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DISSECTING DYSTROPHIN'S ROLES, SUBCELLULAR ORGANIZATION, AND FUNCTIONAL NETWORK IN DROSOPHILA OOGENESIS

MINA AMINI MOGHADDAM

76 Pages

Mutations in the giant actin-membrane linker protein Dystrophin (Dys) are the cause of Duchenne Muscular Dystrophy (DMD). In the *Drosophila* model system, the highly conserved Dys protein is not required for viability but is required for proper wing and ovarian development. *Dys* mutations produce two visible phenotypes: posterior crossveins are detached from the longitudinal veins, and in oogenesis the developing eggs fail to elongate properly. This provides an opportunity to explore the less-understood cellular and developmental roles of Dys and gain a new insight into how tissues adopt their correct shapes.

Animals from worms to flies to humans express multiple Dys isoforms that vary in length; the long forms contain the actin-binding domains (ABD) connecting the cortical F-actin to the extracellular matrix (ECM). The short forms however, lack the ABD and should not be able to act as membrane-actin linkers, but could scaffold and regulate signaling pathways associated with Dys. Probing the functional differences of these isoforms is important not only for basic cell and developmental biology but also for understanding DMD and treating it with truncated or partially restored proteins. We characterized a transposon insert in the Dys coding region which only removes the long isoforms. Remarkably, it behaves as wild type for the egg elongation phenotype but is null for the wing phenotype. A *Dys* allele with a GFP exon, which labels the long forms but not the shortest class of isoforms, shows Dys is organized in a plane polarized striated pattern, and associates with F-actin in a dynamic, stage-dependent manner. A Dys monoclonal antibody labels all the isoforms and detects a similar but extended striated pattern of Dys. Since each tag labels no more than one point along the protein, the pattern implies multiple copies of Dys are aligned, and two or more isoforms are coordinated in these structures.

Dys null mutants lose F-actin plane polarization in the basal follicle cell epithelium, and gain excessive cell surface projections. Germline and muscle RNAi against Dys do not trigger the typical

Dys phenotypes. However, follicle cell-specific depletion of Dys results in short eggs and disorganized actin at the egg's exterior indicating Dys is required in the follicle cells for proper morphogenesis.

Dys and another structurally related member of the spectrin-repeat superfamily called beta-Heavy spectrin (β H-Spec), overlap in their developmental functions. More specifically, *β H-spec*; *Dys* double mutants show high lethality, but also fused cysts, demonstrating a novel functional network at the cell cortex involving the spectrins and Dys. Together, these results highlight previously unknown aspects of functional and subcellular organization for Dys which is critical for our understanding of development and for regenerative medicine.

KEYWORDS: Dystrophin; muscular dystrophy; DGC; spectrin repeat superfamily; *Drosophila*; egg chamber; morphogenesis; ECM; F-actin; stress fiber; Laminin; integrin; focal adhesion

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FUNCTIONAL NETWORK IN DROSOPHILA OOGENESIS

MINA AMINI MOGHADDAM

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Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

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MINA AMINI MOGHADDAM

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M.A.M.

DEDICATION

I would like to dedicate this thesis to my loving family, Shirzad, Nazkhatoun, Shahram, Mohammad, Mehdi, and Mahdieh. I would also like to dedicate my work to those affected by the events of June, 2020. Here is a poem by a Persian poet, Saadi, whom I admire dearly:

*Human beings are members of a whole,
In creation of one essence and soul.
If one member is afflicted with pain,
Other members uneasy will remain.
If you have no sympathy for human pain,
The name of human you cannot retain.*

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CHAPTER I: INTRODUCTION

Dystrophin (Dys) is a large cytoskeletal protein which provides a direct mechanical link between the extracellular matrix (ECM) and cortical actin filaments (Allen et al., 2016; Campbell, 2008). Dys is enriched in muscle cells where it is hypothesized to support muscle integrity during contraction. Two prevalent ideas in the literature support this hypothesis. First, given its actin-to-ECM linking function, Dys is expected to act as a “shock absorber” for the sarcolemma, protecting muscle fibers against contraction-induced injury (Houang et al., 2018; Townsend et al., 2007; Clafin and Brooks, 2008). Second, Dys scaffolds a series of proteins involved in intracellular calcium and oxidative homeostasis and so, its deficiency results in abnormal function of these proteins, triggering damage pathways and eventually myocyte death (Allen et al., 2010). In addition to the muscle, Dys expression in the brain has been characterized in both humans and animal models, and an increasing body of evidence underscores its important roles in the nervous system and cognitive health (Doorenweerd et al., 2017; Pilgram et al., 2010). Decades of research have allowed major breakthroughs into the structural and functional properties of Dys proteins in the context of muscle dystrophies. Yet, the non-muscle roles of Dys and its vertebrate paralog Utrophin have remained largely elusive.

