by cell-cell and cell-extracellular matrix (ECM) interactions (Herszterg et al. 2014, Higashi et al. 2016, Daniel et al. 2018, McKinley et al. 2018, Wang et al. 2018, Osswald and Morais-de-Sa 2019). Understanding these challenges is essential, as cytokinesis failure leads to genome duplication and centrosome amplification, which are key contributors to oncogenesis (Levine et al. 2017, Lens and Medema 2019, Vittoria et al. 2023).

Previous studies of epithelial cytokinesis focused on aspects relevant for animal tissues topology and morphogenesis: E-Cadherin-based adherens junctions (AJ) remodeling, the coordination with *de novo* junction formation and the role of AJ in the direction of ring closure (Founounou et al. 2013, Guillot and Lecuit 2013, Herszterg et al. 2013, Morais-de-Sa and Sunkel 2013, Firmino et al. 2016, Pinheiro et al. 2017, di Pietro et al. 2023). However, few studies have yet examined the impact of the epithelial context on the efficiency of cytokinesis completion. Work in the *Drosophila* pupal notum epithelium has shown that in the absence of septins, AJ promote cytokinesis failure (Founounou et al. 2013). In line with this, increased tension at AJ, due to tight junction defects or excessive neighbor cell contractility, led to cytokinesis failure in *Xenopus* epithelia (Hatte et al. 2018, Landino et al. 2023). Cell-matrix interactions may also interfere with epithelial cytokinesis. Accordingly, in the zebrafish epicardium, focal adhesions that connect the cytokinetic ring to the ECM promote cytokinesis failure when reinforced (Uroz et al. 2019). Thus, while cell adhesion molecules are not inherently essential for cytokinesis, they can potentially interfere with cytokinesis efficiency threatening organismal homeostasis.

The follicular epithelium of the *Drosophila* ovary combines genetic tractability with the power to image epithelial cytokinesis in an adult organ. We designed an RNAi-based genetic modifier screen to test the impact of the major regulators of cell-cell and cell-matrix interactions on cytokinesis efficiency. This uncovered unexpected cytokinetic functions for the Dystrophin-Dystroglycan complex, a transmembrane ECM-cytoskeleton linker with important implications in neuromuscular dystrophies and cancer (Nowak and Davies 2004, Jones et al. 2021, Mirouse 2023).

Results & Discussion

Drosophila modifier screen to test the impact of cell-cell and cell-matrix interactions on cytokinesis efficiency

We performed an RNAi modifier screen in the follicular epithelium to identify modulators of epithelial cytokinesis efficiency. To produce a modifiable phenotype with mild frequency of multinucleated cells, we targeted Anillin, a component of the cytokinetic ring that tethers it, as well as the midbody ring to the plasma membrane (Zhang and Maddox 2010, Kechad et al. 2012). To ensure temporally-controlled and tissue-specific expression of Anillin RNAi, we induced UAS-driven Anillin RNAi with the follicular epithelium specific *tj-GAL4* driver (Olivieri et al. 2010) and blocked the GAL4 transcription factor with its temperature-sensitive repressor Gal80^{ts} (McGuire et al. 2003) until 3 days prior to tissue dissection (Fig 3.1A). We marked the plasma membrane (Myr:GFP) and nuclei (DAPI) to enable automated quantification of the multinucleation ratio (nuclei/cell) in stage 10 egg chambers (Sup Fig 3.S1) as a proxy for cytokinesis failure during earlier proliferative stages. In these conditions, Anillin RNAi led to mild multinucleation, which is suitable to monitor rescue or enhancement of defects.

To validate the strategy to identify modifiers of cytokinesis efficiency, we tested the impact of reinforcing apical cell adhesion. Overexpression of E-Cadherin (ECad) led to a significant increase in multinucleation (Fig 3.1B, C), consistent with earlier findings that AJ challenge cytokinesis (Founounou et al. 2013). We then used RNAi lines to test the impact of the cadherin superfamily members expressed in the follicular epithelium (based on the modENCODE transcriptional profile (Brown et al. 2014)). Controls co-expressing UAS-Anillin RNAi and UAS-mCherry were processed in parallel to each independent experiment,

and multinucleation for each RNAi was compared to its respective control (Δ multinucleation ratio). Intriguingly, ECad depletion had no effect on multinucleation (Fig 3.1D), whereas depleting N-cadherin (NCad) reduced it. This suggests that NCad depletion, but not ECad, effectively reduces apical cell adhesion in follicle cells, which is likely explained by NCad compensating for ECad loss during the proliferative stages ((Tanentzapf et al. 2000) and Sup Fig 3.S3A). Depletion of the apical adhesion molecules Cad74A and Cad99C also reduced the multinucleation ratio (Fig 3.1D). In contrast, depletion of the non-canonical cadherin Fat2, kugelei (kug/fat2), enriched at the basal side (Viktorinova et al. 2009), or of Calsyntenin (Cals), with unknown localization, increased the multinucleation ratio (Fig 3.1D). Altogether, this suggested that in the follicular epithelium cadherin-based adhesion only opposes cytokinesis completion when exerted at the apical level.

A second large family of cell adhesion molecules belongs to the immunoglobulin superfamily (Finegan and Bergstralh 2020). This includes Echinoid (Ed), the functional orthologue of mammalian Nectin, which forms a second adhesion complex at AJ, as well as Neuroglian (Nrg), Fasciclin 2 (Fas2), Fasciclin 3 (Fas3), Lachesin (Lac) and Contactin (Cont), which localize to the lateral membrane in the follicular epithelium prior to septate junction maturation. We did not observe any effect of depleting Fas2, Fas3 and Lac. However, Cont RNAi suppressed multinucleation, whereas Nrg and Ed depletion enhanced it (Fig 3.1E). Nrg interacts heterotypically both with Ed and the single-pass transmembrane protein Neurexin IV (NrxIV) (Islam et al. 2003, Banerjee et al. 2006). NrxIV RNAi also increased multinucleation, suggesting Nrg-NrxIV and Nrg-Ed complexes in the lateral membrane promote epithelial cytokinesis efficiency.

Integrins are heterodimeric receptors formed by α and β subunits that work as physical linkers between the cytoskeleton and the ECM (Kanchanawong and Calderwood 2023). Knockdown of the only subunit in follicle cells, Myospheroid (*mys*/ βPS), or of the α subunits Scab (*scb*/αPS3) and Multiple edematous wings (*mew*/αPS1) did not affect multinucleation (Fig 3.1F). To corroborate this conclusion, we validated integrin protein depletion and its functional impact by reproducing its reported effect on egg chamber elongation (Fig 3.S3B, (Qin et al. 2017)). Interestingly, knocking down the non-integrin ECM receptor Dystroglycan (Dg) increased multinucleation, suggesting it plays a positive role in cytokinesis (Fig 3.1F).

The mechanical properties of the matrix and its ability to signal via focal adhesions modulate cytokinesis efficiency in cell culture (Rabie et al. 2021, Sambandamoorthy et al. 2015). We postulated that ECM composition could impact cytokinesis efficiency and screened the main ECM components in the basement membranes of follicle cells: Collagen IV (*viking*/Col4α2), Laminins, Perlecan (trol) and Nidogen (Diaz-Torres et al. 2021). Depletion of Collagen IV, the major regulator of basement membrane stiffness in the

follicular epithelium (Crest et al. 2017, Topfer et al. 2022), caused previously reported egg chamber elongation defects (Sup Fig 2.S3B), but did not impact multinucleation (Fig 3.1G and Sup Fig 3.S3B). Laminin B2 (LanB2) depletion reduced multinucleation, but RNAi for Laminin A (LanA) or Laminin B1 (LanB1) did not modify the multinucleation ratio, despite of an efficient reduction of protein levels (Sup Fig 3.S2I, J). In contrast, Perlecan depletion increased multinucleation dramatically, suggesting it promotes cytokinesis robustness (Fig 3.1G).

Altogether, the genetic modifier screen indicates that whereas AJ can challenge epithelial cytokinesis efficiency, there are a number of transmembrane proteins involved in cell-cell and cell-matrix interactions that rather promote efficient cytokinesis (screen data overview in Table A1; validation of protein depletion to confirm the main screen results in Sup Fig 3.S2). It is worth noting that depletion of Ed, Nrg, NrxIV, Dg, Kug or Perlecan did not produce multinucleated cells on their own (Sup Fig 3.S3C), which suggests these proteins are dispensable for cytokinesis but contribute to cytokinesis efficiency.

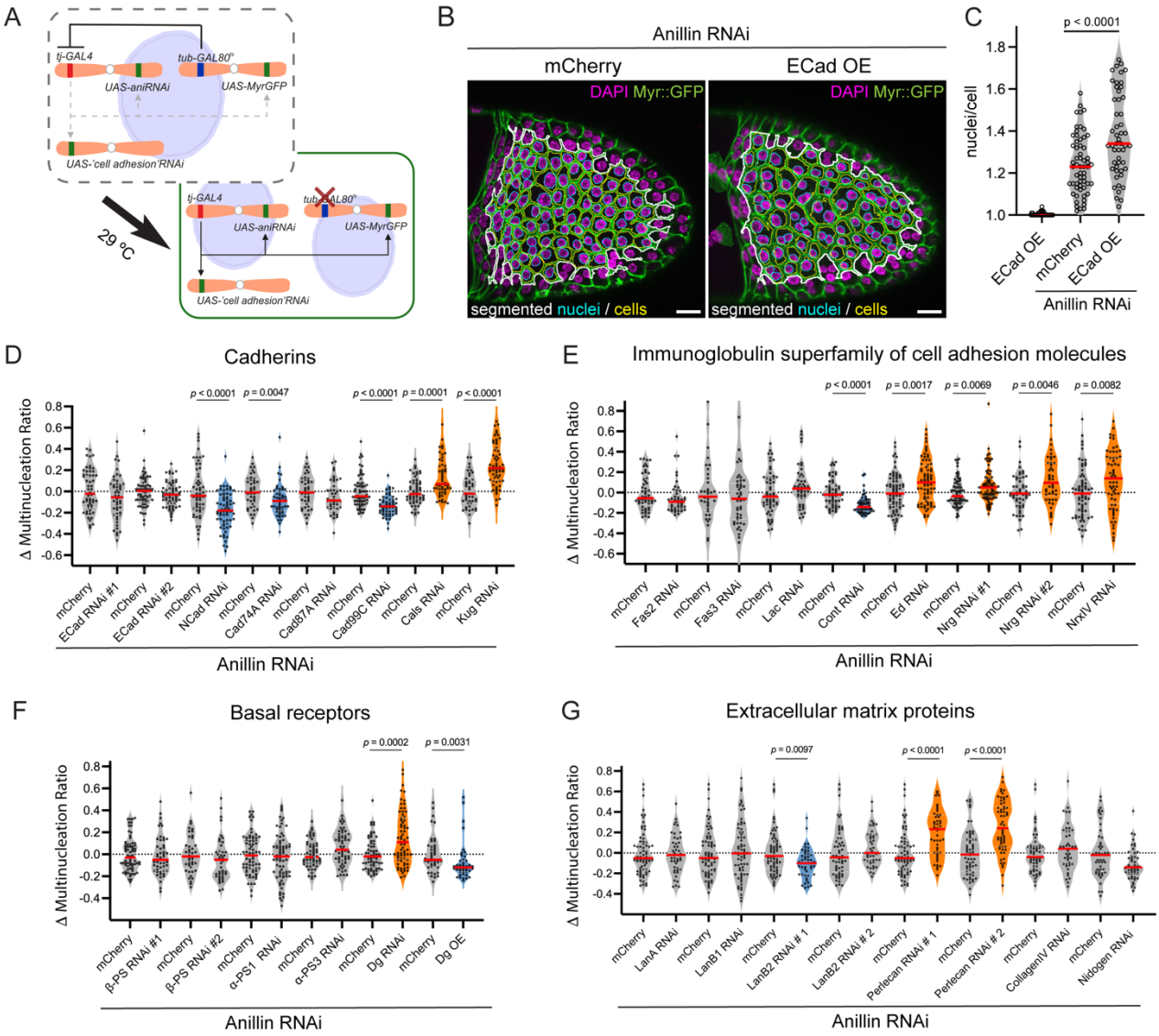


Figure 3. 1. Impact of cell adhesion molecules on epithelial cytokinesis efficiency.

A) Genetic strategy of the RNAi modifier screen in the follicular epithelium.

B) Automated segmentation of cells (yellow) and nuclei (cyan) in the central area (white line) of egg chambers expressing Anillin RNAi simultaneously with a control UAS-mCherry transgene or overexpressing E-cadherin. Myr:GFP marks cell membranes; DAPI labels nuclei. Scale bars: 20 μm.

C) Nuclei/cell in stage 10 egg chambers expressing UAS-Ecad on its own (n = 53) or co-expressing Anillin RNAi with UAS-ECad (n = 52), or with UAS-mCherry (n = 59).

D-G) Modification of the Anillin RNAi multinucleated cell phenotype by co-depletion of proteins from the Cadherin (D) and Immunoglobulin superfamily (E), basal receptors (F) and extracellular matrix components (G). Enhancers (orange) and suppressors (blue) are highlighted. UAS-mCherry was used as a control (Δ Multinucleation Ratio = $(\text{nuclei/cell})_{RNAi}$ - mean $(\text{nuclei/cell})_{mCherry}$).

(C-G) Each dot represents an egg chamber (median in red). *p*-value calculated by non-parametric unpaired Mann-Whitney test.

The Dystrophin-Associated Protein Complex promotes cytokinesis efficiency

The importance of regulating cell-substrate interactions during cytokinesis has been well studied in cell culture (Taneja et al. 2019), but remains unexplored in epithelia. We therefore addressed the role for the basal ECM receptor Dg in cytokinesis efficiency. Dg overexpression reduced cytokinesis defects caused by Anillin RNAi (Fig 3.1F). In addition, Dg RNAi enhanced the multinucleation frequency produced by depletion of Tumbleweed (human RacGAP1, Sup Fig 3.S3D), a centralspindlin complex component that regulates multiple aspects of cytokinesis (White and Glotzer 2012). These findings further support the positive role of Dg in cytokinesis.

Dg is a transmembrane heterodimeric component of the Dystrophin-associated protein complex (DAPC). It links the ECM to the intracellular cytoskeleton by interacting with Dystrophin (Dys), a cytoplasmic actin-binding protein (Fig 3.2A). To investigate if Dys also modulates epithelial cytokinesis, we assessed the genetic interaction between Anillin RNAi and *Dys* mutant alleles. We used a genomic deletion (*Df (3R)Exel6184*, named Dys^{Df}) that spans the entire Dys locus, along with Dys^{E17} (nonsense mutation results in a truncated protein without the Dg-binding domain) or with $Dys^{MI025024}$ (*minos* insertion inserted in the exon that encodes the actin-binding domain; Fig 3.2A). Neither $Dys^{E17/Df}$ nor $Dys^{MI025024/Df}$ egg chambers exhibited a significant number of multinucleated cells, but *Dys* mutant alleles in the background of Anillin depletion led to a dramatic increase in multinucleated cells when compared to Anillin RNAi alone (Fig 3.2B, C). A potential role of Dys on Anillin recruitment to the contractile ring could explain this phenotypic enhancement. However, mRFP:Anillin redistribution during cytokinesis was unaffected by loss of Dys function (Fig 3.2D, E). We conclude that the DAPC promotes cytokinesis efficiency but does not directly contribute for Anillin cytokinetic recruitment.

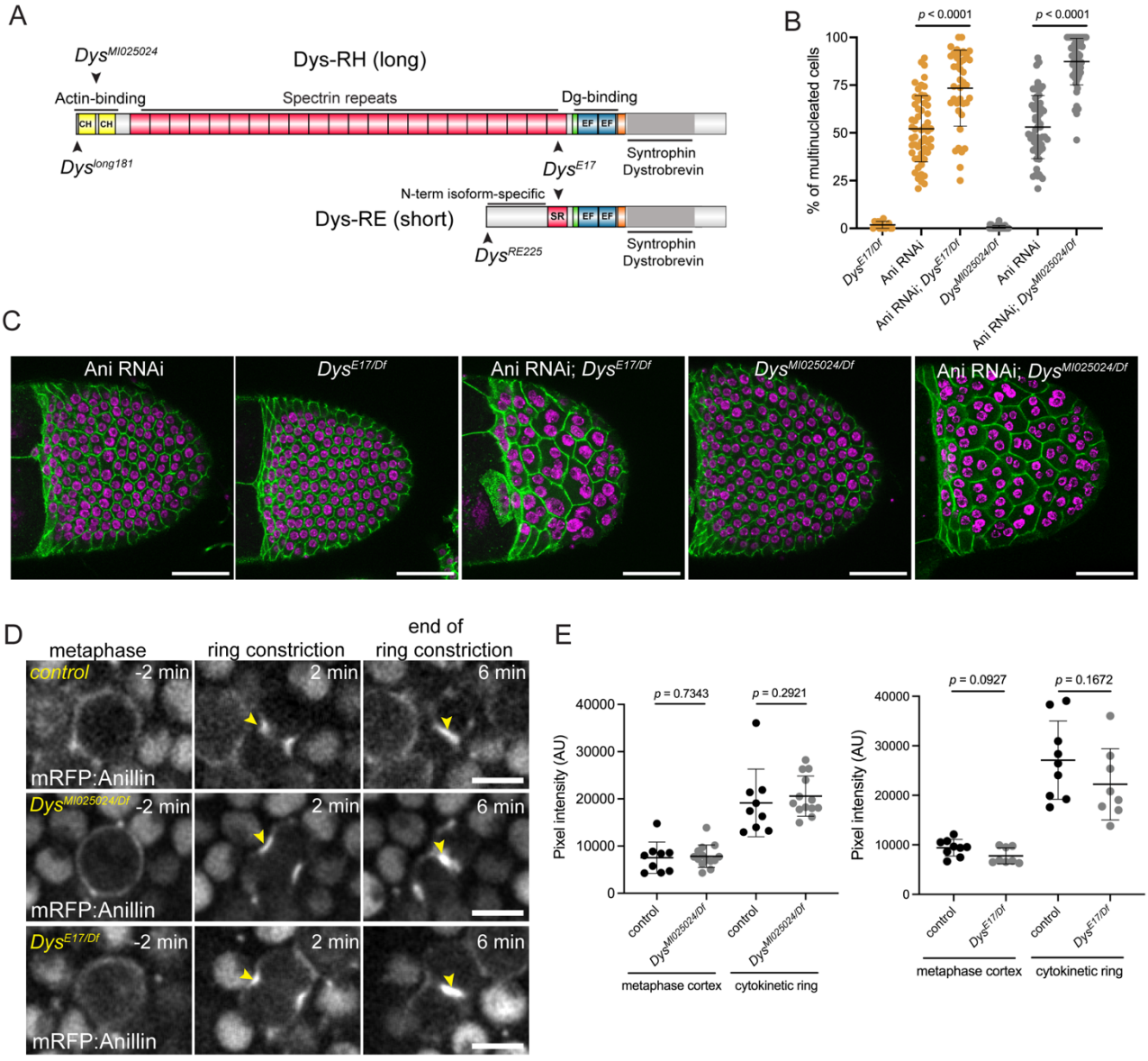


Figure 3. 2. Dystrophin promotes cytokinesis efficiency.

A) Schematic representation of Dys isoforms. The position of the insertion mutant *Dys*^{MI025024} and the truncating mutations *Dys*^{E17} *(*deletes the Dg-binding domain in both isoforms), *Dys*^{long181} (disrupts long isoform) and *Dys*^{RE225} (disrupts short isoform) is shown.

B,C) Frequency of multinucleated cells in egg chambers from Anillin RNAi, *Dys*$^{E17/Df}$ and *Dys*$^{MI025024/Df}$ mutants with unperturbed or sensitized (Anillin RNAi) cytokinesis. (B) Graph shows mean ± SD. Each dot represents an egg chamber. *p*-value calculated by non-parametric unpaired Mann-Whitney test. (C) Surface projections show cell membranes (ECad:GFP) and nuclei (DAPI). Scale bars: 50 μm.

D) Surface projections of dividing control, *Dys*$^{MI025024/Df}$ and *Dys*$^{E17/Df}$ follicle cells expressing mRFP:Anillin. Arrowheads indicate Anillin enrichment during or after ring constriction. Scale bars: 5 μm.

E) Graph shows mean (± SD) pixel intensity of mRFP:Anillin measured in the metaphase cortex (2 minutes before initiation of ring constriction and in the cytokinetic ring (2 minutes after constriction onset). Each dot represents a dividing cell. $n_{control} = 9$; $n_{DysMI025024/Df} = 14$; $n_{control} = 9$; $n_{DysE17/Df} = 8$. *p*-value calculated by non-parametric unpaired Mann-Whitney test.

Dynamic DAPC redistribution during cytokinesis

To investigate how the DAPC functions during epithelial cytokinesis, we examined its distribution in dividing follicle cells expressing UAS-driven Dg:GFP and mRFP:Anillin, which labels first the cytokinetic ring and then the midbody as it is assembled at the end of constriction (Fig 3.3A). Dg:GFP strongly accumulated at the ingressing membrane below the cytokinetic ring while it constricts towards the apical domain until midbody formation. Overexpressed Dg:GFP was not restricted to the basal side in interphase cells, unlike observations by antibody staining (Schneider et al. 2006). To rule out overexpression effects, we examined the mitotic redistribution of endogenously tagged Dg:GFP and Dys:sfGFP (Fig 3.3B, C, Movie 3.S1-S3). These became locally enriched at the basal part of the membrane at the onset of constriction and accumulated at the ingressing membrane below the ring during cytokinesis. After ring constriction, Dys:sfGFP and Dg:GFP accumulated at the new interface between daughter cells, close to the midbody (marked by Sqh:mKate2 upon constriction (Fig 3.3D) or by midbody-associated microtubules labelled with Tubulin:RFP (Fig 3.3E)). The cytokinetic redistribution of these proteins is not common to all cell types, as the main Dys isoforms do not show any cortical accumulation during cytokinesis in *Drosophila* S2 cells (Sup Fig 3.S4B). Nevertheless, the DAPC may have cytokinetic functions beyond epithelia, as Dys and Dg showed cleavage furrow enrichment in rat fibroblasts and non-polarized cell culture models (Higginson et al. 2008, Villarreal-Silva et al. 2011). More importantly, in the *Drosophila* pupal notum epithelium, Dg and Dys also accumulated strongly at the ingressing membranes just below the contractile ring and persisted post-constriction along these membranes below the midbody ring (Sup Fig 3.S4C, D, respectively). Thus, the Dg-Dys complex redistributes dynamically during cytokinesis in different epithelial tissues, in line with a role during epithelial cytokinesis.

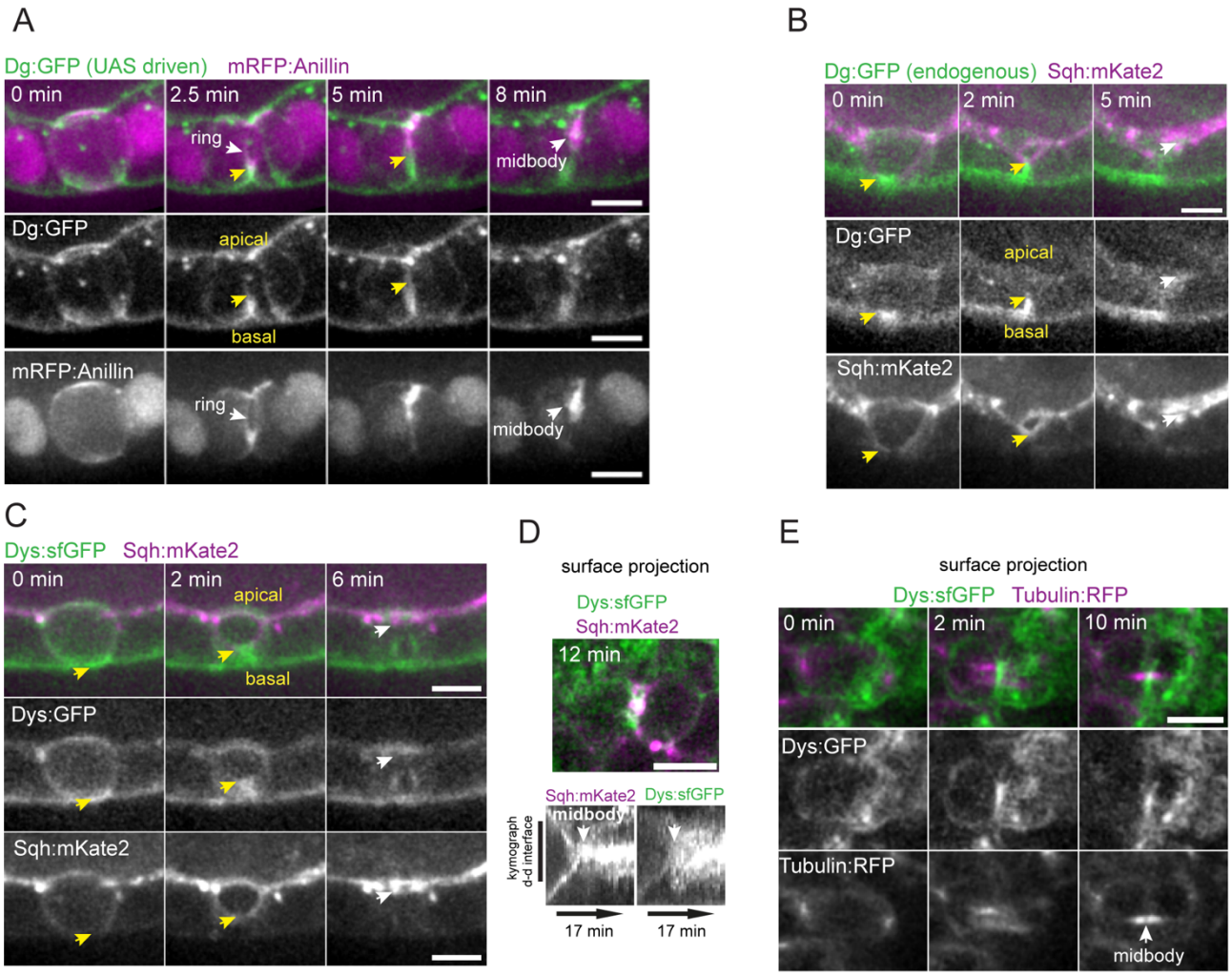


Figure 3. 3. DAPC redistribution during cytokinesis.

A-E) Time-lapse images of follicle cells expressing UAS-driven Dg:GFP (A) or endogenously tagged Dg:GFP (B) and Dys:sfGFP (C-E). Anillin:mRFP (A), Sqh:mKate2 (B-D) or Tub:RFP (E) label the contractile ring and the midbody. Apical-basal views (A-C) show that (A) UAS-driven Dg:GFP becomes enriched in the ingressing furrow (yellow arrows). Endogenous Dg:GFP (B) and Dys:sfGFP (C) accumulate at the basal part of the ingressing membrane from the start of ring constriction (yellow arrows) and become enriched close to the midbody (white arrows). (D) A line kymograph (bottom) along the daughter-daughter (d-d) interface from a surface projection (top) shows that upon midbody formation (arrow), Dys:sfGFP maintains an accumulation at the new d-d interface. (E) Surface projection showing the accumulation of Dys:sfGFP near the midbody associated microtubules (arrow). Scale bars: 5 μm.

Dys encodes several isoforms in both humans and flies. All of them bear a conserved C-term region with the Dg binding domain (Fig 3.2A), but the shorter isoforms lack actin-binding domains (N-term actinin like domain and a second actin-binding domain within a rod domain). Translating ribosome affinity purification experiments show that only one long (Dys-RH) and one short isoform (Dys-RE) are expressed in the follicular epithelium (Vachias et al. 2023). To investigate whether Dys redistribution and function during

cytokinesis rely on direct binding to the actin cytoskeleton, we generated by CRISPR/Cas9 isoform-specific indel mutant alleles in an untagged *Dys* genomic locus (*Dys*^{long181}, i.e. mutating all long isoforms including RH, and *Dys*^{RE225} mutating RE) and in a sfGFP-tagged one. Naming of *Dys*^{long181}:sfGFP and *Dys*^{RE225}:sfGFP was simplified by indicating the tagged isoforms: *Dys*^{short}:sfGFP and *Dys*^{long}:sfGFP, respectively. Western blot confirmed the long isoform's absence in *Dys*^{short}:sfGFP and the short isoform's absence in *Dys*^{long}:sfGFP (Fig 3.4A). Both isoforms showed strong basal enrichment in the proliferative follicular epithelium, even if *Dys*^{short}:sfGFP exhibited a more uniform distribution in the basal cortex that contrasts with *Dys*^{long}:sfGFP planar polarization (Sup Fig 3.S5A, B). More importantly, both isoforms accumulated below the ingressing furrow during constriction, and at the new interface post-constriction, suggesting both contribute to epithelial cytokinesis (Fig 3.4B and Movie 3.S4). Accordingly, *Dys* disruption with either isoform-specific untagged mutant alleles (*Dys*^{long181} and *Dys*^{RE225}) dramatically increased multinucleation upon Anillin depletion (Fig 3.4C, D). Thus, both Dys isoforms, regardless of the actin-binding domains, localize at the ingression furrow and contribute to cytokinesis efficiency.

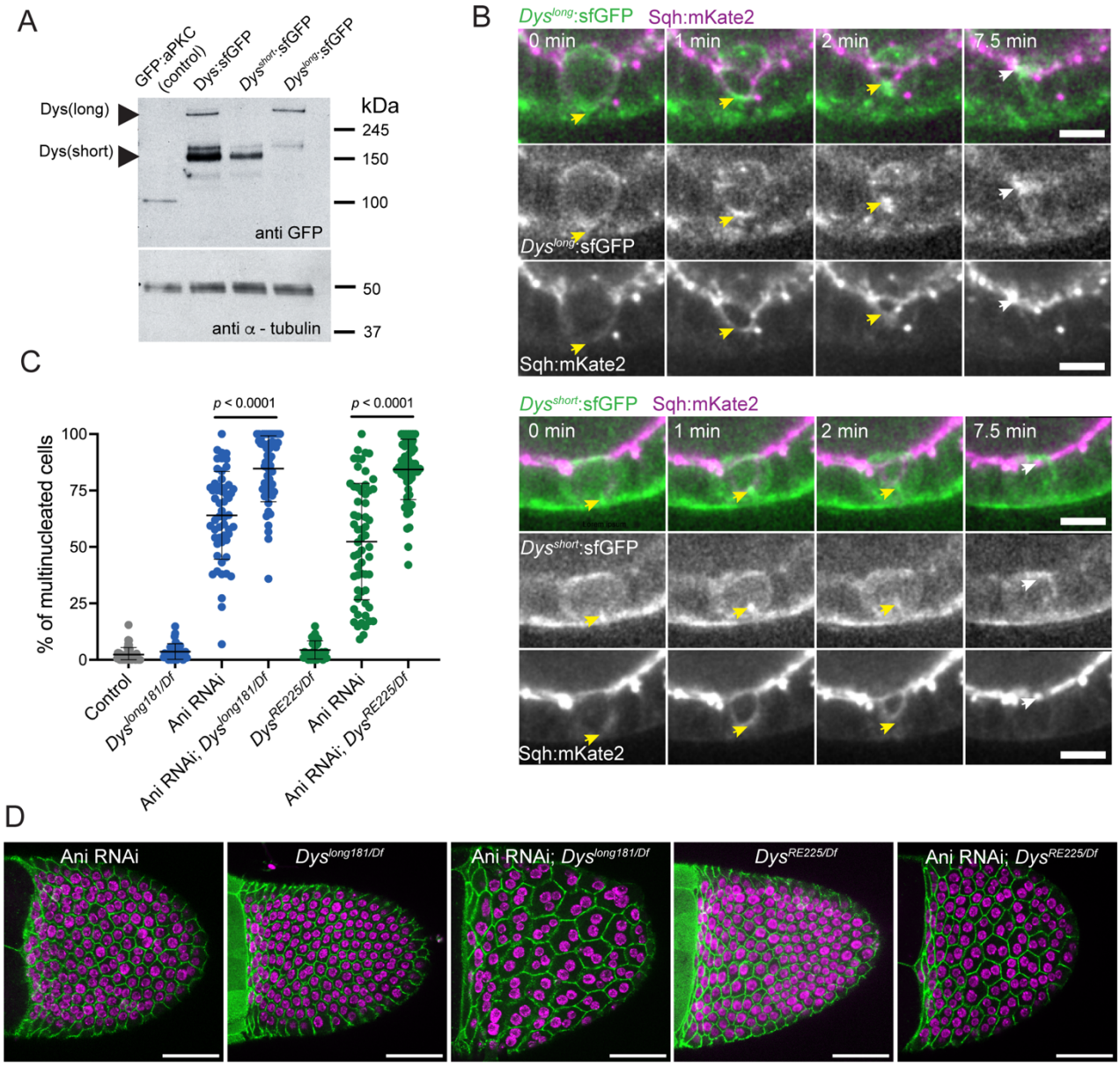


Figure 3. 4. Short and long Dys isoforms contribute for cytokinesis efficiency.

A) Western blots from ovaries of GFP:aPKC (GFP detection control), Dys:sfGFP; *Dys*^{short}:sfGFP and *Dys*^{long}:sfGFP flies probed with anti-GFP or anti-α-tubulin.

B) Endogenously tagged *Dys*^{long}:sfGFP (top) and *Dys*^{short}:sfGFP (bottom) accumulate at the basal ingressing membrane from the start of ring (labelled with Sqh:mKate2) constriction (yellow arrows) and become enriched close to the apically positioned midbody (white arrows).

C-D) Frequency of multinucleated cells in control, *Dys*$^{long181/Df}$ and *Dys*$^{RE225/Df}$ egg chambers with unperturbed or sensitized (Anillin RNAi) cytokinesis. (C) Graph shows mean ± SD. Each dot represents an egg chamber. *p*-value calculated by non-parametric unpaired Mann-Whitney test. (D) Surface projections show cell membranes (ECad:GFP) and nuclei (DAPI). Scale bars: 50 μm.

To identify protein domains involved in Dys enrichment at the ingressing furrow, we generated UAS-driven *Dys*^{short}:GFP truncations that retained the ability to bind Dg, but lacked evolutionarily conserved domains that are common to all isoforms: the N-term spectrin repeat (*Dys*$^{short\Delta SR24}$) or a motif in the C-terminal part that interacts with cytoplasmic adaptors, Syntrophins and Dystrobrevin (*Dys*$^{short\Delta SD}$). *Dys*$^{short\Delta SR24}$ partially mislocalized to the apical side and nuclei (Sup Fig 3.S5C), but it was still enriched at the ingressing membranes during ring constriction and upon midbody formation (Sup Fig 3.S5E). In contrast, *Dys*$^{short\Delta SD}$ had reduced basal enrichment and did not accumulate in the ingressing membranes during cytokinesis (Sup Fig 3.S5C, F). This effect requires the removal of the syntrophins/dystrobrevin binding sites, as a shorter deletion of the most C-terminal region (aa956-1051, *Dys*$^{short\Delta CT}$) did not affect Dys cytokinetic redistribution (Sup Fig 3.S5G). Syntrophins and Dystrobrevin function in epithelial tissues has yet to be understood. Our results show that the cortical and cytokinetic localization of Dys relies on motifs that interact with these scaffolding proteins, a region containing an alpha-helix and adjacent coiled-coil domains (Sadoulet-Puccio et al. 1997, Newey et al. 2000).

Multiple roles of the DAPC complex on epithelial cytokinesis efficiency

Anillin is required for the maturation of the midbody ring, that stabilizes the intercellular bridges during abscission. Accordingly, Anillin RNAi in *Drosophila* S2 cells leads to reopening of the furrow a significant period of time after ring constriction (Kechad et al. 2012). To further understand the genetic interaction between Anillin RNAi and *Dys* in cytokinesis, we monitored follicle cell cytokinesis by live imaging (Fig 3.5A). We could barely detect cytokinesis failure upon Anillin depletion on its own, but additional disruption of *Dys* function increased the frequency of cytokinesis failure (Fig 3.5C). Interestingly, almost all cells reached a semi-stable state after ring closure, but the new interface formed between the daughter cells regressed as late as 30 minutes post-ring constriction (Fig 3.5B, C and Movie 3.S5), suggesting the DAPC contributes to the efficiency of the last stage of cytokinesis.

Since Dys and Dg spatially redistribute in the furrowing membrane during cytokinesis, we also postulated that the DAPC regulates early cytokinetic furrowing. We therefore monitored cytokinetic ring constriction in *Dg* (*Dg*$^{043/086}$) and *Dys* (*Dys*$^{E17/Df}$) egg chambers. *Dg* and *Dys* disruption significantly delayed ring constriction (Fig 3.5D, E and Movie 3.S6) from the beginning of furrowing, as the ring diameter at the onset of the phase of constant ring constriction (1.5 min) is already significantly different. To specifically

examine the impact of long and short *Dys* isoforms, we also imaged egg chambers mutant for each isoform ($Dys^{RE225/Df}$ or $Dys^{long181/Df}$). Both mutants impaired cytokinetic closure (Fig 3.5F). In addition, *Dys* isoforms and *Dg* also contributed to normal ring constriction rate during the phase of constant ring constriction (Fig 3.5G). Hence, we conclude the DAPC ensures normal ring constriction in the follicular epithelium.