In the following chapter, I summarize the structure of the Dys complex in the muscle, and how its various structural domains allow Dys to act both as a membrane stabilizer and as a scaffold protein. Then I will focus on understanding the roles of Dys proteins outside the muscle and nervous tissues, an area that has been mainly understudied. In the current study, we use a 3D model of tissue morphogenesis, the *Drosophila* egg chamber system, in an attempt to shed light on the structure-function properties of Dys in developing egg chambers. I will conclude this chapter by providing a brief summary of our findings on the developmental roles of Dys, and our major discovery that long forms of Dys are dispensable for proper egg chamber morphogenesis.

Loss-of-function mutations in the *DMD* gene, encoding Dystrophin (Dys), are associated with Duchenne Muscular Dystrophy (DMD), which has the second highest incidence of all inherited diseases, affecting 1 in 3600-6000 live male births worldwide (Anderson et al., 2002; Fairclough et al., 2013; Bushby et al., 2010; Passamano et al., 2012; Lim et al., 2017; McGreevy et al., 2015; Pilgram et al., 2010; Zhou and Lu, 2010). DMD is characterized by progressive muscle wasting, cognitive

impairment, cardiac dysfunction, pulmonary complication, and premature death (Anderson et al., 2002; Sacco et al., 2010; Fairclough et al., 2013; Passamano et al., 2012; Lim et al., 2017; Zhou and Lu, 2010; Adamo et al., 2010; Pilgram et al., 2010). In contrast, Becker Muscular Dystrophy (BMD) is usually associated with much milder symptoms compared to DMD and is caused by in-frame mutations of *DMD*, resulting in expression of a truncated but partially functional Dys protein (Doorenweerd et al., 2017; Nelson et al., 2016; Fairclough et al., 2013; Lim et al., 2017; McGreevy et al., 2015).

While Dys has been molecularly characterized for 30 years, there is currently no cure for DMD (Kunkel et al., 1985; Ray et al., 1985; Koenig et al., 1988; Anderson et al., 2002; Deconinck and Dan, 2007; Chamberlain and Benian, 2000; Sacco et al., 2010; Miura and Jasmin, 2006). Because of the genetic nature of the disease, gene therapy is a promising option for its treatment. However, the enormous size of the Dys cDNA presents a challenge to gene delivery. As a result, some therapeutic strategies aim to generate BMD-like Dystrophins such as mini/micro-dystrophin genes which are compatible with the packaging capacity of adeno-associated virus (AAV) vectors for gene delivery (Harper et al., 2002; Banks et al., 2007; Nelson et al., 2016; Gao and McNally, 2011; Fairclough et al., 2013; McGreevy et al., 2015). To properly evaluate such modified Dys proteins, we need a better understanding of the basic cellular and developmental roles of Dys and how these functions map to its structure.

Dys belongs to the spectrin repeat superfamily of proteins (Koenig and Kunkel, 1990; Liem, 2016) and contains 4 structural regions, as diagrammed in Figures 1 and 2: (i) The amino-terminal Actin-Binding Domain (ABD) contains a pair of calponin homology (CH) modules that can bind filamentous actin (F-actin) (Winder et al., 1995; Korenbaum and Rivero, 2002; Gao and McNally, 2011; Constantin, 2014); (ii) The central rod domain contains 24 homologous triple helical spectrin-like repeats that are interrupted by four flexible hinge regions and are thought to confer flexibility to the protein and act as “shock absorbers”. However, these spectrin repeats are not merely structural components and can anchor signaling molecules such as neuronal nitric oxide synthase (nNOS) (Lapidos et al., 2004; Allen et al., 2016; Gao and McNally, 2011; Nichols et al., 2015; Potikanond et al., 2018; Constantin, 2014); (iii) The third region bears a WW domain involved in intracellular signalling through the recognition of proline-rich or phosphorylated linear peptide sequences. Adjacent to the WW domain, there is a cysteine-rich domain with two EF-hand motifs