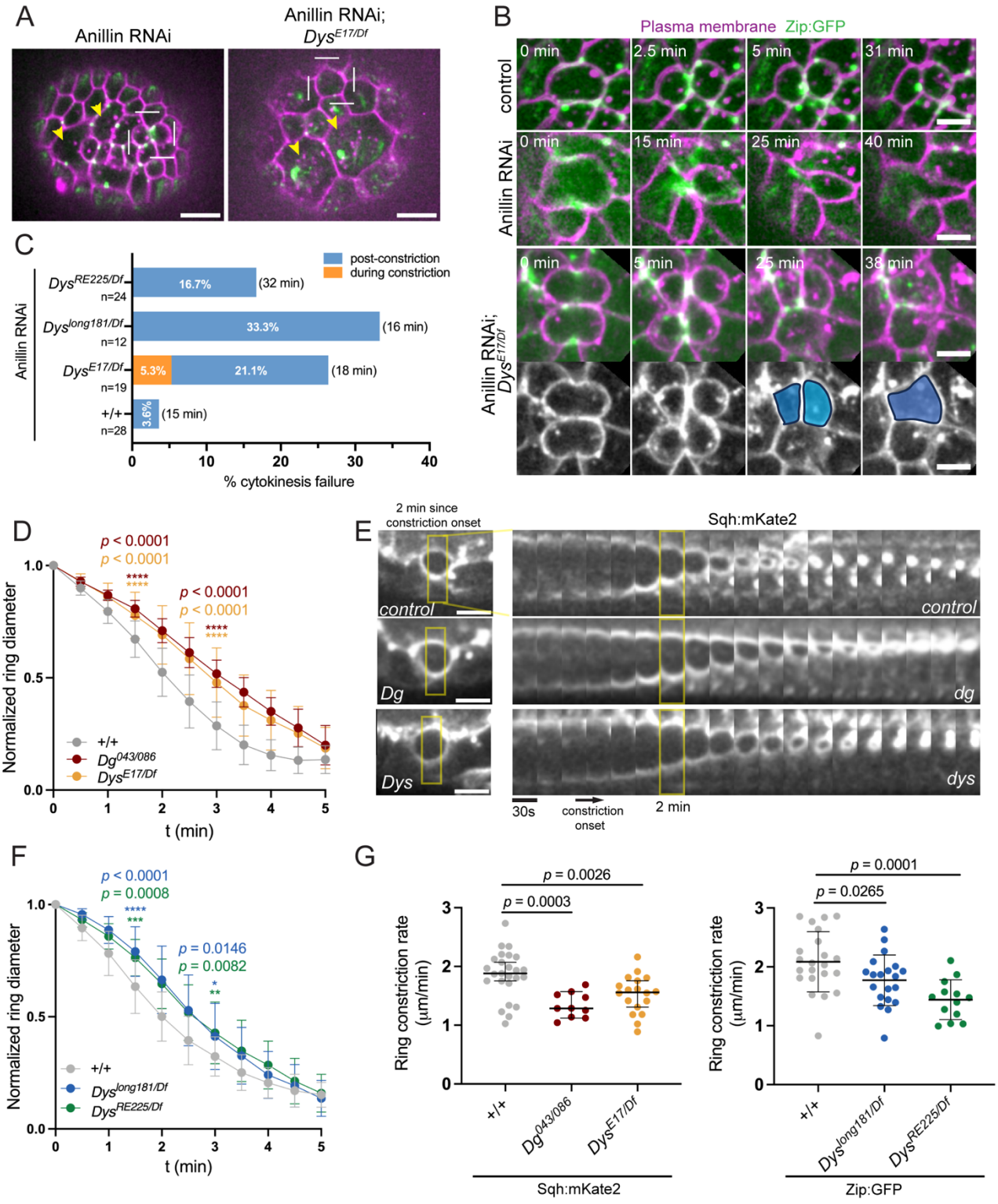


Figure 3. 5. Roles of the DAPC in epithelial cytokinesis efficiency.

A) Surface view of Anillin RNAi and *Dys*$^{E17/Df}$, Anillin RNAi egg chambers expressing Zip:GFP (green) with stained membranes (CellMask, magenta). Note large cells produced by cytokinesis failure (yellow arrows). Live cytokinesis failure was quantified (B, C) only in cells without previous cytokinesis failure (white box). Scale bars: 10 μm.

B) Time-lapse images (surface view) of Zip:GFP egg chambers with stained membranes captured from control, Anillin RNAi and $Dys^{E17/Df}$, Anillin RNAi. *Dys* disruption in Anillin-depleted follicle cells leads to cytokinesis failure due to membrane regression after ring closure. Scale bars: 5 μm.

C) Frequency of cytokinesis failure in Anillin-depleted egg chambers on its own (+/+) or with concomitant *Dys* disruption (allelic combinations indicated). For post-constriction failure, average time elapsed from ring constriction end until membrane regression is shown.

D-G) Contractile ring diameter was measured during cytokinesis in control (n=25), $Dg^{043/086}$ (n=10) and $Dys^{E17/Df}$ (n=18) egg chambers expressing Sqh:mKate2 (D, E, G left) and in control (n=24), $Dys^{long181/Df}$ (n=20) and $Dys^{RE225/Df}$ (n=13) egg chambers expressing Zip:GFP (F, G right). (E) Stills show 2 minutes since constriction onset (left) and a close-up (region defined within the yellow box) of cytokinesis progression in control, $Dg^{043/086}$ and $Dys^{E17/Df}$ mutant (right) from a time-lapse of ring constriction viewed along the apical-basal axis. Scale bars: 5 μm. Graphs in (D, F) show mean ± SD through time. Ring diameters were normalized to frame before constriction onset (t = 0). *p*-values correspond to the difference in ring diameter between $Dg^{043/086}$ (D red), $Dys^{E17/Df}$ (D yellow), $Dys^{long181/Df}$ (F blue) or $Dys^{RE225/Df}$ (F green) and the respective controls at t=1.5 and t=3 min. Graphs in (G) show ring constriction rate during the constant constriction phase (mean ± SD). *p*-value calculated using non-parametric unpaired Mann-Whitney test.

In conclusion, this study provides new insights into the mechanisms that ensure epithelial cytokinesis efficiency, and uncovers new cytokinetic functions for the DAPC in *Drosophila* tissues. Previous *in vivo* studies have shown that cell-cell and cell-matrix adhesion could potentiate cytokinesis failure and delay ring constriction (Founounou et al. 2013, Higashi et al. 2016, Hatte et al. 2018, Uroz et al. 2019). Our RNAi modifier screen now identifies a number of cell-cell and cell-matrix interaction mediators that promote, rather than oppose, cytokinesis completion. These include Perlecan, a Dg ligand (Talts et al. 1999), Fat2, a genetic interactor of Dys (Cerqueira Campos et al. 2020), and cell-cell adhesion molecules from the immunoglobulin superfamily. Building on this screen, we show that the DAPC, through long and short *Dys* isoforms, functions during cytokinetic furrowing and contributes to efficient daughter cell separation after ring constriction.

How the DAPC contributes to each step of cytokinesis remains an important open question. We show that Dg and Dys accumulate below the contractile ring during furrowing, which may contribute to remodel cell-matrix interactions to facilitate basal plasma membrane invagination. Indeed, the DAPC contributes to the dynamic organization of the basement membrane and underlying cytoskeleton in different morphogenetic processes (Buisson et al. 2014, Cerqueira Campos et al. 2020, Villedieu et al. 2023). DAPC enrichment in late cytokinesis near the midbody ring suggests it could also regulate the abscission machinery. Interestingly, Dys binds microtubules and pauses their polymerization (Prins et al. 2009, Belanto et al. 2014), so it could be involved in the disassembly of midbody-associated microtubules. Alternatively, the DAPC could facilitate cytokinesis completion by linking the cell cytoskeleton to the ECM to enhance cell-matrix

adhesion. This would be consistent with cell culture studies suggesting that maintaining adhesion to the substrate facilitates cytokinesis in cells with a compromised actomyosin ring (Dix et al. 2018). Although Dys cytokinetic function require isoforms lacking known actin-binding domains, adaptor proteins that interact with conserved Dys C-terminal motifs could link the DAPC to the cytoskeleton. Future work can focus on these hypotheses to elucidate how the DAPC coordinates the intracellular cytoskeleton and the extracellular ECM to promote cytokinesis fidelity in different tissues.

Methods

The list of reagents used in this study is found in **Table 3.1**.

Table 3. 1. Reagents and Tools Table.

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Experimental Models		
Drosophila (*D. melanogaster*)	List of transgenic lines and alleles is shown in Table 3.2/Table A1	N/A
S2 cells (*D. melanogaster*)	*Drosophila* Genomics Resource Center	FlyBase: FBtc0000179
Recombinant DNA		
DysEcDNA	*Drosophila* GOLD cDNA collection	RE11449
pHWG	*Drosophila* Genomics Resource Center	Gateway Collection
pH-DysE:GFP	This study	N/A
pCFD6	(Port and Bullock 2016)	Cat# AddGene-73915
pUASz	(DeLuca and Spradling 2018)	Cat# DGRC-1431

pUAS-Dys^{short}	This study	N/A
pUAS-Dys$^{short\Delta SD}$	This study	N/A
pUAS-Dys$^{short\Delta SR24}$	This study	N/A
pUAS-Dys$^{short\Delta CT}$	This study	N/A
pUAS-GFP:DysRH	This study	N/A
pActinGal4	(Potter et al. 2010)	Cat# AddGene 24344
Antibodies		
rat anti-N-cadherin	DSHB	Cat# DN-Ex #8
mouse anti-Fas2	DSHB	Cat# 1D4
mouse anti-Fas3	DSHB	Cat# 7G10
mouse anti-βPS-integrin	DSHB	Cat# CF.6G11-s
rabbit anti-Perlecan	González-Reyes lab, (Diaz-Torres et al. 2021)	N/A
mouse anti-Armadillo	DSHB	Cat# N2.7A1
goat anti-rat Alexa 568	Invitrogen	Cat# A11077
goat anti-mouse Alexa 488	Invitrogen	Cat# A11029
goat anti-rabbit Alexa 488	Invitrogen	Cat# A11008
goat anti-mouse Alexa 568	Invitrogen	Cat# A11031
rabbit anti-GFP	i3S core facility	N/A
goat anti-rabbit HRP	Jackson ImmunoResearch	Cat# 111-035-003
Oligonucleotides and other sequence-based reagents		
gRNAs	This study	Methods (Section “Molecular biology and transgenesis”)
Chemicals, Enzymes and other reagents		
Phalloidin-TRITC	Merck	Cat# P1951
Vectashield Mounting Medium with DAPI	Vector Laboratories	Cat# H-1200-10
CellMask Orange Plasma Membrane Stain	Thermo Fisher	Cat# C10045
Poly-D-lysine	Sigma-Aldrich	Cat# P7405

Schneider's insect medium	Sigma-Aldrich	Cat# S0146
Insulin solution from bovine pancreas	Sigma-Aldrich	Cat# I0516
Fetal Bovine Serum (FBS), heat inactivated	Thermo Fisher	Cat# 10500-064
Paraformaldehyde 20%	Electron Microscopy Sciences	Cat# 15713
Tween 20	Sigma-Aldrich	Cat# P9416
Effectene Transfection Reagen	QIAGEN	Cat# 301425
NEB Builder HiFi DNA Assembly Cloning Kit	New England Biolabs	Cat# E5520S
LR Clonase II	Thermo Fisher	Cat# 11791020
Software		
FIJI	(Schindelin et al. 2012)	N/A
GraphPad Prism 9	GraphPad Software (La Jolla, CA, USA)	N/A
Other		
Laser Scanning Confocal Microscope TCS SP5 II	Leica Microsystems	N/A
Spinning disk confocal microscope Andor Revolution XD	Andor Technology	N/A

Drosophila lines and maintenance

Fly stocks and genetic crosses were raised in standard fly media (cornmeal/agar/molasses/yeast) at 18ºC or 25ºC, with 60% humidity and 12h/12h dark light cycle, unless stated otherwise. The details of the fly lines used throughout this study are listed in Table 3.2. A detailed list of the fly genotypes for each experiment can be found in Table 3.3. We used *traffic jam-Gal4* to drive the expression of UAS constructs in the *Drosophila* follicular epithelium.

Table 3. 2. Mutant alleles and transgenes.

Mutant allele or transgene	Citation	Stock number or lab of origin
Dys^{Df}	(Christoforou et al. 2008)	BDSC #7663
Dys^{E17}	(Christoforou et al. 2008)	BDSC #63047
Dg^{086}	(Christoforou et al. 2008)	BDSC #63049
Dg^{043}	(Christoforou et al. 2008)	BDSC #63048
Dys^{MI025024}	(Bellen et al. 2011)	BDSC #24182
Dys^{long181}	this study	Vincent Mirouse
Dys^{RE225}	this study	Vincent Mirouse
Dys^{short}:sfGFP	this study	Vincent Mirouse
Dys^{long}:sfGFP	this study	Vincent Mirouse
Dys:sfGFP	(Dennis et al. 2024)	Vincent Mirouse
UAS-*Dys*^{short}:GFP	this study	Vincent Mirouse
UAS-*Dys*$^{short\Delta SR24}$:GFP	this study	Vincent Mirouse
UAS-*Dys*$^{short\Delta SD}$:GFP	this study	Vincent Mirouse
UAS-*Dys*$^{short\Delta CT}$:GFP	this study	Vincent Mirouse
Dg:GFP	(Villedieu et al. 2023)	Yohanns Bellaiche
UAS-Dg:GFP	(Bogdanik et al. 2008)	Marie-Laure Parmentier
GFP:aPKC	(Chen et al. 2018)	Daniel St Johnston
Zip:GFP	(Lowe et al. 2014)	DGGR #115082
ECad:GFP	(Pinheiro et al. 2017)	Yohanns Bellaiche
Sqh:3xmKate2	(Pinheiro et al. 2017)	Yohanns Bellaiche
Ed:GFP	(Lowe et al. 2014)	DGGR # 115114
Collagen IV:GFP	(Morin et al. 2001)	BDSC: #98343
LanB1:GFP	(Sarov et al. 2016)	VDRC #318180
LanA:GFP	(Sarov et al. 2016)	VDRC: #318155
Nrg:GFP	(Morin et al. 2001)	BDSC: #6844
Ubq-Tub:RFP	(Dobbelaere et al. 2008)	Jordan Raff
tub-Gal80^{ts}	(McGuire et al. 2003)	BDSC #7018
tj-Gal4	(Olivieri et al. 2010)	DGGR #104055
UAS-mRFP:Anillin	Donated to BDSC by Andy Wilde	BDSC #52220
UAS-ECad	(Pacquelet and Rorth 2005)	BDSC #58494
UAS-AnillinRNAi$^{(KK107556)}$	www.vdrc.at	VDRC #104674
UAS-TumRNAi$^{(TRIP.JF01639)}$	(Perkins et al. 2015)	BDSC # 28982
UAS-Myr:GFP	(Pfeiffer et al. 2010)	BDSC #32197
UAS-mCherry	http://www.flyrnai.org/TRiP-HOME.html	BDSC #35787
UAS-mCherry RNAi	http://www.flyrnai.org/TRiP-HOME.html	BDSC #35785

Table 3. 3. List of *Drosophila* genotypes.

Figure	Genotype
3.1B, C	*tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-mCherry *tj-Gal4*, UAS-Anillin RNAi/UAS-ECad; UAS-Myr:GFP, tub-Gal80^{ts}/+ *tj-Gal4*/UAS-ECad; UAS-Myr:GFP, tub-Gal80^{ts}/+
3.1D, E, F, G	*tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-mCherry *tj-Gal4*, UAS-Anillin RNAi/UAS-CAM or UAS-ECM-protein RNAi; UAS-Myr:GFP, tub-Gal80^{ts}/+ or *tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-CAM or UAS-ECM-protein RNAi
3.2B, C	*tj-Gal4*, ECad:GFP/+; *Dys$^{E17/Df}$* *tj-Gal4*, ECad:GFP/UAS-Anillin RNAi *tj-Gal4*, ECad:GFP/UAS-Anillin RNAi; *Dys$^{E17/Df}$* *tj-Gal4*, ECad:GFP/+; *Dys$^{MI025024/Df}$* *tj-Gal4*, ECad:GFP/UAS-Anillin RNAi; *Dys$^{MI025024/Df}$*
3.2D, E	*tj-Gal4*/UAS-mRFP:Anillin *tj-Gal4*/UAS-mRFP:Anillin; *Dys$^{MI025024/Df}$* *tj-Gal4*/UAS-mRFP:Anillin; *Dys$^{E17/Df}$*
3.3A	*tj-Gal4*, UAS-Dg:GFP/UASp-mRFP:Anillin
3.3B	Dg:GFP/Sqh:3xmKate2
3.3C, D	Sqh:3xmKate2/+; Dys:sfGFP
3.3E	Tub:RFP/+; Dys:sfGFP
3.4A	GFP:aPKC/CyO IF/CyO; Dys:sfGFP *Dys^{short}*:sfGFP/TM3 *Dys^{long}*:sfGFP/TM3
3.4B	Sqh:3xmKate2/+; *Dys^{short}*:sfGFP Sqh:3xmKate2/+; *Dys^{long}*:sfGFP
3.4C, D	*tj-Gal4*, ECad:GFP/+; MKRS/TM6 *tj-Gal4*, ECad:GFP/+; *Dys$^{long181/Df}$* *tj-Gal4*, ECad:GFP/UAS-Anillin RNAi *tj-Gal4*, ECad:GFP/UAS-Anillin RNAi; *Dys$^{long181/Df}$* *tj-Gal4*, ECad:GFP/+; *Dys$^{RE225/Df}$* *tj-Gal4*, ECad:GFP/UAS-Anillin RNAi; *Dys$^{RE225/Df}$*
3.5A	*tj-Gal4*, Zip:GFP/UAS-Anillin RNAi *tj-Gal4*, Zip:GFP/UAS-Anillin RNAi; *Dys$^{E17/Df}$*

3.5B	*tj-Gal4*, Zip:GFP/+ *tj-Gal4*, Zip:GFP/UAS-Anillin RNAi *tj-Gal4*, Zip:GFP/UAS-Anillin RNAi; $Dys^{E17/Df}$
3.5C	*tj-Gal4*, Zip:GFP/UAS-Anillin RNAi *tj-Gal4*, Zip:GFP/UAS-Anillin RNAi; $Dys^{E17/Df}$ *tj-Gal4*, Zip:GFP/UAS-Anillin RNAi; $Dys^{long181/Df}$ *tj-Gal4*, Zip:GFP/UAS-Anillin RNAi; $Dys^{RE225/Df}$
3.5D, E	*tj-Gal4*, Sqh:3xmKate2/+ $Dg^{086/043}$; Sqh:3xmKate2/+ Sqh:3xmKate2/+; $Dys^{E17/Df}$
3.5F	*tj-Gal4*, Zip:GFP/+ *tj-Gal4*, Zip:GFP/+; $Dys^{long181/Df}$ *tj-Gal4*, Zip:GFP/+; $Dys^{R225/Df}$
3.5G	*tj-Gal4*, Sqh:3xmKate2/+ $Dg^{043/086}$; Sqh:3xmKate2/+ Sqh:3xmKate2/+; $Dys^{E17/Df}$ *tj-Gal4*, Zip:GFP/+ *tj-Gal4*, Zip:GFP/+; $Dys^{long181/Df}$ *tj-Gal4*, Zip:GFP/+; $Dys^{RE225/Df}$
3.S1A, B	*tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP, tub-$Gal80^{ts}$/UAS-mCherry
3.S2A	*tj-Gal4*, ECad:GFP/+; UAS-mCherry RNAi/+ *tj-Gal4*, ECad:GFP/+; UAS-ECad RNAi/+
3.S2B	*tj-Gal4*/+; UAS-mCherry/+ *tj-Gal4*/+; UAS-NCad RNAi/+
3.S2C	*tj-Gal4*/+; tub-$Gal80^{ts}$/UAS-mCherry RNAi *tj-Gal4*/+; tub-$Gal80^{ts}$/UAS-Fas2 RNAi
3.S2D	*tj-Gal4*/+; tub-$Gal80^{ts}$/UAS-mCherry RNAi *tj-Gal4*/+; tub-$Gal80^{ts}$/UAS-Fas3 RNAi
3.S2E	*tj-Gal4*, Ed:GFP/+; UAS-mCherry RNAi *tj-Gal4*, Ed:GFP/UAS-Ed RNAi; +/+
3.S2F	Nrg:GFP/+; *tj-Gal4*/+ Nrg:GFP/+; *tj-Gal4*/+; UAS-Nrg RNAi/+
3.S2G	*tj-Gal4*/+; UAS-mCherry/+ *tj-Gal4*/+; UAS-*βPS-integrin* RNAi/+