and two ZZ-type zinc finger (ZZ) modules in series. The WW domain and cysteine-rich domain bind to proline residues on the carboxy-terminal cytoplasmic side of β -Dystroglycan (β -DG) (Ilsley et al., 2002; Rentschler et al., 1999; Ponting et al., 1996; Gao and McNally, 2011; Constantin, 2014); (iv) The carboxy-terminal domain of Dys contains two α -helical coiled coil regions forming the binding site for Dystrobrevin (Dbr) (Sadoulet-Puccio et al., 1997; Blake et al., 1995; Gao and McNally, 2011; Constantin, 2014; Allen et al., 2016).

In muscle, Dys is a cortical component of a large protein complex known as dystrophin-glycoprotein complex (DGC) which serves as an important link between the cortical cytoskeleton and the extracellular matrix (ECM) (Fig 1). More specifically, Dys is linked to the ECM through its β -DG binding site and to the cytoskeleton through its amino-terminal ABD (Ervasti and Campbell, 1991; Sadoulet-Puccio et al., 1997; Pilgram et al., 2010; Gao and McNally, 2011; Constantin, 2014; Allen et al., 2016). The protein β -DG has a single transmembrane domain spanning the plasma membrane and an amino-terminal extracellular domain binding to the highly glycosylated extracellular protein α -Dystroglycan (α -DG) (Kanagawa et al., 2004). The glycoepitope of α -DG mediates the binding of ECM components such as Laminin (Kanagawa et al., 2004). The ABD of Dys binds the cortical F-actin and thus completes the bridge between the cytoskeleton and ECM (Way et al., 1992; Constantin, 2014; Allen et al., 2016). Due to this linkage, the DGC is thought to have a “mechanical” role providing structural stability to muscle fibers during muscle contraction. This complex is also proposed to act as a hub for multiple regulatory and cell communication functions, due to bearing multiple binding sites and domains which scaffold a variety of cell signaling proteins (Constantin, 2014; Lapidus et al., 2004; Gao and McNally, 2011; Allen et al., 2016). For instance, the ZZ modules in the cysteine-rich domain of Dys may represent a functional calmodulin-binding site which could modulate the binding of other Dys-associated proteins in a calcium-dependent manner (Anderson et al., 1996; Tommasi di Vignano et al., 2000; Constantin, 2014). Moreover, the DG complex might participate in the transduction of extracellular-derived signals or transfer of information between DG and other signalling pathways (Constantin, 2014). β -DG is also shown to be involved in mitogen-activated protein kinase (MAPK) and Rac1 small GTPase signalling (Spence et al., 2004; Yang et al., 1995; Oak et al., 2003). In addition, the DGC associates with 4 Syntrophin proteins through syntrophin binding sites located at the carboxy-termini of Dys and Dbr, providing a scaffold for multiple signaling proteins. Interactions between syntrophins and

many other proteins such as ion channels, G protein-coupled receptor, and NOS occur through the Pleckstrin homology (PH) and PDZ domains of Syntrophin, resulting in multi-protein complexes for efficient signal transduction (Brenman et al., 1996; Hasegawa et al., 1999; Iwata et al., 1998; Oak et al., 2001; Nakamori and Takahashi, 2011; Constantin, 2014; Allen et al., 2016).

In humans, the *DMD* gene contains seven independent tissue-specific promoters which produce multiple isoforms, with their names reflecting their length and splicing patterns. More specifically, three independent 5' promoters and four internal promoters give rise to three long transcripts and four shorter carboxy-terminal transcripts, respectively. In contrast to shorter isoforms which lack the amino-terminal ABD, Dys Longer isoforms bear ABD and a variable number of spectrin repeats. However, all isoforms invariably bear the DG-binding site and the highly conserved carboxy-terminal region (Pilgram et al., 2010; Jin et al., 2007; Constantin, 2014; Neuman et al., 2005; Doorenweerd et al., 2017). Thus, short isoforms should not be able to act as membrane-actin linkers, in the manner of full length Dys, but could scaffold or regulate signaling pathways associated with the DGC. Understanding this diversity of isoforms is critically important both for basic cell/developmental biology and to understand and treat DMD. Yet, our understanding of the functions of individual Dys isoforms is very limited, mainly due to the complexity of the *DMD* gene expression and the presence of multiple isoforms.