3.S2H	*tj-Gal4*/Dg:GFP; UAS-mCherry RNAi/+ *tj-Gal4*/Dg:GFP; UAS-Dg RNAi/+
3.S2I	*tj-Gal4*/+; LanA:GFP/UAS-mCherry *tj-Gal4*/+; LanA:GFP/UAS-LanA RNAi
3.S2J	*tj-Gal4*/+; LanB1:GFP/UAS-mCherry RNAi *tj-Gal4*/+; LanB1:GFP/LanB1 RNAi
3.S2K	*tj-Gal4*/+; UAS-mCherry/+ *tj-Gal4*/+; UAS-Perlecan RNAi/+
3.S2L	*tj-Gal4*/Collagen IV:GFP; tub-Gal80^{ts}/+ *tj-Gal4*/Collagen IV:GFP; tub-Gal80^{ts}/UAS-Collagen IV RNAi
3.S3A	*tj-Gal4*, ECad:GFP/+; UAS-mCherry RNAi/+ *tj-Gal4*, ECad:GFP/+; UAS-ECad RNAi/+
3.S3B	*tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP tub-Gal80^{ts}/UAS-mCherry *tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP tub-Gal80^{ts}/UAS-αPS1 RNAi #1 *tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-βPS RNAi #1 *tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-βPS RNAi #2 *tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-Dg RNAi *tj-Gal4*, UAS-Anillin RNAi/UAS-Kug RNAi; UAS-Myr:GFP, tub-Gal80^{ts}/+ *tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-Collagen IV RNAi *tj-Gal4*, UAS-Anillin RNAi/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-Perlecan RNAi
3.S3C	*tj-Gal4*/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-mCherry *tj-Gal4*/UAS-Kug RNAi; UAS-Myr:GFP, tub-Gal80^{ts}/+ *tj-Gal4*/UAS-Ed RNAi; UAS-Myr:GFP, tub-Gal80^{ts}/+ *tj-Gal4*/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-Nrg RNAi #1 *tj-Gal4*/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-NrxIV RNAi *tj-Gal4*/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-Dg RNAi *tj-Gal4*/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-Perlecan RNAi *tj-Gal4*/+; UAS-Myr:GFP, tub-Gal80^{ts}/UAS-Cals RNAi
3.S3D	*tj-Gal4*, ECad:GFP/Gal80^{ts}; UAS-mCherry RNAi/UAS-Dg RNAi *tj-Gal4*, ECad:GFP/Gal80^{ts}; UAS-mCherry RNAi/UAS-Tum RNAi *tj-Gal4*, ECad:GFP/Gal80^{ts}; UAS-Dg RNAi/UAS-Tum RNAi
3.S4C	Sqh:3xmKate2/Dg:GFP
3.S4D	Sqh:3xmKate2/+; Dys:GFP/+
3.S5A	*Dys^{short}*:sfGFP/+

3.S5B	*Dys*^{long}:sfGFP/+
3.S5C	*tj-Gal4*, Sqh:3xmKate2/+; *Dys*^{short}:GFP/+ *tj-Gal4*, Sqh:3xmKate2/+; *Dys*$^{short\Delta SR24}$:GFP/+ *tj-Gal4*, Sqh:3xmKate2/+; *Dys*$^{short\Delta SD}$:GFP/+ *tj-Gal4*, Sqh:3xmKate2/+; *Dys*$^{short\Delta CT}$:GFP/+
3.S5D	*tj-Gal4*, Sqh:3xmKate2/+; *Dys*^{short}:GFP/+
3.S5E	*tj-Gal4*, Sqh:3xmKate2/+; *Dys*$^{short\Delta SR24}$:GFP/+
3.S5F	*tj-Gal4*, Sqh:3xmKate2/+; *Dys*$^{short\Delta SD}$:GFP/+
3.S5G	*tj-Gal4*, Sqh:3xmKate2/+; *Dys*$^{short\Delta CT}$:GFP/+

In vivo genetic modifier screen

For the genetic modifier screen, the following fly stock was generated: *tj-Gal4*, UAS-Anillin RNAi/CyO; UAS-Myr:GFP, tub-Gal80^{ts}/TM6. The RNAi lines for the analyzed cell adhesion molecules were obtained from *Drosophila* Bloomington Stock Center (DBSC), and are listed in detail in Table A1. To fully suppress premature UAS-driven RNAi expression, we used Gal80^{ts}, the temperature-sensitive repressor of Gal4, and kept the crosses at 18ºC. 0-4 days after hatching, adult offspring was transferred to 29ºC for 3 days, to boost the efficiency of RNAi depletion. After these 3 days, ovaries were dissected and fixed. At least 3 fully independent experiments were performed for each RNAi line used and, for each of these experiments, a minimum of 10 egg chambers (randomly selected from a pool of 6-8 dissected animals) were analyzed.

Drosophila Schneider (S2) cell culture and transfection

Drosophila S2 cells were cultured at 25°C in Schneider's Insect medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher). Transient transfection of mCherry:Tubulin, pH-DysE:GFP or pUAS-DysH:GFP and pActinGAL4 was performed using the Effectene Transfection Reagent (QIAGEN), according to manufacturer's instructions. After transfections, cells were incubated at 25 °C for at least 3 days and maximum 5 days prior to the induction of gene expression. 5x10^5 cells were plated in MatTek culture disks (MatTek; No 1.5; P35G-1.5-7-C) previously coated with poly-D-lysine (Sigma-Aldrich) before performing live imaging.

Molecular biology and transgenesis

Isoform-specific mutants were generated by CRISPR. gRNAs GGAGGAGCTGAATCTGCAGG and GTGGAGCTGCTCCTCCGACG, targeting long (RA,C,F,G,H,I and K) and RE isoforms, respectively, were cloned in PCFD6 vector and transgenic lines were generated at attP40 landing site. Then, these lines were crossed with a line expressing Cas9 in the germline and with or without the sfGFP KI at *Dys* locus. Indel mutations were isolated in the progeny by sequencing. Alleles selected for this work were $Dys^{long181}$ (4bp deletion, STOP at amino acid position 181 on RH), Dys^{RE225} (2bp deletion, STOP at amino acid position 225 on RE), $Dys^{long181}$:sfGFP (4bp deletion, STOP at amino acid position 181 on RH) and Dys^{RE225}:sfGFP (deletion of 2 bp, STOP at amino acid position 225).

Dys isoform E cDNA sequence was amplified by PCR from clone RE11449 (*Drosophila* GOLD cDNA collection) and cloned into pENTR. The Gateway cloning system was then used to create pH-DysE:GFP by recombining pENTR-DysE into pHWG through LR Clonase II (Thermo Fisher) mediated recombination. In frame cloning of the pUAS-Dys^{short} transgenes was performed using NEB Builder HiFi DNA Assembly Cloning Kit (New England Biolabs) in pUASz vector in which EGFP in C-terminal position was previously added. Dys^{short}, $Dys^{short\Delta SR24}$, $Dys^{short\Delta SD}$, $Dys^{short\Delta CT}$ encodes amino acids 1-1051, 371-1051, 1-956, 1-714 of the RE isoform, respectively. Transgenes were generated at attP2 site. To generate pUAS-GFP:DysRH: Dys-RH cDNA was synthetized *in vitro* (Integrated DNA Technologies) in several fragments before to be cloned in pUASz:GFP vector using NEB Builder HiFi DNA.

Fixation and staining of egg chambers

Drosophila ovaries were dissected in Schneider's Insect Medium (Sigma-Aldrich) supplemented with 10% FBS (fetal bovine serum, heat inactivated; Thermo Fisher) and fixed using a 4% paraformaldehyde solution (prepared in PBS with 0.05% Tween 20 (Sigma-Aldrich)) for 20 minutes. After washing three times for 10 minutes with PBT, samples were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories). Alternatively, for antibody staining, after the post-fixation washes, egg chambers were blocked for 1 hour at room temperature with 10% BSA prepared in PBT and incubated overnight at room temperature with the primary antibody diluted in PBT + 1% BSA. Samples were then washed with PBT + 1% BSA and incubated again for at least two hours at room

temperature with the secondary antibody diluted in PBT + 0.1% BSA. After three washing steps with PBT, samples were mounted with Vectashield with DAPI (Vector Laboratories). For F-actin staining, we added Phalloidin-TRITC (Merck, 1:250) to the fixative solution and increased the incubation time to 30 min. The following primary antibodies were used: rat anti-N-cadherin (DSHB DN-Ex #8, 1:50), mouse anti-Fas2 (DSHB 1D4, 1:50), mouse anti-Fas3 (DSHB 7G10, 1:100), mouse anti-βPS-integrin (DSHB CF.6G11-s, 1:10), rabbit anti-Perlecan ((Diaz-Torres et al. 2021), gift from González-Reyes, 1:850) and mouse anti-Armadillo (DSHB N2.7A1, 1:100). Respectively, the following secondary antibodies were used: goat anti-rat Alexa 568 (Invitrogen A11077, 1:300) for N-cadherin, goat anti-mouse Alexa 488 (Invitrogen A11029, 1:300) for Fas2, Fas3 and βPS-integrin, goat anti-rabbit Alexa 488 (Invitrogen A11008, 1:300) for Perlecan and goat anti-mouse Alexa 568 (Invitrogen A11031, 1:300) for Armadillo.

Imaging

Images of fixed *Drosophila* egg chambers were acquired on an inverted laser scanning confocal microscope Leica TCS SP5 II (Leica Microsystems), with HC PL APO CS 20x/0.70 NA water, 40x/1.10 NA water or 63x/1.30 NA glycerine objectives, using the LAS 2.6 software. For live imaging of Drosophila egg chambers, individual ovarioles were dissected in ex vivo culture medium (Schneider's medium (Sigma-Aldrich) supplemented with 10% FBS (fetal bovine serum, heat inactivated; Thermo Fisher) and 200 µg/µL insulin (Sigma-Aldrich)) and the enveloping muscle removed. Ovarioles were transferred to new culture medium and imaged on glass bottom dishes (MatTek; No 1.5; P35G-1.5-7-C) with an Andor XD Revolution Spinning Disk Confocal system equipped with two solid state lasers – 488nm and 561nm –, an iXonEM+ DU-897 EMCCD camera and a Yokogawa CSU-22 unit built on an inverted Olympus IX81 microscope with a PLAPON 60x/1.42 NA objective, using iQ software (Andor). When indicated in the figures, to mark the cell membrane, ovarioles were stained with CellMask Orange Plasma membrane Stain (ThermoFisher; diluted 1:10 000 in culture medium) for 15 minutes and washed with ex vivo culture medium before imaging. Live imaging was performed at 25ºC, with the exception for the experiments where UAS-Anillin RNAi was expressed, which were performed at 29ºC. Midsagittal egg chamber cross-sections were used to image the follicular epithelium along the apical-basal axis and z-stacks at the surface of the egg chamber to cross-section the follicular epithelium along the apical-basal axis. Z stacks were collected with serial optical sections separated

by 1 µm. Live imaging of *Drosophila* S2 cells was performed with the same Andor XD Revolution Spinning Disk Confocal system, using with a UPLSAPO 100x/NA 1.40 objective.

Live imaging of *Drosophila* pupa was conducted as described in (Bosveld et al. 2012), during the first round of cell division in the anterior scutum region of the notum epithelium. Pupa imaging was performed using an inverted spinning disk wide homogenizer confocal microscope (CSU-W1, Roper/Zeiss) equipped with a sCMOS camera (Orca Flash4, Hamamatsu) and using a 63x/1.4 NA oil DICII PL APO objective. A 40 slices Z-stack was collected with serial optical sections separated by 0.5 µm, and captured every 30 seconds. To obtain apical-basal side views of cytokinesis, a maximum projection was generated from a 2 µm resliced region, centered at the cytokinetic ring.

Protein extracts and Western blot

We prepared protein extracts from *Drosophila* ovaries (at least 25 flies per genotype) of endogenously-GFP-tagged Dys^{long} and Dys^{short} mutants, as well as Dys:sfGFP. Protein extracts of endogenously GFP-tagged aPKC were used as a control to detect an unrelated GFP-tagged protein. Dissected ovaries were transferred to lysis buffer (150mM KCl, 75mM HEPES pH 7.5, 1.5 mM EGTA, 1.5mM MgCl2, 15% glycerol, 0.1% NP-40, 1x protease inhibitors cocktail (Roche) and 1x phosphatase inhibitors cocktail 3 (Sigma-Aldrich)), and disrupted through sonication. Protein extracts were collected from the supernatant after centrifugation. Samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen), according to manufacturer's instructions. Transferred proteins were confirmed by Ponceau staining (0.25% Ponceau S in 40% methanol and 15% acetic acid). The membrane was blocked for at least 1 hour at room temperature with 5% dry milk prepared in PBT, and subsequently incubated overnight with the primary antibody (rabbit anti-GFP (i3S core facility, 1:1000)) diluted in blocking solution, at 4ºC. The membrane was then washed three times for 10 minutes with PBT, and incubated for 1 hour with the secondary antibody conjugated to HRP (Jackson ImmunoResearch, 1:5000) diluted in blocking solution, at room temperature. After washing the membrane again three times for 10 minutes with PBT, blots were developed with Clarity Western ECL Substrate (Bio-Rad) and detected on X-ray films (Fuji Medical).

Data processing and analysis

Image processing and quantifications were done with FIJI (Schindelin et al. 2012). For representative midsagittal images from egg chambers, we used a single optical section or maximum intensity projections of 2-5 planes. Surface or bottom images of egg chambers correspond to maximum intensity projections of the sections encompassing the epithelial region of interest. For live imaging processing of egg chambers, two FIJI features were always applied: the *StackReg* plugin (EPFL; Biomedical Imaging Group), to correct for the egg chamber movement, and the *Gaussian Blur 3D* filter, to remove background noise. For live imaging of pupal notum, the *Bleach Correction* plugin was applied to correct bleaching.

Statistical analysis and graphs were generated using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA).

Quantification of multinucleation rate for the Anillin RNAi modifier screen

For the *in vivo* genetic modifier screen, we scored the amount of multinucleated follicle cells in surface z-projections of non-proliferative stage 10 egg chambers ($\Delta z = 1\ \mu m$). We selected stage 10 egg chambers to evaluate the multinucleation ratio for two main reasons. First, follicle cells are no longer undergoing mitotic division, which allows us to directly associate the number of nuclei with the presence of a multinucleated cell. Second, follicle cells are larger at these stages, which facilitates automated segmentation of nuclei and cell membranes. To segment epithelial cells, we developed a set of automated macros for FIJI. The first macro projects a 1μm thick section from the acquired z-stack centered around the nucleus; since the nucleus position along the z-axis is variable due to egg chamber curvature, the optimal z-planes to project are determined locally based on the closest nucleus. To extract the multinucleation ratio, the second macro selects a central region of interest (ROI) of the epithelium (to avoid quantification in the borders of egg chambers whose nuclei/membrane could be missed from the projection), segments the cells (marked with Myristoylated-GFP) within this ROI, segments nuclei in the acquired image (marked with DAPI) and then counts the number of nuclei within the previously segmented cells (see Fig 3.S1 for further details). Results from individual images are then summarized in a results table. Manual validation of the segmentation was performed for each image to correct any errors prior to the quantification of the nuclei/cell ratio (multinucleation ratio). In the context of the modifier screen (Fig 3.1D–G), the multinucleation ratio for each egg chamber was compared to the mean of a control group

of cytokinesis-sensitized (Anillin RNAi) egg chambers expressing UAS-mCherry. This control group was incubated during the same period of time of each of the three independent experiments performed for each RNAi line. Δ multinucleation ratio for each egg chamber is calculated as $(\text{nuclei/cell})_{\text{cell adhesion RNAi}}$ - $(1/n)\ \Sigma(\text{nuclei/cell})i_{\text{control}}$, where *n* represents the number of control egg chambers within each replicate.

Quantification of multinucleation frequency for interaction experiments with Dys mutants and Tum RNAi

Due to genetic constraints, we used a different genetically encoded marker to visualize the epithelial cell cortex (ECad:GFP) than the one used in the *in vivo* genetic modifier screen. The number of mono and multinucleated cells was quantified in cross-sections in non-proliferative stage 10 egg chambers. Similar to the approach used for the screen, this quantification was restricted to the central area of the follicular epithelium. Due to the strong multinucleation phenotype produced by Tum RNAi, we used a more restricted period of incubation at 29ºC (2 days prior to dissection) than the one used for experiments with Anillin RNAi.

Quantification of ring constriction rate

Analysis of cytokinetic ring constriction was performed in egg chambers expressing a tagged version of non-muscle myosin light chain, Sqh:mKate2 (Fig 3.5D, E and G), or non-muscle myosin heavy chain, Zip:GFP (Fig 3.5F, G). The diameter of the contractile ring along the apical-basal axis of the epithelium was manually measured using FIJI, from cytokinesis onset (t_0) to complete constriction (t_f). To depict the change in ring diameter through time (Fig 3.5D, F), these values were then normalized to the length of the ring at t_0 (defined as the frame prior to a change in ring diameter) and plotted as a function of time, using Prism (GraphPad Software). To measure constriction rate during the linear phase of constriction (~ 80%- 40% of the original diameter), we selected four consecutive time points for which the change in absolute ring diameter could fit to a linear regression (R^2>0.95 was set as the threshold for the linear fit to assume constant constriction rate). We determined the constriction rate as the slope (β) of a linear regression ($d = \alpha+\beta t$ computed with Prism (GraphPad Software).

Quantification of cytokinesis failure in time-lapse movies

To understand when epithelial cells failed cytokinesis upon Anillin depletion and/or Dys loss-of-function, time-lapse movies were generated in egg chambers expressing Zip:GFP and labelled with a plasma membrane marker. We limited the analysis to small cells to prevent misleading results from defects accumulated from prior cytokinesis failure.

Moreover, we only included in the analysis cells that were imaged at least 20 minutes after ring constriction. Cells were scored in three categories: 1) not failing cytokinesis, 2) failing cytokinesis during ring constriction or 3) failing cytokinesis post-constriction.