Hundreds of studies have focused on determining the functions of Dys in muscle and neurons, in an attempt to elucidate whether the DGC only plays a mechanical role in stabilizing the sarcolemma during normal contractions, or its docking sites for signaling proteins impart functions important in muscular dystrophies (Gumerson and Michele, 2011). More recent works used transcriptomic data and analyzed the expression patterns of Dys isoforms in the brain to characterize the role of Dys in DMD-associated cognitive phenotypes (Doorenweerd et al., 2017). Although understanding the distinct roles of Dys isoforms in muscle and neurons remains an active area of investigation, it is also crucial to investigate these roles outside muscle and neurons, an area that has been mostly unappreciated.

Metazoans including *Drosophila melanogaster* have a single gene for Dys protein whereas humans have 3 genes: Dys, its close paralog Utrophin, and a further paralog, DRP2 (Roberts and Bobrow, 1998; Wang et al., 1998; Neuman et al., 2005; Pilgram et al., 2010). The *utrophin* gene was discovered as an autosomal paralog of the *DMD* gene (Love et al., 1989). Similar to Dys, Utrophin can interact

with F-actin through its amino-terminal ABD containing two tandem CH domains (Prochniewicz et al., 2009). It also contains a middle rod domain with 22 spectrin repeats (Rybakova and Ervasti, 2005). In Utrophin, the amino-terminal ABD and the first 10 spectrin repeats make a single continuous actin-binding site, whereas in Dys the spectrin repeats 11-15 form a second ABD in the rod domain (Prochniewicz et al., 2009). The carboxy-terminal ends of Utrophin and Dys also show structural resemblance. Thus, Utrophin is capable of binding the same proteins as Dys interacting partners such as beta-DG, Dbr, and Syntrophins (Matsumura et al., 1992; Winder et al., 1995; Ervasti, 2007). Due to the structural similarities and common binding partners, Dys and Utrophin are thought to have some functional redundancy in membrane stabilization. In support of this notion, mice lacking both Utrophin and Dys exhibit a more severe muscle dystrophy. Moreover, the transgenic overexpression of Utrophin in Dystrophin-deficient mice is shown to prevent the development of muscular dystrophy (Deconinck et al., 1997; Grady et al., 1997; Tinsley et al., 1998).

Although it is suggested that Utrophin can functionally compensate for the lack of Dys, there are evidence for significant differences in their functional capacities. For instance, as opposed to Dys, Utrophin cannot anchor nNOS to the sarcolemma (Li et al., 2010). Utrophin expression is regulated by two different promoters, A and B, which give rise to two full-length isoforms, named A-Utrophin and B-Utrophin with unique N-termini (Weir et al., 2002). Isoform A is present at the sarcolemma of normal fetal muscle fibers and in DMD muscle, form A is expressed throughout the sarcolemma while the expression of isoform B is confined to the vascular endothelium (Weir et al., 2002; Sewry et al., 2005; Janghra et al., 2016). Understanding of Utrophin roles in the skeletal muscle has gained a prominent attention. Nonetheless, clear functional roles for Utrophin in non-muscle tissues remain largely unclear. Given invertebrate animal models such as *C.elegans* and *Drosophila* have a single Dystrophin/Utrophin ortholog, their functions may reflect roles played by both Dystrophin and Utrophin in mammals. In *Drosophila*, while Dys protein is not required for viability and fertility, it is critical for proper wing and ovarian development (Christoforou et al., 2008; Mirouse et al., 2009). Collectively, these features make *Drosophila* a promising model system to better understand the cellular and developmental roles of Dys.

In *Drosophila*, the *Dys* locus produces multiple isoforms (Fig. 2 & Table 1), analogous to human Dys proteins. For instance, the three distinct skeletal muscle-specific (Dp427M), brain-specific (Dp427B), and Purkinje cells-specific (Dp427P) long isoforms in human/mice are similar to long

isoforms in *Drosophila* (Dys-PA, Dys-PC, Dys-PF, Dys-PG, Dys-PH, Dys-PI, Dys-PK). The 4 shorter isoforms in human/mice include Dp260, Dp140, Dp116, and Dp71 (Pilgram et al., 2010). The short isoforms in *Drosophila* include Dys-PL, Dys-PB, Dys-PD, Dys-PJ, and Dys-PE (for more details, see Table 1).

The *Drosophila* Dys-PA isoform is full-length and expected to carry out both mechanical and signaling functions. Previous studies show Dys-PA is expressed in the muscle but is not required for muscle integrity. Instead, it is required at the post-synaptic side of the neuromuscular junction for retrograde control of neurotransmitter release (van der Plas et al., 2006). *Drosophila* Dp186, a CNS-specific Dys isoform, retains the carboxy-terminal scaffolding region but lacks the amino-terminal ABD. Notably, generation of Dp186-specific deletions revealed its role in retrograde modulation of presynaptic release at motorneuron-interneuron synapse, independent of Dys-PA (Fradkin et al., 2008). Surprisingly, the muscle-specific Dys-PE/Dp117, lacking the ABD (see Figure 2), plays an important role in stabilizing the muscle structure (van der Plas et al., 2007). Strikingly, mesodermal expression of the carboxy-terminal non-muscle mammalian Dys isoform, Dp116, rescues the dilated cardiomyopathy in flies lacking Dys long forms (Dystrophins with ABDs) but retaining the naturally occurring muscle-specific Dp117 (Taghli-Lamalle et al., 2008). In contrast, other studies suggest the expression of Dp116 does not prevent and/or ameliorate the severe dystrophic muscle phenotype in mice (Judge et al., 2011). It was further shown that either ‘mechanical’ or ‘signaling’ Dys constructs rescue systolic function and heart contractility in *Drosophila* (Taghli-Lamalle et al., 2014). These findings not only indicate that the behaviors of Dys isoforms are complex but also call for more extensive research on distinct functions of these isoforms outside muscle and neurons to further our understanding of Dys structure-function, isoform diversity, and smaller dystrophins including those used for DMD therapy purposes.

The *Drosophila* egg chamber system, composed of a germline cyst encased by a single layer of somatic follicle cell epithelium (FCE) (Gates, 2012; Horne-Badovinac, 2014; Jia et al., 2016), serves as an elegant epithelial model to study Dys roles, subcellular organization, and functional network during development (see Figure 3). Each egg chamber buds from the germarium near the ovary’s anterior to join an array of progressively older egg chambers termed the ovariole, and proceeds through 14 morphological stages (Gates, 2012; Horne-Badovinac, 2014). Although initially spherical, egg chambers elongate along their anterior-posterior (A-P) axes as they mature through

stages 5-10 (Horne-Badovinac, 2014; Andersen and Horne-Badovinac, 2016). The follicle cells (FCs) are polarized as their apical sides contact the germ cells (15 nurse cells (NCs) and one oocyte) while their basal sides face the basement membrane (BM) ECM (Horne-Badovinac, 2014; Isabella and Horne-Badovinac, 2016). These follicle cells themselves produce the underlying BM (Horne-Badovinac, 2014; Crest et al., 2017) which surrounds the entire egg chamber and is shown to change its architecture coincident with egg chamber elongation. Indeed, between stages 5 and 8, newly-synthesized BM proteins such as Type IV Collagen (Col IV), Laminin, and Perlecan exit through a basal region of FC lateral membranes and aggregate in the pericellular space between FCs before they incorporate into the preexisting planar BM as fibrils, perpendicular to the elongation axis (Isabella and Horne-Badovinac, 2016).

The formation of aligned BM fibrils is reported to be dependent on a collective migration of FCs along the stationary BM which rotates the entire egg chamber in the direction of fibril polarity (Haigo and Bilder, 2011). In addition to polarizing the BM, egg chamber rotation is also required for parallel alignment of basal actin bundles at each FC's basal surface (Cetera et al., 2014). Due to the epithelium's closed topology, the tissue-level organization of BM fibrils and parallel actin bundles is thought to form a circumferential "molecular corset" around the egg's exterior, perpendicular to the A-P axis (see Figures 3 & 4).