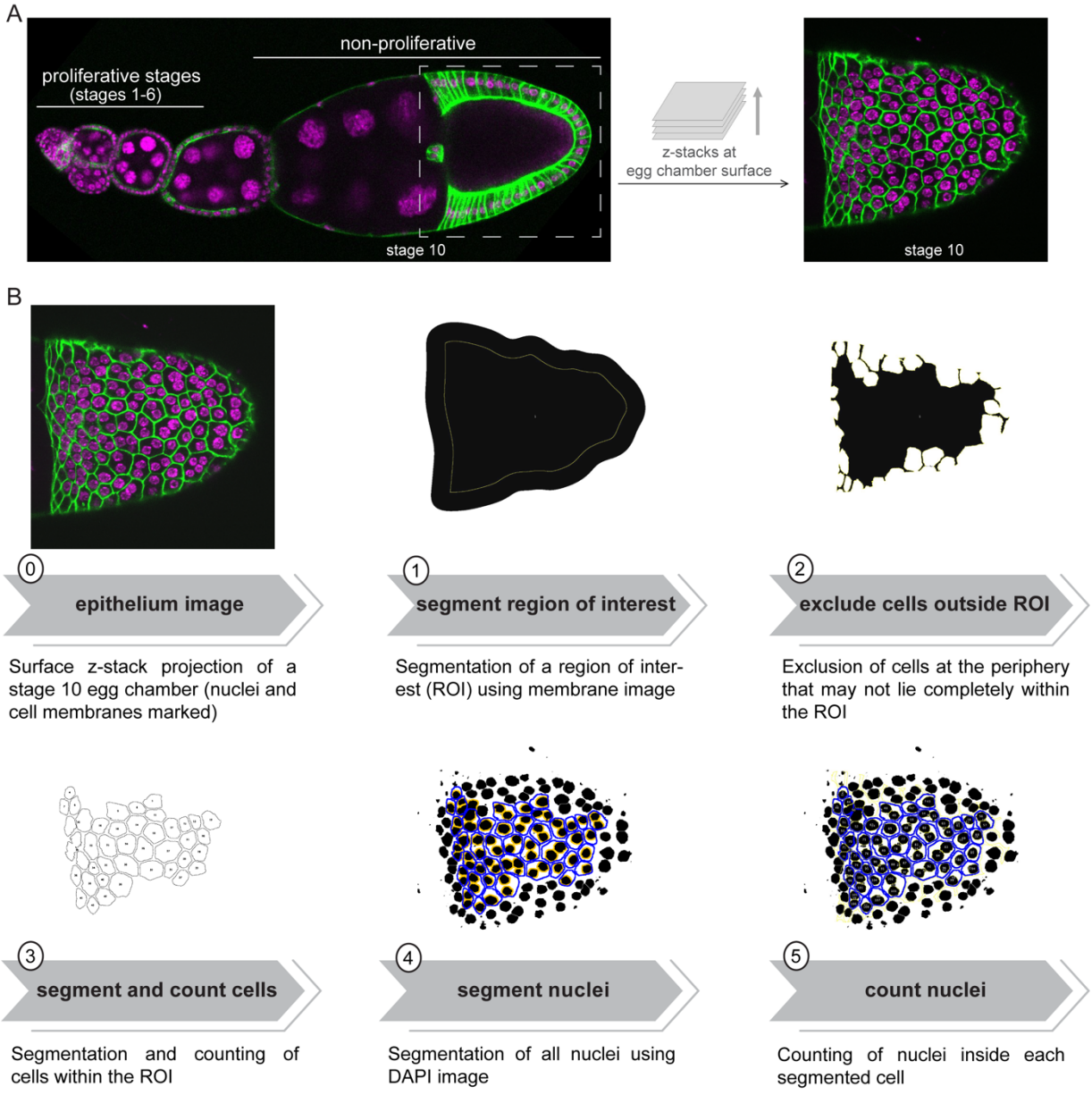
Quantification of RFP:Anillin fluorescence intensity

Z-stacks at the surface of control, $Dys^{E17/Df}$ and $Dys^{MI025024/Df}$ egg chambers were acquired by live imaging to quantify mRFP:Anillin fluorescence intensity during cell division. Z-sum projections encompassing 2 planes separated by 1 μm that cross-sectioned the mitotic cell were used for manual segmentation and quantification of mean pixel intensity at two timepoints: 2 min before the beginning of ring constriction (metaphase) and cytokinesis (2 min after initiation of ring constriction). In metaphase, mRFP:Anillin signal was quantified in the whole cell cortex segmented with a circular ROI (3 pixel width). During cytokinesis, mRFP:Anillin signal was quantified at the cytokinetic ring using a segmented line with ~10 pixel in length and 3 pixel width. The mean cytoplasmic signal measured with a circular ROI with 10 pixel diameter was used for background subtraction of the respective mean pixel intensity of metaphase or cytokinesis signals.

Acknowledgments

The authors thank M-L Parmentier, the Bloomington *Drosophila* Stock Center, the Kyoto *Drosophila* Stock Center and the Vienna *Drosophila* Resource center for fly stocks, and C Sunkel for support with supervision. Work in the EM lab was funded by National Funds through FCT—Fundação para a Ciência e a Tecnologia, IP, under the projects UIDB/04293/2020 and PTDC/BIA-CEL/1511/2021, EM is funded by "FCT Scientific Employment Stimulus - Individual Call" program (CEECIND/00622/2017). MG was supported by a PhD fellowship from FCT (SFRH/BD/130708/2017) and by IPATIMUP through CANCER_CHALLENGE2022, which also supported CL. The authors also acknowledge the support of the i3S Scientific Platform ALM, member of the national infrastructure PPBI - Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122) and the Institut Curie PICT-IBiSA@BDD imaging facility (member of the French National Research Infrastructure France-BioImaging, ANR-10-INBS-04). Work in the VM lab was supported by the Association Française contre les Myopathies (AFM-Téléthon) (MyoNeurAlp2 network) and by the French government IDEX-ISITE initiative 16-IDEX-0001 (CAP 20-25). Work in the YB lab is supported by the Institut Curie, the CNRS, the INSERM as well as ARC (SL220130607097), ANR (TiMecaDiv 20CE13000801), CANCERO-INCA (PLBIO2020/BELLAICHE) grants.

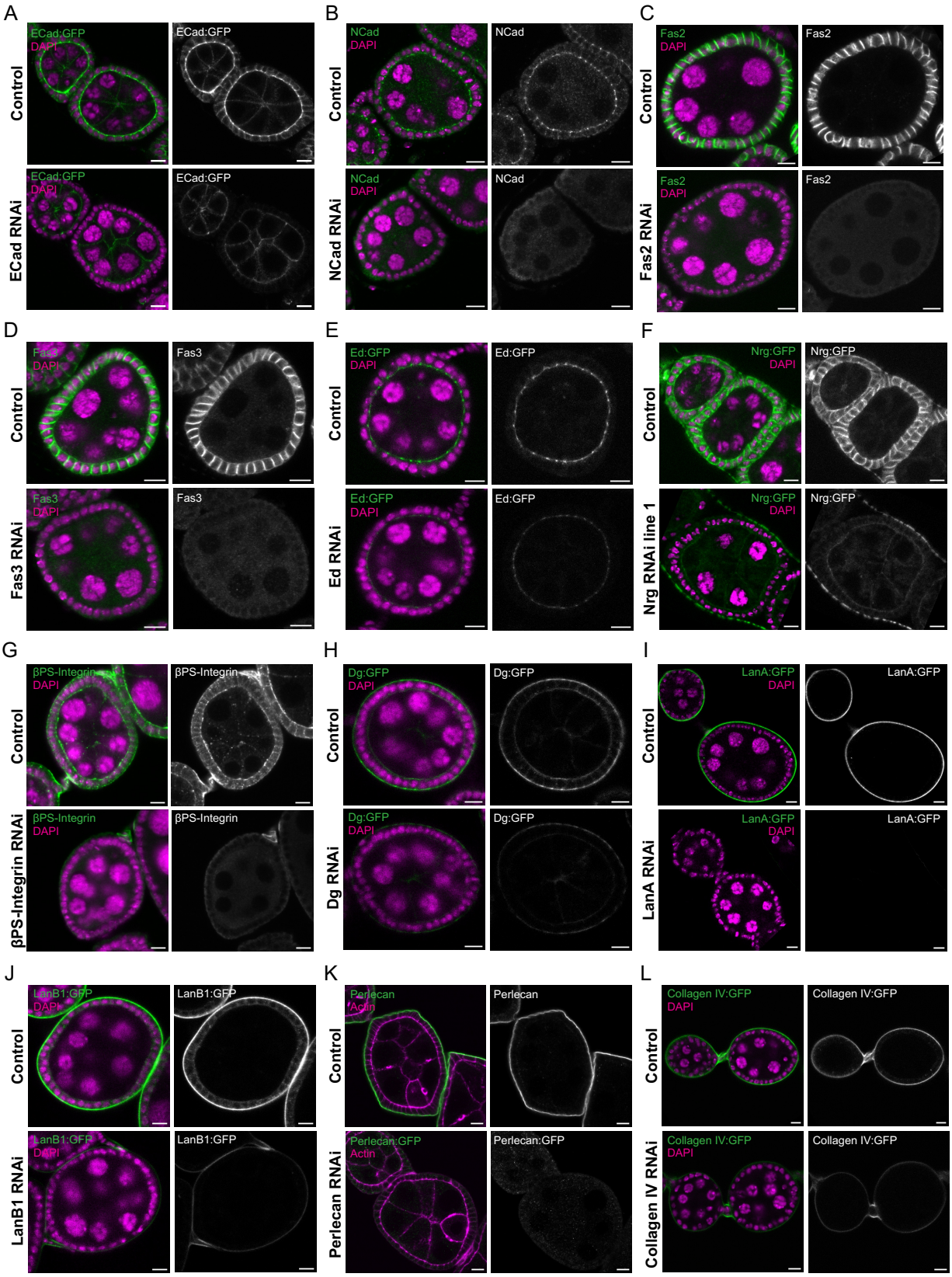
Supplementary Material



Supplementary Figure 3.S1. Pipeline for the quantification of cytokinesis defects in the follicular epithelium of stage 10 egg chambers.

A) Surface z-projections of non-proliferative stage 10 egg chambers were used to quantify the cytokinesis defects from the in vivo genetic modifier screen (nuclei marked with DAPI (magenta) and cell membranes endogenously expressing Myr:GFP (green)). At this stage of oogenesis, follicle cells are no longer undergoing mitosis, allowing us to directly correlate multinucleation with cytokinesis failure, and are larger than at younger stages, facilitating the automated segmentation of nuclei and cell membranes.

B) A macro for FIJI was developed for the automated segmentation and counting of nuclei and cells in two-channel images of the follicular epithelium (nuclei in magenta and cell membranes in green). Note that this was restricted to the central area of the egg chamber, to avoid misleading quantifications from nuclei/cells at the periphery of the egg chambers. After running the macro, a manual validation of the segmentation of both nuclei and cells was performed for all images. The obtained values were used to calculate the Multinucleation Ratio, as explained in detail in the Methods section.



Supplementary Figure 3.S2. Validation of RNAi-mediated depletion of proteins from the genetic modifier screen.

A) Midsagittal images of control and ECad RNAi egg chambers endogenously expressing ECad:GFP (green) and DAPI (magenta).

B) Midsagittal images of control and NCad RNAi egg chambers, stained for NCad (green) and DAPI (magenta).

C) Midsagittal images of control and Fas2 RNAi egg chambers, stained for Fas2 (green) and DAPI (magenta).

D) Midsagittal images of control and Fas3 RNAi egg chambers, stained for Fas3 (green) and DAPI (magenta).

E) Midsagittal images of control and Ed RNAi egg chambers endogenously expressing Ed:GFP (green) and stained for DAPI (magenta).

F) Midsagittal images of control and Nrg RNAi (line 1) egg chambers endogenously expressing Nrg:GFP (green) and stained for DAPI (magenta).

G) Midsagittal images of control and βPS-integrin RNAi egg chambers, stained for βPS-integrin (green) and F-actin (magenta).

H) Midsagittal images of control and Dg RNAi egg chambers endogenously expressing Dg:GFP (green) and stained for DAPI (magenta).

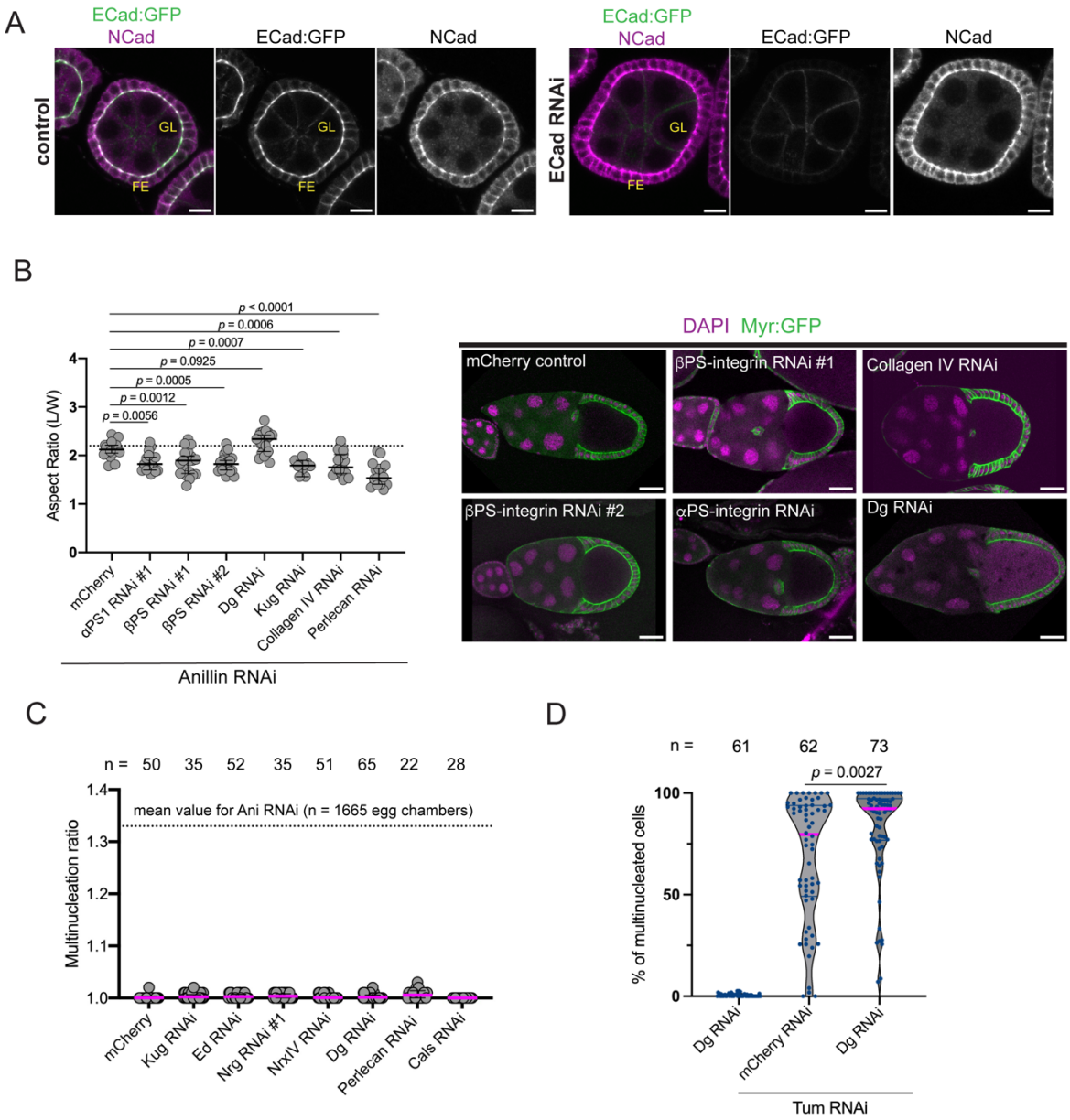
I) Midsagittal images of control and LanA RNAi egg chambers endogenously expressing LanA:GFP (green) and stained for DAPI (magenta).

J) Midsagittal images of control and LanB1 RNAi egg chambers endogenously expressing LanB1:GFP (green) and stained for DAPI (magenta).

K) Midsagittal images of control and Perlecan RNAi egg chambers, stained for Perlecan (green) and F-actin (magenta).

L) Midsagittal images of control and Collagen IV RNAi egg chambers endogenously expressing Collagen IV:GFP (green) and stained for DAPI (magenta).

A-L – Scale bars: 10 μm.



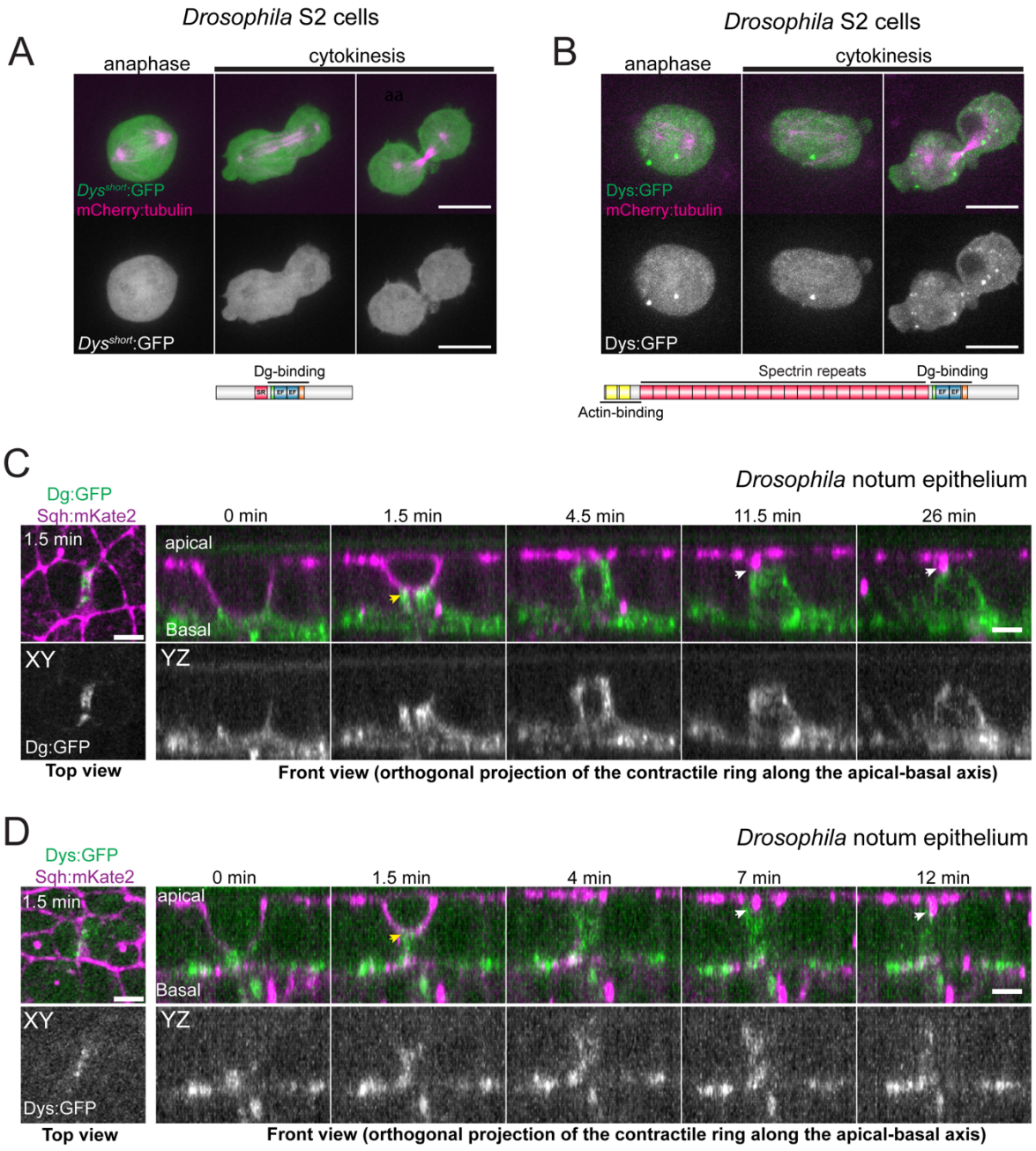
Supplementary Figure 3.S3. Complementary analysis of RNAi-mediated depletion and their effects on cytokinesis.

A) Midsagittal images of control and ECad RNAi egg chambers endogenously expressing ECad:GFP (green) and stained for NCad (magenta). Expression of RNAi for ECad in the follicular epithelium (FE) induces efficient protein depletion. As anticipated, reduction of ECad:GFP fluorescence by ECad RNAi is restricted to the follicular epithelium (FE) and ECad levels are normal in the germline (GL). Scale bar: 10 μm.

B) Midsagittal images and measurement of the aspect ratio in stage 10 egg chambers co-depleted for Anillin and the indicated basal proteins, in comparison with control (UAS-mCherry). Aspect ratio was calculated as the ratio between egg chamber length and width, as depicted in the bottom image (DAPI in magenta and cell membrane in green (Myr:GFP)). Each dot represents the aspect ratio of an analyzed egg chamber. The dashed line represents the anticipated aspect ratio value for this egg chamber stage, as calculated in (Jia et al, 2016). Median ± 95 Confidence Interval is shown. *p*-value was calculated by an ordinary one-way ANOVA. Scale bar: 50 μm.

C) Multinucleated ratio in egg chambers depleted for the regulators of cell-cell and cell-matrix interactions that enhanced multinucleation in the Anillin RNAi modifier screen (Fig 1D-F), in comparison with control (UAS-mCherry). Depletion of these molecules on their own (in an unperturbed cytokinetic background) does not cause multinucleation. Each dot represents the Multinucleation Ratio of an analyzed egg chamber. Median is indicated. The dashed line represents the mean value of the multinucleation ratio for co-expression of Anillin RNAi with the control line (UAS-mCherry).

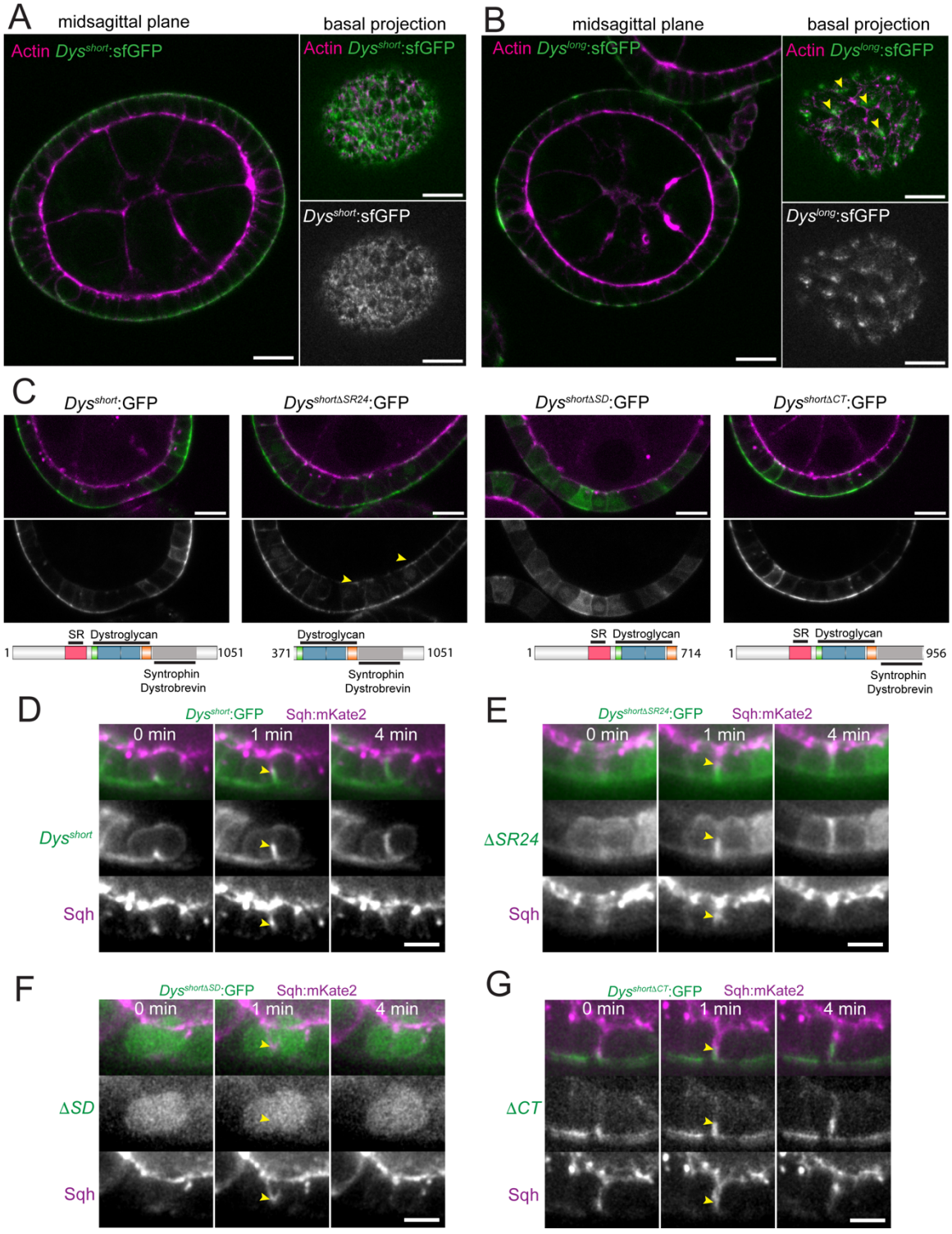
D) Frequency of multinucleated cells in egg chambers either co-expressing UAS-Dg RNAi with UAS-mCherry RNAi (UAS titulation control), UAS-Tum RNAi with UAS-mCherry RNAi or UAS-TumRNAi with UAS-Dg RNAi. UAS-driven depletion of Tum causes strong multinucleation frequency in the follicular epithelium, which is significantly increased by the concomitant depletion of Dg. Violin plots and median (magenta) is indicated. Each dot represents an egg chamber. Sample size (n) is indicated on top. *p*-value calculated by non-parametric unpaired Mann-Whitney test.



Supplementary Figure 3.S4. Dynamic redistribution of the DAPC during epithelial cytokinesis in *Drosophila* S2 cells and pupal notum epithelium.

A, B) The short Dys isoform E (A) and the long isoform H (B) do not show any local accumulation during cytokinesis in *Drosophila* S2 cells. Dys isoforms are GFP-tagged versions (green) and mCherry:Tubulin labels microtubules (magenta). Scale bars: 10 μm.

C, D) Redistribution of endogenously tagged Dg:GFP (A) and Dys:GFP (B) during epithelial cytokinesis in the Drosophila pupal dorsal thorax (notum). During ring (labelled with Sqh:mKate2) constriction, Dg:GFP (A) and Dys:GFP (B) accumulate at the basal part of the ingressing membrane (yellow arrows). After ring closure, both proteins become enriched close to the midbody (white arrows). Scale bars: 5 μm.



Supplementary Figure 3.S5. Spatial distribution of Dystrophin isoforms and truncated versions.

A) Midsagittal images (left) and basal projection (right) of the proliferative follicular epithelium endogenously expressing *Dys*^{short}:sfGFP (green) and stained for actin (magenta). *Dys*^{short} mainly localizes at the basal domain of epithelial cells. Scale bar: 10 μm.

B) Midsagittal images (left) and basal projection (right) of the proliferative follicular epithelium endogenously expressing *Dys^{long}:sfGFP* (green) and stained for actin (magenta). *Dys^{long}* mainly localizes to the basal domain in a planar polarized manner. Scale bar: 10 μm.

C) Midsagittal images of egg chambers expressing UAS-driven *Dys^{short}*:GFP, *Dys^{shortΔSR24}*:GFP, *Dys^{shortΔSD}*:GFP or *Dys^{shortΔCT}*:GFP and expressing Sqh:mKate2 (magenta). The short *Dys* isoform mainly localizes at the basal domain of epithelial cells, which is not altered by the deletion of the most downstream C-term region (*Dys^{shortΔCT}*). Partial mislocalization to the apical domain (yellow arrows) is observed in egg chambers expressing *Dys^{shortΔSR24}*:GFP. Deletion of syntrophins/dystrobrevin interaction sites leads to Dys mislocalization and only weak basal enrichment (*Dys^{shortΔSD}*). Note that these UAS-driven constructs show mosaic expression levels in the follicular epithelium. Scale bar: 10 μm.

D-G) Time-lapse images of follicle cells expressing UAS-driven *Dys^{short}*:GFP, *Dys^{shortΔSR24}*:GFP, *Dys^{shortΔSD}*:GFP or *Dys^{shortΔCT}*:GFP. *Dys^{short}* (A) becomes enriched at the ingressing membrane during ring constriction, which is labelled with Sqh:mKate2 (position of the basal part of the ring is marked with arrows). *Dys^{shortΔSR24}* (E) and *Dys^{shortΔCT}* (G) present a similar cytokinetic redistribution. In contrast, removal of the syntrophins/dystrobrevin binding sites prevents Dys accumulation in the ingressing membrane during epithelial cytokinesis (F).

Scale bar: 5 μm.

Supplementary Movie 3.S1. Dystrophin redistribution during epithelial cytokinesis.

Time-lapse movie of an egg chamber expressing endogenous Dys:sfGFP (green) and Cell Mask Plasma Membrane stain (magenta) (left). An inset of a follicle cell dividing perpendicularly to the plane of imaging allows the visualization of Dys accumulating in the ingressing membrane during ring (labelled with Sqh:mKate2) constriction (right). Scale bar: 5 μm. Stills in Figure 3.3.

Supplementary Movie 3.S2. Dystroglycan redistribution during epithelial cytokinesis.

Time-lapse movie of an egg chamber expressing endogenous Dg:GFP (green) and Sqh:mKate2 (magenta) (left). An inset of a follicle cell dividing parallelly to the plane of imaging allows the visualization of Dys accumulating in the ingressing membrane, below the contractile ring (labelled with Sqh:mKate2) (right). Scale bar: 5 μm. Stills in Figure 3.3.

Supplementary Movie 3.S3. Accumulation of Dystrophin at the newly-formed daughter cell interface.

Time-lapse movie of surface projections of an egg chamber expressing endogenous Dys:sfGFP (green) and Sqh:mKate2 (magenta). After ring closure, Dys:sfGFP (middle) becomes enriched at the new daughter cell interface. Scale bar: 5 μm. Stills in Figure 3.3.

Supplementary Movie 3.S4. Imaging of Dystrophin isoforms during epithelial cytokinesis.

Time-lapse movies of dividing follicle cells endogenously expressing *Dys*^{short}:sfGFP (top) or *Dys*^{long}:sfGFP (bottom) (green) and Sqh:mKate2 (magenta). The dividing plane (parallel to the plane of imaging) allows for an end-on view of the contractile ring (labelled with Sqh:mKate2). Both isoforms display a local enrichment at the ingressing membrane during ring constriction, and close to the midbody (at the apical side of follicle cells) after ring closure. Scale bar: 5 μm. Stills in Figure 3.4.

Supplementary Movie 3.S5. Dys contributes for the efficiency of the last stage of cytokinesis.

Time-lapse movie of surface projections of dividing follicle cells endogenously expressing Zip:GFP (green) and stained for a membrane marker (magenta), in *Dys* mutant egg chambers depleted of Anillin by RNAi. Cells fail cytokinesis due to membrane regression after ring closure. Scale bar: 5 μm. Stills in Figure 3.5.

Supplementary Movie 3.S6. DAPC ensures normal contractile ring constriction in the follicular epithelium.

Time-lapse movies of cytokinetic ring constriction viewed along the apical-basal axis in control, *Dg*$^{043/086}$ and *Dys*$^{E17/Df}$ mutant egg chambers, expressing Sqh:mKate2. Disruption of either Dg or Dys function delays ring constriction in the follicular epithelium. Scale bar: 5 μm. Stills in Figure 3.5.

Chapter IV



Reflections on the journey

General discussion

É necessário sair da ilha para ver a ilha, não nos vemos se não nos saímos de nós.
— José Saramago, *O Conto da Ilha Desconhecida*

Cytokinesis is the process that allows a dividing cell to give rise to two physically-independent daughter cells. This process entails several shape changes that the dividing cell has to deal with – from the remodeling of the actin cytoskeleton that makes the dividing cell usually rounder, to the bisection of the cytoplasm and, ultimately, the physical separation of the daughter cells at the end of cytokinesis. In fact, the molecular mechanisms underlying cytokinesis have been thoroughly dissected in the last decades, mainly using single-cell systems. Despite the knowledge that these studies provided, it remains unclear how cells in a multicellular context coordinate extensive cytoskeleton remodeling with the presence of adhesions to its neighbors and to the underlying substrate. One striking example is the epithelial tissue, whose function relies on the polarized positioning of adhesion molecules along its apical-basal axis, which dynamically modulate cell-cell and cell-matrix interactions to ensure tissue integrity and cohesiveness. The *in vivo* studies performed so far on the impact of these adhesion complexes on epithelial cytokinesis have mainly focused on E-cadherin-based cell-cell adhesion, overlooking the contribution of lateral and basal adhesion proteins. Therefore, the primary aim of my PhD was to perform a systematic *in vivo* RNAi modifier screen in the *Drosophila* follicular epithelium, to assess the impact of cell-cell and cell-matrix interactions on the efficiency of epithelial cytokinesis. In this chapter, I will outline the main findings of this work, integrating them with the current knowledge on epithelial cytokinesis and on the modulation of cell-cell and cell-matrix adhesion during cell division.

The impact of cell-cell adhesion on epithelial cytokinesis efficiency

Cell-cell adhesion has been shown to play a role during cell division in the epithelial context, with increasing evidence for the importance of a multicellular regulation of epithelial cytokinesis (Founounou et al. 2013, Herszterg et al. 2013, Pinheiro et al. 2017, Hatte et al. 2018, Wang et al. 2023, Landino et al. 2023). In the planar divisions of the *Drosophila* pupal notum, *Septin* mutant cells failed cytokinesis, when the dividing cell has to pull on the surrounding Adherens Junctions (AJs), but completed cytokinesis when AJs were removed from the neighboring cells (Founounou et al. 2013). Similarly, in the *Xenopus* embryonic

gastrula, AJs strengthening through depletion of Tight Junctions (TJs)-associated proteins led to cytokinesis failure (Hatte et al. 2018), suggesting that AJs challenge cytokinesis, both in flies and vertebrates. In line with this, enhancing the binding of α-catenin, a core AJs component, to cortical actin through α-catenin mechanosensitive mutants was shown to promote cytokinesis failure in MDCK cells (Wang et al. 2023), indicating that tension-dependent alterations on AJs contribute to a successful cytokinesis. Noteworthy, these α-catenin mechanosensitive mutants did not cause cytokinesis failure in isolated cells, further supporting the importance of modulating neighboring cell-cell adhesion to ensure cytokinesis efficiency in epithelia. Recently, a non-autonomous regulation of epithelial cytokinesis by the neighboring cells was also reported in vertebrates (Landino et al. 2023). Non-dividing neighboring cells accumulate contractility factors near the cleavage furrow which, when hyper-activated, restrain furrow ingression and lead to cytokinesis failure (Landino et al. 2023). In agreement with this, our data shows that, in the *Drosophila* follicular epithelium, cadherin-based adhesion at the apical level challenges cytokinesis completion. This suggests that the contractile ring needs to exert sufficient pulling forces to overcome the resisting forces of its neighbors, applied by the AJs, in order to efficiently complete cytokinesis. Strikingly, this opposing role of AJs on cytokinesis efficiency might be specific for epithelial tissues, since a positive effect was reported in non-epithelial cells of both vertebrates and invertebrates (Goldbach et al. 2010). In both *Drosophila* spermatocytes and mouse fibroblasts, strengthening of AJs through E-cadherin overexpression rescued the cytokinesis defects caused upon Anillin depletion, highlighting a possible tissue-specific effect of AJs on cytokinesis efficiency (Goldbach et al. 2010).

Interestingly, our modifier screen also uncovered an unanticipated role for lateral adhesion on cytokinesis completion. Our results show that the depletion of either Neuroglian, Echinoid or Neurexin IV increase the multinucleation defects caused by Anillin depletion. Notably, Neuroglian heterotypically interacts with both Echinoid, the functional homologue of Nectins that is present at the Nectin/Afadin complexes in most epithelial cells, and with Neurexin IV (Islam et al. 2003, Banerjee et al. 2006). In the *Drosophila* follicular epithelium, these molecules localize laterally, enrolling in Septate Junctions formation during late stages of vitellogenesis (Tiklova et al. 2010). Thus, despite a role for these molecules on cytokinesis has not been reported so far, it is possible that, similar to what happens in the *Xenopus* embryo (Hatte et al. 2018), these Neuroglian-based complexes at the lateral membrane negatively regulate the tension applied to the AJs. If this is the case, depletion of these proteins would trigger adhesion strengthening at the AJs, which would most likely induce cytokinesis failure, in light with the results from our screen and from previous works (Founounou et al. 2013, Wang et al. 2023).

Modulation of cell-substrate adhesion on epithelial cytokinesis efficiency

It has been shown that the mitotic remodeling of the actomyosin cytoskeleton requires a previous remodeling of the cell-matrix adhesive contacts (Dao et al. 2009, Lancaster et al. 2013). This foreshadows a role for basal adhesion on the efficiency of cytokinesis, although only few studies addressed this question. Strengthening basal adhesion in the migrating zebrafish epicardium restrains cytokinetic ring constriction, leading to cytokinesis failure (Uroz et al. 2019). A similar phenotype was observed on mammary epithelial cells, in which reinforcing cell-extracellular matrix (ECM) adhesion promotes cytokinesis failure in cells cultured on soft substrates (Rabie et al. 2021). On the other hand, mitotic mammalian cells require adhesive contacts with the substrate to successfully divide (Sambandamoorthy et al. 2015, Dix et al. 2018), suggesting that a fine-tuned modulation of the cell-matrix adhesion is necessary for cells to successfully divide. We observed no effect on cytokinesis efficiency when depleting the different integrin subunits present in the *Drosophila* follicular epithelium. One possibility is that the depletion of one integrin subunit could be compensated for another subunit of the same family. Nevertheless, this is unlikely to be the case, as βPS is the only subunit expressed in the *Drosophila* follicle cells, thus suggesting a negligible role for integrins during epithelial cytokinesis. Recently, it was reported in mammalian cells in culture that integrin-mediated cell-ECM adhesion is drastically reduced upon mitotic entry, as integrins cannot bind the actin cytoskeleton (Huber et al. 2023). Instead, mitotic cells employ integrins, particularly β1-integrin, and adhesome proteins, as Talin, Kindlin or Vinculin, to further enhance cell-cell adhesion, in order to prevent cell delamination due to mitotic rounding. It would thus be interesting to address if this remodeling of integrin-mediated adhesion also occurs in the *Drosophila* follicular epithelium. Although our screen data does not support a role for integrins on cytokinesis efficiency, it is possible that some integrin-driven adhesion reinforcement at the apico-lateral level (and concomitant weakening at the basal level) might occur, despite not being sufficient to be translated in a significant change on the multinucleation defects caused by Anillin depletion.

Remarkably, a non-integrin basal receptor – Dystroglycan (Dg) – was shown to promote cytokinesis efficiency in the *Drosophila* follicular epithelium. Most strikingly, disruption of both its high-affinity extracellular binding partner, Perlecan, or of its intracellular interactor, Dystrophin (Dys), also revealed a positive role for these proteins on epithelial cytokinesis efficiency. In fact, from the four core structural proteins forming the ECM, Perlecan was the only one that promoted cytokinesis efficiency. Interestingly, Perlecan disruption in cultured human skin and cutaneous cells induced shedding of Dg from the plasma membrane (Herzog et al. 2004), suggesting a destabilization of Dg in the absence

of Perlecan. It would thus be interesting to assess if this destabilization also occurs in the follicular epithelium, as this would most likely explain the effect of Perlecan on ensuring epithelial cytokinesis efficiency. The fact that 1) Dystroglycan and Dystrophin are part of a well-known complex, the Dystrophin-associated protein complex (DAPC), 2) that Perlecan is the extracellular ligand with more affinity for Dystroglycan, 3) that Perlecan depletion might destabilize Dystroglycan, and 4) that the individual depletion of these three proteins increased the cytokinesis defects initially caused by Anillin loss, fostered our interest on the role of the Dystroglycan-Dystrophin complex during epithelial cytokinesis. To rule out the possibility that the enhancement on cytokinesis failure observed upon concomitant disruption of either Dystroglycan or Dystrophin function with Anillin depletion was due to an impairment on Anillin recruitment to the cleavage furrow, rather than on an autonomous role of the Dystroglycan-Dystrophin complex, we imaged Anillin redistribution during epithelial cytokinesis in wild-type and *Dys* mutant egg chambers. Anillin localization was unaffected by the disruption of Dystrophin function, further supporting an alternative mechanism involving the cell-substrate adhesion machinery through which epithelial cells ensure an efficient cytokinesis.