This "molecular corset" is thought to resist an internal pressure conferred by the germline cyst growth to bias elongation through the A-P axis (Andersen and Horne-Badovinac, 2016; Cetera et al., 2014). In addition to the BM fibril formation, the stage-specific regulation of BM protein levels also modulates egg chamber elongation as Col IV and Perlecan levels in the BM are shown to increase and decrease during egg chamber elongation, respectively (Isabella and Horne-Badovinac, 2015). Prior works suggest mechanical anisotropy along individual BM fibrils per se is not sufficient to resist the isotropic tissue growth in an anisotropic fashion but it is the circumferentially even distribution of fibrils that promotes egg elongation (Crest et al., 2017). Yet, follicles that are wild type for BM fibril polarity but are deficient in differential A-P BM levels also fail to elongate (Crest et al., 2017). Thus, for elongation to occur properly, the BM needs to be anisotropic on a 'global' tissue-wide scale, resulting from morphogen-regulated mechanical properties (Crest et al., 2017).

Along with the "molecular corset", a tube of muscle known as the ovariole muscle sheath surrounds each ovariole within which the egg chambers freely move toward the oviduct via its

rhythmic contractions (Andersen and Horne-Badovinac, 2016). This tissue is also shown to play a role in egg elongation by promoting germ cell growth. For example, a hypo-contractile muscle sheath results in defective yolk uptake by the oocyte and thus egg chamber shortening. In contrast, a hyper-contractile muscle sheath is likely to exert increased exogenous circumferential compression to the epithelium and thus lead to premature and enhanced egg elongation (Andersen and Horne-Badovinac, 2016). Together, egg chamber elongation occurs through cellular influences provided by different cell types including somatic FCs, germ cells, and ovarian muscles, and also through extracellular influences conferred by a globally anisotropic polarized BM.

In the present study, we use the *Drosophila* egg chamber system to explore cellular and developmental roles of Dys and its isoforms, and gain a new insight into how tissues adopt their correct shapes. We provide evidence that Dys long isoforms and their actin-ECM linker function are not required for egg elongation, contrary to expectations. Instead, the presence of carboxy-terminal short forms which act as scaffolds at the DGC, is sufficient to promote elongation. In contrast, we find that short isoforms fail to rescue the wing phenotype associated with *Dys* null mutations, the partial loss of posterior crossvein (PCV). This suggests Dys long isoforms are critical for proper fly wing vein patterning, a developmental program that was previously reported to involve Dys roles in a Notch and TGF β -dependent completion of PCVs (Christoforou et al., 2008).

Prior to this study, a detailed analysis of Dys localization in *Drosophila* oogenesis was lacking. Schneider et al. (2006) generated an antibody against Dys and found Dys was basally localized in the FC layer imaged in a cross section. Here, to probe for the subcellular organization of Dys and its isoforms in more details, we used two different visualizing methods : (i) a Dys[GFP] protein trap in the endogenous *Dys* locus with its GFP exon labeling all isoforms except for Dys-PE/PJ, and (ii) a mammalian Utrophin antibody, detecting the conserved second coiled coil domain of the carboxy-terminal region and thus all isoforms (Table 1, Fig 2).

We find Dys[GFP] signals show a striking plane-polarized striated pattern at the basal FCE, co-oriented with but distinct from nearby actin bundles in a stage-dependent manner. Moreover, in egg chambers co-expressing Dys[GFP] and RFP-tagged Zyxin which labels focal adhesions, RFP-Zyxin colocalizes with the termini of plane-polarized integrin-dependent F-actin stress fibers, and associates with Dys[GFP] signals in a dynamic matter. Anti-Dys signals show a broader but similar pattern of basal striations within which the Dys[GFP] signals are located. The striated

distribution of the carboxy-terminal Mab epitope suggests the carboxy-termini are engaged with Dg complexes that spread out evenly along strands of ECM in the BM. The observation that GFP labels only a portion of these strands could mean that the longer, GFP-tagged isoforms have a more limited distribution, while the shortest class Dys-PE/PJ is found all along the strands. Alternatively, the carboxy-termini are arranged along the strands while the central portions of the same proteins are “gathered” by interactions with each other or with binding partners.