Redistribution of the DAPC during epithelial cytokinesis

Although an enrichment of Dg and Dys at the cleavage furrow and the midbody was observed in cultured cells (Higginson et al. 2008, Villarreal-Silva et al. 2011), an active role for these proteins on cytokinesis was never reported. We demonstrated that, during cytokinesis in the *Drosophila* follicular epithelium, the Dg-Dys complex is also redistributed in the plasma membrane, with both proteins accumulating at the ingressing membrane and at the new interface of daughter cells. Interestingly, live imaging of GFP-tagged versions of both Laminin A and Perlecan did not show a redistribution of these ECM proteins during cytokinesis on the follicular epithelium (data not shown), hinting that the ECM remains physically stable during this process. The cell autonomous redistribution of Dg and Dys observed during epithelial cytokinesis, together with the results from our *in vivo* screen, unveil an unanticipated role for the DAPC in promoting cytokinesis efficiency in the *Drosophila* follicular epithelium. Of note, a strong accumulation of both Dg and Dys at the ingressing membrane, that persisted post-constriction, was also observed in the *Drosophila* pupal notum, further suggesting that the DAPC has cytokinetic functions in different *Drosophila* epithelial tissues. In line with this, it would be interesting to evaluate whether the

Dg-Dys complex also promotes cytokinesis efficiency in the pupal notum, similar to what happens in the follicular epithelium.

As Dystrophin directly interacts with the actin cytoskeleton (Way et al. 1992), one possibility would be that its cytokinetic redistribution occurs in response to cytoskeleton remodeling and, therefore, would rely on its actin-binding domains. Nevertheless, *Dys* mutant flies only expressing a short *Dys* isoform, lacking the canonical actin-binding domains at the N-terminal, exhibited a similar distribution during epithelial cytokinesis than its full-length counterpart, suggesting that Dystrophin cytokinetic relocalization occurs regardless of its interaction with the actin cytoskeleton. We thus focused our attention on the C-terminal end of the protein, which is common to both isoforms, and is known to contain both a Dystroglycan- and an adjacent Syntrophins/Dystrobrevin-binding domain. Expression of two short *Dys* isoforms harboring both domains, but differing in the last spectrin repeat of the rod-domain, fully recapitulated the relocalization of the full-length Dystrophin during cytokinesis. Surprisingly, expression of a short *Dys* isoform lacking the Syntrophins/Dystrobrevin-binding domain, although still harboring the Dystroglycan-binding one, prevented its cytokinetic relocalization, hinting that Dystrophin redistribution during epithelial cytokinesis depends on its interaction with Syntrophins/Dystrobrevin. Although the role of Syntrophins/Dystrobrevin in the epithelial tissue or during cell division remains unexplored, it may provide a mechanistic link towards the understanding of the cytokinetic functions of the DAPC. It would also be interesting to understand how this particular domain is guiding Dystrophin redistribution during epithelial cytokinesis, as some Syntrophins and Dystrobrevin may provide alternative connections to the actin cytoskeleton (Iwata et al. 1998, Iwata et al. 2004).

A role for the DAPC during ring constriction

Strengthening AJs-mediated adhesion between the mitotic and the neighboring cells in the *Drosophila* pupal notum (Founounou et al. 2013) and *Xenopus* embryo (Higashi et al. 2016, Hatte et al. 2018) seems to resist ring furrowing, delaying cytokinesis. Recently, it was further shown a direct role for neighbor cells contractility in regulating furrow closure in the *Xenopus* embryonic epithelium, as the speed of ring constriction in the mitotic cell becomes delayed when increasing tissue stiffness in its neighbors (Landino et al. 2023). Here, we observe that disruption of either Dg or Dys function, core components of the basal DAPC, significantly delays ring constriction from the beginning of furrowing and impairs ring constriction rate during the phase of constant ring constriction. These findings highlight that

basal proteins that may act as cell-ECM adhesion molecules can also regulate the kinetics of ring constriction, uncovering a new role for the DAPC in ensuring ring furrowing in the *Drosophila* follicular epithelium.

Notwithstanding, the mechanism underlying this role of the DAPC during ring constriction remains an important open question. The accumulation of DAPC components – both Dystroglycan and Dystrophin – below the contractile ring during furrowing, may contribute to the remodeling of the cell-matrix interactions at early cytokinesis, in order to facilitate the invagination of the basal membrane. This is in agreement with a role for this complex on the dynamic organization of the basement membrane and the underlying cytoskeleton during morphogenesis (Buisson et al. 2014, Cerqueira Campos et al. 2020, Villedieu et al. 2023). Additionally, as aforementioned in this chapter, it was recently observed in cultured mammalian cells that adhesion to the ECM is reduced upon entry in mitosis (Huber et al. 2023), possibly to reduce the resisting forces of the basal attachments with the substrate and allow the contractile ring to exert effective pulling forces that drive its constriction. It is thus possible that, in our system, the concomitant redistribution of the DAPC with ring constriction occurs, at least in part, to sustain a reduction in the adhesion of the dividing cell to the ECM, to facilitate the constriction of the contractile ring.

As Dystroglycan and Dystrophin are redistributed near the contractile ring during cytokinesis, another possibility is that the DAPC might play a role in ring contractility. To test this hypothesis, we assessed if the recruitment of two major contractile ring components – Anillin and Myosin – to the cleavage furrow was impaired in *Dys* mutant flies. Anillin accumulation in both the metaphase cortex and the contractile ring was not affected by the disruption of Dystrophin function. Similarly, the rate of Myosin accumulation in the contractile ring was not impaired in *Dys* mutants (data not shown), hinting that the DAPC is not involved in the recruitment of major contractile ring components. Despite this, the DAPC might still be indirectly affecting ring contractility. One possibility is that the close localization of Dystroglycan and Dystrophin to the contractile ring allows them to modulate the tension sensed by the ring, facilitating its constriction. Nevertheless, current evidences implying DAPC in mechanotransduction are so far indirect (Wilson et al. 2022, Mirouse 2023), as it has not been ruled out if the observed changes in response to mechanical stress directly derive from the disruption of the DAPC or if they are an indirect consequence of an impairment on the cell-ECM interface/mechanical properties. Alternatively, an upstream involvement of the DAPC on the regulation of ring contractility remains to be addressed. In mouse and rat interphasic myoblasts, it was shown that RhoA activation up-regulates the levels of Utrophin, the mouse autosomal homologue of Dystrophin, and enhances its localization at the plasma membrane (Bonet-Kerrache et al. 2005). In line with a role for RhoA in the activation of different cytokinetic components, one possibility is that RhoA also

might regulate Dystrophin relocalization near the contractile ring during epithelial cytokinesis, to facilitate its constriction.

A role for the DAPC during late cytokinesis

Although the molecular mechanism by which the DAPC contributes for cytokinesis completion is yet to be understood, we provide evidence that loss of Dystrophin function in cytokinesis-sensitized cells impairs late cytokinesis, since the majority of cells complete ring closure but show subsequent regression of the new interface formed between daughter cells. A similar phenotype was observed in MDCK cells expressing α-catenin mechanosensitive mutants, binding with more affinity to cortical actin, which failed cytokinesis during its latest stage (Wang et al. 2023). In our system, it is likely that the concomitant disruption of Dystrophin and Anillin impairs the connection of the DAPC to the actin cytoskeleton, which could destabilize the new daughter-daughter interface, leading to cytokinesis failure during the abscission phase. This hints that the actin cytoskeleton must maintain a tightly regulated coupling with its binding partners to ensure a faithful cytokinesis, as both impairing (through Dys disruption) or strengthening (through α-catenin mutants (Wang et al. 2023)) it results in late cytokinesis failure. Remarkably, *Dys* mutant flies expressing only the short isoform (which lacks the known actin-binding domains) also failed cytokinesis during its latest stages, due to membrane regression after ring closure. This suggests that both isoforms are required to efficiently maintain membrane separation between the daughter cells, leaving the mechanism by which DAPC promotes the efficiency of the latest stages of cytokinesis as a question to be addressed.

The Dystrophin enrichment observed near the midbody ring during late cytokinesis suggests a possible role for the DAPC in regulating the abscission machinery. In fact, in the *Drosophila* follicular epithelium, cytokinesis is incomplete. This leads to interconnected sibling cells through cytoplasmic bridges termed ring canals (Brown and King 1964, McLean and Cooley 2013). Nevertheless, despite not being completed, the abscission process is initiated, with the stabilization of midbody-associated microtubules during 30 minutes post-constriction (Morais-de-Sa and Sunkel 2013) and the recruitment of ESCRT-III components to the midbody, such as Shrub (the *Drosophila* orthologue of CHMP4B). In line with this, it would be interesting to assess if Dystrophin might regulate the disassembly of the midbody-associated microtubules during the abscission phase. In fact, it has been reported that Dystrophin is able to bind microtubules and pause their polymerization in mice (Prins et al. 2009, Belanto et al. 2014), leaving the question if this role is conserved across other species

to be explored. Another possibility is that Dystrophin might be involved in Shrub recruitment to the midbody, where it is required for Spastin recruitment (Yang et al. 2008) and to induce membrane fission (Guizetti et al. 2011). Interestingly, the pulling forces exerted by the daughter cells on the intercellular bridge (ICB) inhibit Shrub recruitment to the midbody and, ultimately, delay abscission (Lafaurie-Janvore et al. 2013). Counterintuitively, abscission proceeds when ICB tension is released, triggering the assembly of the ESCRT-III complex and subsequent membrane fission. As Dystrophin localizes near the midbody ring for a long period after complete ring constriction, it would be interesting to determine if it might regulate the tension sensed by the ICB, or even transmit tension from the ECM to the ICB, and, consequently, modulate the recruitment of the abscission machinery to the midbody.

Another possibility is that the DAPC could foster cytokinesis completion by enhancing cell-matrix adhesion at the end of cytokinesis, linking the intracellular cytoskeleton to the ECM, to facilitate the reintegration of the daughter cells in the epithelial monolayer. In line with this, it has been shown in human cells that cell-substrate adhesion facilitates cytokinesis and drives abscission in dividing cells with a compromised actomyosin ring (Dix et al. 2018). Although the newly-formed daughter cells do not have the space to crawl away from each other in the follicular epithelium, the basal attachments at the end of cytokinesis might produce traction forces that promote cell separation and facilitate cytokinesis completion in a context where the midbody ring is weakened.

Linking the cytokinetic roles of the DAPC to cancer

Understanding the molecular mechanisms underlying the role of the DAPC on epithelial cytokinesis will bring valuable insights, not only to the current knowledge on fundamental biology, but also to different pathological contexts, such as muscular dystrophies and cancer. By causing whole-genome duplication and centrosome amplification, a single round of cytokinesis failure can act as a starting point to generate aneuploidy, which is known to drive tumorigenesis *in vivo* (Ganem et al. 2007, Godinho and Pellman 2014, Lens and Medema 2019). This is particularly relevant, as these events (whole-genome duplication, centrosome amplification and aneuploidy) are highly frequent in tumorigenesis, which may all be triggered by cytokinesis failure: 1) a genomic study in a cohort of 9692 patients with a wide range of cancer types identified whole-genome doubling events in nearly 30% of the tumors (Bielski et al. 2018); 2) centrosome amplification has been detected in a variety of tumors, (an extensive study to quantify numerical and structural centrosome amplification showed centrosome amplification in 68% of triple-

negative breast cancer samples and 25-30% in pancreatic ductal adenocarcinomas (Mittal et al. 2021)); and 3) nearly 70% of all solid tumors are aneuploid (Duijf et al. 2013). Therefore, it is crucial to understand how cytokinesis efficiency is ensured in a multicellular context, in order to avoid the formation of aneuploid cells and, subsequently, tumor initiation and progression. This is particularly relevant in an epithelial context, as more than 90% of malignant tumors have epithelial origin (Cairns 1975). Interestingly, the lack of a clear and effective checkpoint that monitors cytokinesis fidelity, as well as the scarcity of mutations in cytokinesis regulators in the majority of tumors (Lens and Medema 2019), hints that cells within a tissue must have evolved safeguard mechanisms to ensure a faithful cytokinesis – which may occur, at least partially, through regulation of the adhesion to their neighbors and to the underlying substrate. Remarkably, Dystrophin has been recently shown to be a functional constituent of centrosomes in human and mouse myoblasts, whose depletion causes centrosome amplification and misorientation (Winter et al. 2022). It would therefore be interesting to evaluate if Dystrophin is also a component of the centrosomes in epithelial cells, and what is its functional relevance.

Increasing evidence also points to a correlation between a mutation in the Duchenne muscular dystrophy (*DMD*) gene and/or its protein product and the development of all major cancer types (Jones et al. 2021). DMD mutations are known to cause degenerative neuromuscular disorders, as Duchenne and Becker muscular dystrophies, which lead to the progressive weakness of the muscles. However, a number of studies in muscular dystrophy mouse models about 15 years ago showed that they were more prone to the development of soft tissue sarcomas. Since then, several reports of an association between mutations in the *DMD* gene/protein and the development, progression and/or overall survival of patients with a wide range of tumors have emerged. This increasing evidence has been pointing Dystrophin as a tumor suppressor, since the full-length protein is usually inactivated in neoplastic cells, while the short one is preserved (Jones et al. 2021). Similarly, disruption of the long Dystrophin isoform in the *Drosophila* follicular epithelium (only maintaining the short one) increases the cytokinesis defects observed upon Anillin depletion, which could highlight the significance of this long isoform in cytokinesis, similar to what happens in the tumor context. However, neither Dystrophin redistribution during epithelial cytokinesis is affected when the long isoform is disrupted, nor cytokinesis defects are rescued when the long isoform is expressed without the short one, suggesting a more complex mechanism by which Dystrophin suppresses tumorigenesis.

In addition, it will also be valuable to dissect whether a specific molecular Dys domain is required for its role during epithelial cytokinesis, and subsequently determine if these domain(s) contain mutations causing DMD. In fact, mutations in the N-terminal actin-

binding domain of Dys have been observed in meningiomas (tumors in the meninges) (Juratli et al. 2018), even if according to our results, this domain is dispensable for cytokinetic redistribution in the follicular epithelium. Since we have identified the C-terminal domain of Dys, which interacts with the adaptor proteins of the DAPC Syntrophins/Dystrobrevin, as critical for its localization during epithelial cytokinesis, it will be interesting to explore if there is an association between mutations in this domain and specific types of tumors.

Chapter V



The curtain falls

Concluding remarks

There is always something left to love.
— Gabriel García Márquez, *One Hundred Years of Solitude*

In this thesis, I performed a genetic modifier screen to identify modulators of cytokinesis efficiency in the *Drosophila* follicular epithelium. With this approach, I pinpointed a number of cell-cell and cell-matrix interaction mediators that, rather than opposing epithelial cytokinesis, surprisingly promote its completion. These findings emphasize the emerging notion that cytokinesis in the epithelial context relies on a multicellular coordination between the dividing cells and its surrounding environment, instead of merely being a cell autonomous process. Building on this genetic screen, I also unveiled an unanticipated role for the Dystrophin-Dystroglycan complex in epithelial cytokinesis, both during and after ring constriction. Altogether, our results uncover not only a role for the epithelial adhesion apparatus on safeguarding the fidelity of cytokinesis, but also provide new insights on the multistep role of the Dystrophin-Dystroglycan complex during cytokinesis in epithelial tissues.

As in a roller-coaster, in which after one loop there are many more to overcome, after one challenge in science becomes solved, plenty of others arise. This work opened the crucial question of *how* the Dystrophin-Dystroglycan complex contributes to each step of cytokinesis. An avenue of research would be to assess if the Dystrophin-Dystroglycan complex might be involved in the abscission of epithelial cells, due to its localization at late cytokinesis, near the midbody. This is particularly relevant as it has been previously shown that Dystrophin is able to bind microtubules and pauses its polymerization (Prins et al. 2009), hinting that it might play a role on the disassembly of the microtubules that remain associated to the midbody at the end of cytokinesis. It would also be interesting to understand if remodeling the adhesion to the extracellular matrix contributes for cytokinesis, both at the beginning of cytokinetic ring constriction and during the abscission period. In line with this, the redistribution of the Dystrophin-Dystroglycan complex might contribute for the remodeling of the cell-matrix interactions in opposite directions, in different periods of cytokinesis. First, its release from the basal side could remodel cell-ECM adhesion to facilitate the invagination of the ingressing membrane, whereas, later, its presence at the cell-ECM contacts could reinforce adhesion of the newly formed daughter cells to the substrate, which has been shown to be particularly relevant when the actomyosin ring is compromised (Dix et al. 2018). Finally, it would be interesting to address if the cytokinetic roles of the Dystrophin-Dystroglycan complex are conserved in other fly and vertebrate

epithelia, which is encouraged by the observation of a similar redistribution of these proteins during cytokinesis in the notum and in mammalian cell culture.

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Appendixes



Appendix A

Table A 1. Screen overview.

Table with an overview of the data from the *in vivo* modifier screen. The following information is provided (sub-divided by family of proteins): in column A, the name of each depleted protein and, in column B, the RNAi stock line used for protein depletion in the screen. In column C, the sample size (number of independent experiments, dissected egg chambers and quantified cells). In column D, the effect of protein depletion on the multinucleation ratio caused by co-depletion of Anillin. In column E, the median value and, in column F, the standard deviation of the multinucleation ratio (number of nuclei/number of cells) from all experiments for each depleted protein. In column G, the statistical significance of the *t*-test for each depleted protein against the respective experimental control (UAS-mCherry). In column H, the median value of the D multinucleation ratio (= $(\text{nuclei/cell})_{RNAi}$ - mean $(\text{nuclei/cell})_{mCherry}$) from all the replicates for each protein RNAi. In column I, the anticipated sub-cellular localization on the follicular epithelium of each protein and, in column J, the respective human orthologue(s).

Protein	Protein RNAi					Control (UAS-mCherry)			*t*-test against control*	Δ multinucleation ratio (median)	Localization	Human orthologues
	Stock (BDSC)	Modifier of Anillin RNAi	Sample: replicates/egg chambers/cells	Nuclei/cell (median)	Nuclei/cell (sdtDev)	Sample: replicates/egg chambers/cells	Nuclei/cell (median)	Nuclei/cell (sdtDev)				
UAS-E-Cadherin	58494	enhancer	3/52/2947	1,34	0,223	3/59/3874	1,23	0,164	0.0004	0,07	apical junction	CDH1/ CDH20
E-Cadherin line 1	27689	no effect	3/48/3646	1,30	0,200	3/54/3098	1,37	0,217	ns	-0,06	apical junction	CDH1/ CDH20
E-Cadherin line 2	32904	no effect	3/61/4747	1,21	0,117	3/60/3874	1,23	0,164	ns	-0,03	apical junction	CDH1/ CDH20
N-Cadherin	41982	suppressor	3/64/4057	1,25	0,160	3/56/2873	1,40	0,264	<0.0001	-0,18	apical junction	DSC1/ CDH15

Cad74A	27485	suppressor	3/51/3074	1,23	0,142	3/41/2226	1,31	0,161	0.0047	-0,09	apical	CDHR1
Cad87A	28716	no effect	3/42/2487	1,26	0,133	3/41/2226	1,31	0,161	ns	-0,09	Apical (other tissues)	CDH16/ CDHR1/ CDHR4
Cad99C	35037	suppressor	4/54/4075	1,12	0,094	4/62/4011	1,24	0,158	<0.0001	-0,14	apical	PCDH15
Cals	25839	enhancer	3/51/3305	1,30	0,194	3/56/4236	1,19	0,140	0.0001	0,07	unkown	CLSTN2
Kug/Fat2	40888	enhancer	3/61/3045	1,50	0,202	3/45/2740	1,26	0,157	<0.0001	0,22	basal	FAT3
Fas2	34084	no effect	3/43/1433	1,14	0,183	3/50/2011	1,22	0,181	ns	-0,09	lateral	NCAM1/2
Fas3	77396	no effect	3/47/2054	1,48	0,255	3/37/1301	1,48	0,290	ns	-0,06	lateral	CADM3/ NECTIN3/ PVR
Lachesin	28940	no effect	3/44/2400	1,46	0,232	3/55/3485	1,41	0,227	ns	-0,01	lateral	NEGR1
Contactin	28923	suppressor	3/56/3957	1,09	0,084	3/58/2903	1,22	0,132	<0.0001	-0,14	lateral	CNTN4/6
Echinoid	38243	enhancer	4/72/3630	1,46	0,216	4/76/3495	1,32	0,193	0.0017	0,10	apical junction	NPHS1
Neuroglian line 1	28724	enhancer	4/78/4536	1,25	0,171	4/70/3964	1,20	0,142	0.0069	0,05	lateral	L1CAM
Neuroglian line 2	37496	enhancer	3/46/1891	1,43	0,225	3/55/2082	1,34	0,197	0.0046	0,10	lateral	L1CAM
Neurexin IV	32424	enhancer	4/64/2544	1,51	0,264	4/67/2671	1,38	0,255	0.0082	0,14	lateral	CNTNAP2
betaPS/mys line 1	33642	no effect	4/48/2973	1,21	0,179	4/68/4275	1,26	0,169	ns	-0,06	basal	ITGB1

betaPS/mys line 2	27735	no effect	3/43/2602	1,24	0,182	3/42/2478	1,31	0,182	ns	-0,05	basal	ITGB1
alphaPS1/mew	27543	no effect	4/73/5180	1,38	0,210	4/69/4138	1,47	0,227	ns	-0,04	basal	ITGA6/ ITGA7
alphaPS3/scab	38959	no effect	3/58/4225	1,26	0,162	3/56/4236	1,19	0,140	ns	0,04	basal	ITGA4
Dystroglycan	34895	enhancer	4/73/2802	1,35	0,272	4/66/3514	1,22	0,147	0.0002	0,11	basal	Dg
UAS-Dg-C	63052	suppressor	3/42/3186	1,14	0,1624	3/43/2387	1,20	0,170	0.0031	-0,12	basal	Dg
LanA	28071	no effect	3/45/2198	1,29	0,160	3/72/3603	1,26	0,235	ns	-0,02	extracellular	LAMA5
LanB1	42616	no effect	4/68/3543	1,44	0,243	4/67/3696	1,40	0,246	ns	-0,01	extracellular	LAMB2
LanB2 line 1	55388	suppressor	4/47/3516	1,30	0,161	4/69/4247	1,36	0,215	0.0097	-0,10	extracellular	LAMC1
LanB2 line 2	62002	no effect	3/43/2894	1,36	0,167	3/56/3098	1,33	0,244	ns	-0,01	extracellular	LAMC1
Perlecan line 1	29440	enhancer	4/46/1905	1,50	0,251	4/72/4405	1,26	0,235	<0.0001	0,23	extracellular	HSPG2
Perlecan line 2	VDRC – 110494 (Vienna) **	enhancer	3/59/2190	1,74	0,251	3/60/3363	1,45	0,258	<0.0001	0,24	extracellular	HSPG2
Collagen IV	50895	no effect	3/43/2956	1,28	0,206	3/55/3987	1,25	0,216	ns	0,04	extracellular	COL4A1
Nidogen	62902	no effect	3/49/1821	1,23	0,199	3/50/1471	1,34	0,267	ns	-0,14	extracellular	NID1

Appendix B

Contribution to additional publications

Alterations in the expression or function of cell adhesion molecules are associated with tumorigenesis and tumor progression (Makrilia et al. 2009, Paredes et al. 2012). Therefore, to complement my studies on the function of cell adhesion molecules, I have also contributed to studies addressing the impact of P-Cadherin (CDH3) overexpression and E-Cadherin (CDH1) mutant versions that have been respectively identified in breast and gastric cancer. Here, I describe my specific contribution and summarize these publications, which were not included in the main text.

Germline *CDH1* G212E Missense Variant: Combining Clinical, *In Vitro* and *In Vivo* Strategies to Unravel Disease Burden

Figueiredo, J., Mercadillo, F., Melo, S., Barroso, A., Gonçalves, M., Díaz-Tasende, J., Carneiro, P., Robles, L., Colina, F., Ibarrola, C., Perea, J., Morais-de-Sá, E., Seruca, R. and Urioste, M. (2021), *Cancers (Basel)*, 13(17):4359. doi: 10.3390/cancers13174359.

Several *CDH1* germline mutations are known to be associated with a higher risk of developing a hereditary form of diffuse gastric cancer (Hereditary Diffuse Gastric Cancer) (Guilford et al. 1998, Corso et al. 2020). In this publication, we identified a novel *CDH1* mutation – G212E – in a large family strongly affected by diffuse gastric cancer, and developed a pipeline to characterize the deleterious phenotype induced by this genetic alteration, in order to provide the most appropriate clinical management for the affected subjects.

To evaluate the effect of the expression of the G212E mutation in epithelia, I generated transgenic flies expressing either human wild-type E-cadherin or the G212E mutation. Briefly, site-directed mutagenesis (c.635G>A) was performed to generate the pENTR-*CDH1* G212E vector, using the pENTR-*CDH1* vector as a template. Through recombination, *CDH1* WT and *CDH1* G212E from pENTR were inserted into a pPW-attB destination vector, constructed from the pPMW-attB and pPW vectors. *CDH1* WT and

CDH1 G212E transgenes were then inserted into the attP40 landing site via PhiC31 site-specific transgenesis (BestGene Inc), to ensure similar expression regulation of WT and mutated cadherin in *Drosophila.* I then performed clonal analysis in the *Drosophila* follicular epithelium, which showed that the G212E variant is weakly expressed at the plasma membrane, in comparison with wild-type human E-cadherin (Figure 5A in Figueiredo *et al.*, 2021, reproduced in Figure B1A). In addition, I found that egg chambers with G212E clones displayed a compromised architecture, with the epithelial tissue forming invaginations, even in clones with only two or three cells (Figure 5B in Figueiredo *et al.*, 2021, reproduced in Figure B1B). The disruption of epithelial architecture occurred in a large proportion of clonal cells expressing the G212E variant, whereas this was rarely observed upon expression of the wild-type E-cadherin (Figure 5B and 5C in Figueiredo *et al.*, 2021, reproduced in Figure B1B and B1C, respectively).

Furthermore, I assessed if the apical-basal organization of the *Drosophila* follicular epithelium was impaired in flies expressing the G212E variant. To this end, I performed immunofluorescence for an apical marker of the follicular epithelium – aPKC –, in egg chambers with clones for the G212E variant of E-cadherin. aPKC was shown to be decreased in G212E-expressing cells, in comparison with non-expressing cells within the same egg chamber (Figure 5D in Figueiredo *et al.*, 2021, reproduced in Figure B1D), which corroborates the damaging signature of this E-cadherin variant.

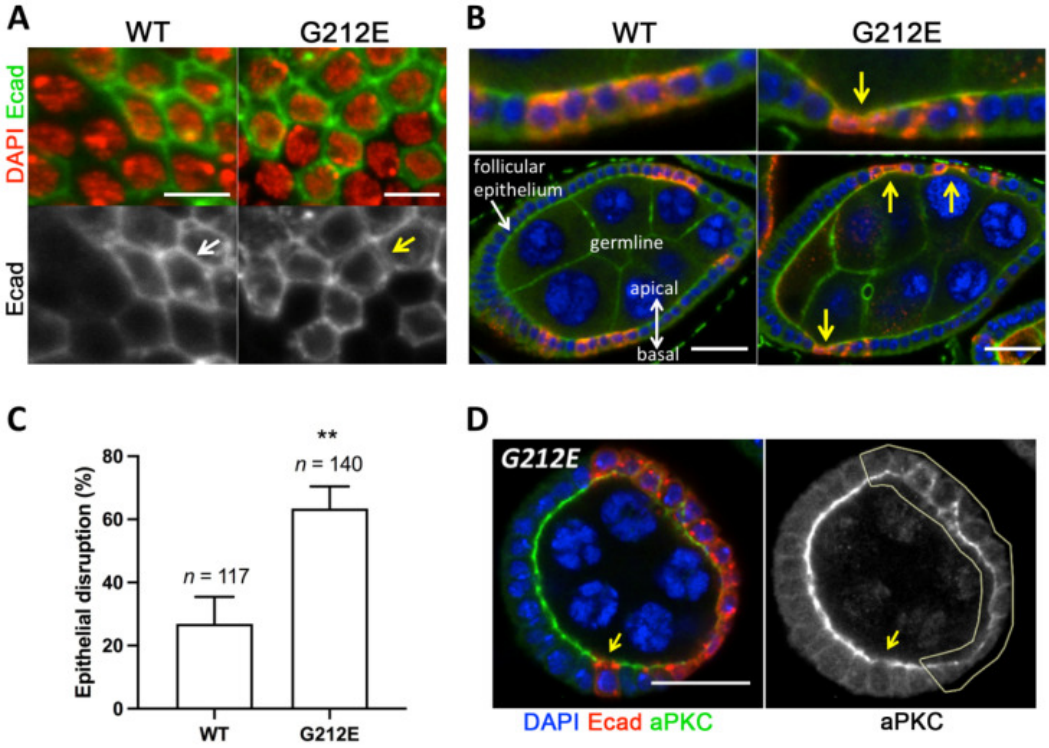


Figure B1. Structural features of human G212E variant in *Drosophila* epithelia. – *Figure 5 in Figueiredo et al., 2021.*

A) Surface views of the *Drosophila* follicular epithelium with mosaic clones expressing human wild-type *CDH1* and the G212E variant using the FLPout/tub-GAL4 system. Cells expressing G212E E-cadherin display weaker accumulation at the plasma membrane (arrows). E-cadherin is shown in green, and DAPI (red) labels the nucleus. Scale bars represent 5 µm.

B) Longitudinal view of egg chambers stained for E-cadherin (red), actin (green) and DAPI (blue). Clones expressing G212E lead to epithelial invaginations with consequent tissue disruption (arrows). Upper images are close-ups of the lower ones. Scale bars represent 20 µm.

C) Quantification of disrupted epithelial architecture caused by the overexpression of wild-type and mutant E-cadherin in the *Drosophila* follicular epithelium. Data derives from four independent experiments. For each experiment, around 30 clones were quantified per genotype (total number in the graph). Graph displays mean + SE; ** represents $p \leq 0.01$ for a two-tailed paired t-test.

D) Longitudinal section of an egg chamber with a large clone of cells expressing the G212E mutant (red) and stained for the apical marker aPKC (green). Loss of apical-basal polarized localization is evident even in a single cell overexpressing G212E (arrow). Scale bar represents 20 µm.

Integrin β1 orchestrates the abnormal cell-matrix attachment and invasive behaviour of E-cadherin dysfunctional cells

Figueiredo, J., Ferreira, R.M., Xu, H., Gonçalves, M., Barros-Carvalho, A., Cravo, J., Maia, A.F., Carneiro, P., Figueiredo, C., Smith, M.L., Stamenović, D., Morais-de-Sá, E. and Seruca, R. (2022), *Gastric Cancer*, 25(1):124-137. doi: 10.1007/s10120-021-01239-9.

In cancer, alterations in the E-cadherin gene (*CDH1*) or aberrant protein expression results in loss of cell–cell adhesion, increased cell invasion and metastasis, being associated with poor prognosis (Paredes et al, 2012). Besides the deregulation in cell-cell adhesion caused by direct alterations in E-cadherin, the invasive ability of cancer cells must also require an imbalance of adhesion with the extracellular matrix (ECM). Thus, in this publication, we aimed to understand if three E-cadherin variants known to be causative of a hereditary form of diffuse gastric cancer (Hereditary Diffuse Gastric Cancer) – A634V, R749W and V832M (Suriano et al. 2003, Kaurah et al. 2007, More et al. 2007) – affect the interplay of cancer cells with the ECM. Following a high-content screening approach to identify the ECM components playing a role in cell-matrix interactions in E-cadherin mutant cells, its functional significance was addressed *in vitro* and also *in vivo* using a *Drosophila* model I generated. Integrin β1 was identified as a key mediator of invasion in gastric cancer cells with E-cadherin dysfunction, and proposed as a potential biomarker of poor prognosis in gastric carcinomas.

For this work, I generated transgenic flies expressing either human wild-type E-cadherin or the R749W variant. Briefly, site-directed mutagenesis (c.2245C>T) was performed to generate the pENTR-*CDH1* R749W vector, using the pENTR-*CDH1* vector as a template. Through recombination, *CDH1* WT and *CDH1* R749W from pENTR were inserted into a pPW-attB destination vector, constructed from the pPMW-attB and pPW vectors. *CDH1* WT and *CDH1* R749W transgenes were then inserted into the attP40 landing site via site-specific transgenesis (BestGene Inc). These fly lines were used to assess the impact of expressing human E-cadherin in border cells (Figure 5A-5C in Figueiredo *et al.*, 2022), a cluster of cells that migrate from the anterior tip of the egg chamber to the border of the oocyte in stage 9 *Drosophila* egg chambers (Dai and Montell 2016), and also in the developing *Drosophila* eye (Figure 5D and 5E in Figueiredo *et al.*, 2022).

Taken together, the experiments on this paper suggest a crosstalk between βPS integrin/integrin β1 and E-cadherin, and pinpoint integrin β1 as a key player in cadherin-mediated invasion. This crosstalk was further validated in an *in vitro* gastric

adenocarcinoma cell model, that showed that an inverse correlation of E-cadherin/integrin β1 levels (low levels of E-cadherin and high levels of integrin β1) is associated with increased tumor grade and poor survival.

Activation of an actin signaling pathway in pre-malignant mammary epithelial cells by P-cadherin is essential for transformation

Faria, L., Canato, S., Jesus, T.T., Gonçalves, M., Guerreiro, P.S., Lopes, C.S., Meireles, I., Morais-de-Sá, E., Paredes, J. and Janody, F. (2023), *Disease Models & Mechanisms*, 16(2):dmm049652. doi: 10.1242/dmm.049652.

P-cadherin is known to be overexpressed in basal-like breast carcinomas and has been associated with aggressive tumor behavior in different types of carcinomas, namely breast, pancreatic and colorectal (Vieira and Paredes 2015). In this publication, we aimed to identify P-cadherin effectors relevant to its functional role in cancer and characterize the downstream signaling network of P-cadherin. To this end, we used a humanized *Drosophila* model with conditional expression of human P-cadherin for the identification of P-cadherin effectors *in vivo*. Two actin nucleators – Mrtf and Srf – were identified as suppressors of the P-cadherin-expressing wing phenotype in flies, and their role on the signaling cascade that induces malignancy was further characterized using a human mammary epithelial cell line with conditional activation of the SRC oncogene.

In basal-like breast carcinomas, P-cadherin overexpression is accompanied by E-cadherin over-expression (Paredes et al. 2005), two major cell adhesion molecules. Nevertheless, *Drosophila* epithelia only expresses E-cadherin, since flies possess the *CDH1* (that encodes for E-cadherin) but not the *CDH3* (that encodes for P-cadherin) gene. As a collaboration with Janody's and Parede's labs, I generated the transgenic fly strains carrying human P-cadherin inducible with the Gal4-UAS system, which are used in Figures 1-3 in Faria *et al.*, 2023. Briefly, the *CDH3* coding sequence was cloned into a pENTR vector, and subsequently inserted into a pPW-attB destination vector (as previously described). The *CDH3* transgene was then inserted into the attP40 landing site in *Drosophila*, via site-specific transgenesis (BestGene Inc).

Furthermore, I performed an immunofluorescence in the *Drosophila* follicular epithelium to assess the localization of both P-cadherin and E-cadherin, since we aimed to primarily understand if P-cadherin was able to establish cell-cell interactions similar to E-cadherin, as an approach to validate the humanized P-cadherin fly model (Figure 1A in

Faria *et al.*, 2023, reproduced in Figure B2). Indeed, we observed that P-cadherin co-localized with E-cadherin at the apical junctions. Clonal analysis showed that P-cadherin concentrates specifically at the cellular membranes juxtaposed to other P-cadherin-expressing cells, establishing trans-junctional homophilic interactions. Moreover, E-cadherin levels or localization do not seem to be affected by P-cadherin expression in the follicular epithelium.

This collaboration allowed for the use of the humanized P-cadherin fly model as a platform for the functional exploration of P-cadherin effectors *in vivo*, identifying Mrtf and Srf as P-cadherin effectors in *Drosophila*. The P-cadherin-downstream signaling pathway was then dissected using a human breast epithelial cell line, which recapitulates the molecular events that occur during the transition from a normal to a malignant state. Overall, the authors were able to demonstrate that P-cadherin plays a central role in the very early stages of breast carcinogenesis, through the two identified effectors, being essential for the acquisition of pre-malignant and malignant phenotypes.

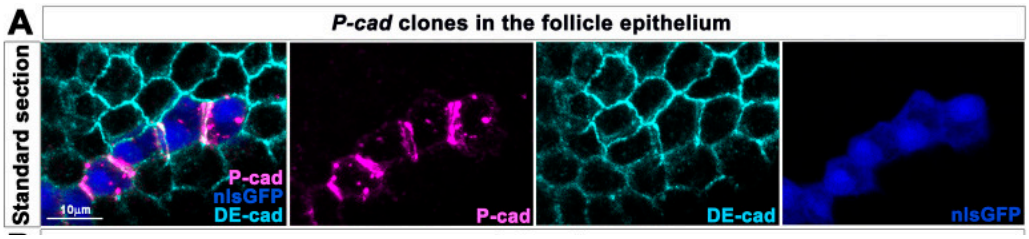


Figure B2. Human P-cadherin (P-cad) colocalizes with *Drosophila* E-cadherin (DE-cad) apically in *Drosophila* epithelia and affects wing development through DE-cad, Src64B and αPS2 integrin. *– adapted from Figure 1 in Faria et al., 2023*

A) Standard confocal sections of P-cad-expressing clones marked with nuclear GFP (nlsGFP; blue) in the follicle epithelium of a stage 6 egg chamber, stained with anti-DE-cad (cyan) and anti-P-cad (magenta). Scale bar: 10 µm.

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Appendix C

This appendix contains the original publication resulting from the work of this thesis, which can be found in EMBO Reports:

Gonçalves, M., Lopes, C., Alégot, H., Osswald, M., Bosveld, F., Ramos, C., Richard, G., Bellaiche, Y., Mirouse, V. and Morais-de-Sá, E. (2024) 'The Dystrophin-Dystroglycan complex ensures cytokinesis efficiency in *Drosophila* epithelia', 26(2):307-328. doi: 10.1038/s44319-024-00319-y.