In addition to the basal sides of FCs, we show that Dys[GFP] signals also localize to the NC membranes but do not overlap with the cytoplasmic actin cables involved in NC dumping. Dys[GFP] is also detected in the membranes of ovariole muscle sheath although much less abundant than in the basal membrane of FCE. Midway through oogenesis, FCs start to migrate posteriorly to transform a homogenously cuboidal epithelium to a columnar and squamous epithelium over the oocyte and NCs, respectively. Intriguingly, we find that Dys[GFP] signals accumulate in novel trailing-edge projections in the spreading FCs overlaying NCs (NCFCs hereafter) which have minimal F-actin. Hence, in *Drosophila* oogenesis, Dys is a membrane-bound protein but does not generally colocalize with all F-actin.

We and others show *Dys* loss-of-function mutations result in rounded rather than elongated eggs. A recent study finds that the elongation defect in *Dys* null mutants does not stem from defective egg chamber rotation as mutant epithelia rotate normally with a velocity comparable to wild type (WT) follicles. Instead, it shows loss of Dys decreases the number and length of BM fibrils although the lateral secretion of ECM proteins seem to be normal in *Dys* nulls (Campos et al., 2020). Therefore, how Dys contributes to BM fibril deposition remains to be determined. The same study finds that in early oogenesis, F-actin stress fibers maintain their global orientation perpendicular to the A-P axis in *Dys* nulls, but take longer to achieve their maximum alignment at stage 8. In late oogenesis, *Dys* null follicles show a loss of stress fiber planar polarization similar to WT follicles, but fail to regain the proper orientation as WT follicles do at stage 13. Furthermore, their results show that inhibition of Dg in FCs in a time frame after BM fibril deposition and before regaining the correct stress fiber orientation at later stages, disrupts the late phase of stress fiber orientation and results in short eggs (Campos et al., 2020). These data indicate BM fibrils alone are insufficient to promote elongation. Early depletion of Dg in FCs also affects the late phase of F-actin alignment and elongation. Interestingly, the authors find that late expression of Dg in a *Dg*

mutant background fails to rescue the late phase of stress fiber orientation. Instead, late expression of *Dys* (25°C to 18°C, *Dys*-RNAi) can rescue egg elongation and stress fiber orientation only if the BM fibrils are restored (by Rab10 overexpression) (Campos et al., 2020). Together, they conclude the early function of DGC in BM fibril deposition serves as a planar polarity cue for its late function in F-actin stress fiber orientation.

Our results using different combinations of *Dys* null alleles recapitulate these defects in egg elongation, basal F-actin alignment, and BM fibril deposition. Yet here, we attribute these phenotypes to distinct *Dys* isoforms and provide evidence that *Dys* short isoforms are sufficient to promote these morphological events. In addition, we observe a novel phenotype in *Dys* null epithelia which is increased cytoskeletal activity. We find that in *Dys* nulls, NCFCs display signs of wound-healing related cell motility with excessive actin-based membrane sheets (lamellipodia) and spikes (filopodia) projecting from cell surfaces. Of note, NCFCs are rich in F-actin and normally spread to become squamous midway through oogenesis but are otherwise quiescent cells, showing no motility or cell division. Strikingly, WT egg chambers can display the same phenotype when the egg chamber becomes damaged during dissection, altering its distribution of tension (Martinelli et al., 2013). The ectopic production of cell surface projections may be due to a requirement for *Dys* either cell-autonomously, or in the underlying NCs. For example, either damage or loss of *Dys* could alter the tension and cortical stiffness in the germ cells which in turn signals “distress” to the overlying FCs to promote a wounding response. In this study, we test this hypothesis using tissue-specific knockdown of *Dys*.

Finally, given the important roles of *Dys* in oogenesis, we will explore its functional network during egg chamber development. We will demonstrate that *Dys* and a structurally related member of the spectrin-repeat superfamily, β -heavy-spectrin (β H-Spec), overlap in their developmental functions. Our findings and other works point to a novel functional network in cortical organization involving the spectrins, *Dys*/Utrophin, and PTP-Pez (Hippo/Yorkie pathway) (Edwards et al., 2001) (see Discussion).

Figure 1: The dystrophin glycoprotein complex (DGC) in skeletal muscle.

Dystrophin forms two binding sites for cortical F-actin: an amino-terminal actin-binding domain (ABD) and spectrin repeats 11-15 located at the middle rod domain. The rod domain contains 24 spectrin repeats (numbered) and 4 hinges (H1-H4). Repeats 20-23 interact with microtubules. The DBD of dystrophin is composed of a WW domain and a cysteine-rich domain and links dystrophin to the cytoplasmic tail of β -dystroglycan, (β -DG). (β -DG) binds the extracellular subunit of dystroglycan, (α -DG), which mediates the binding of ECM proteins such as Laminin and Perlecan. The carboxy-terminal domain of dystrophin forms binding sites for additional DGC subcomplexes including dystrobrevin (Dbr) and syntrophins through the coiled coil domains (CC) and syntrophin-binding sites, respectively. The DGC association with syntrophins provides a scaffold for multiple signaling proteins such as neuronal nitric oxide synthase (nNOS) which recruits to spectrin repeats 16-17. Other DGC components include the sarcoglycan complex (α , β , δ , and γ subunits) and sarcospan. Sarcoglycans and sarcospan do not bind to dystrophin directly but strengthen the entire DGC. The transmembrane protein integrin (α/β subunits) is not a part of the DGC, and connects actin filaments to the ECM at focal adhesion sites. Zyxin protein is a component of focal adhesions where it interacts with cortical F-actin but does not bind it directly.

Figure 2: Dystrophin schematic sequence map and isoforms in *Drosophila*.

CH, twin Calponin Homology (CH) domain that binds F-actin. The triangles match the 24 human spectrin repeats with darker green indicating higher levels of conservation. Bracket indicates the main block of non-conservation versus human Dys. The flexible hinge regions are numbered (h1, h2, and h3). The blue graph shows the output of the Coils program with the peaks representing coiled coil propensity. Asterisks correspond to the helix-breaking motifs containing closely spaced Pro/Pro or Pro/Gly common in turns and flexible regions. The region matching spectrin repeats 14 and 15 shows no evidence of homology and does not conform to predictions for α -helical triple coil and might be instead selected for flexibility. Dys isoforms contain unique sequences at the amino-terminal region but carry a conserved carboxy-terminal region. In contrast to the long isoform (Dys-PA/DLP2), short isoforms (Dys-PB/Dp186, Dys-PD/Dp205, and Dys-PE, PJ/Dp117) lack the amino-terminal actin-binding domain. Short isoforms bear a variable number of spectrin repeats and unique large exons (blue, magenta, and cyan blocks). All isoforms contain the binding site for Dystroglycan (a WW domain followed by a cysteine-rich domain (CR)) and coiled coil (CC) domains. In the *Dys*[GFP] allele, GFP coding region is inserted into the beginning of a conserved region in spectrin repeat 22 (indicated). Among all the isoforms, only the shortest forms, Dys-PE and Dys-PJ, bypass the GFP exon in *Dys*[GFP]. Insertion of MB02524 destroys a large exon encoding most of the amino-terminal third of long isoforms and thus does not affect the short isoforms. The monoclonal anti-Dys Mab MANCHO17 binds the second coiled coil domain (CC2) and therefore detects all isoforms.

Figure 3: Overview of *Drosophila* oogenesis.

(A) Each *Drosophila* ovary consists of 16-18 ovarioles. (B) Each ovariole is a developmental array of egg chambers. At the anterior tip of the ovariole, a region called the germarium is the site for egg chamber assembly. Here, different cell types arise from stem cell populations and then organize into an egg chamber with a cluster of germ cells (1 oocyte and 15 nurse cells (NCs)) ensheathed by a monolayer of follicle cells (FCs). The budding of egg chamber from the germarium marks stage 1 of oogenesis which is divided into 14 morphologically distinct stages. The budding egg chambers are spherical through stage 5 when they start elongating along their anterior-posterior (A-P) axes. Tissue-level actin bundle alignment is established in the germarium and inherited by the budding egg chamber. The early rotation of the egg chamber through stages 1-5 maintains this alignment. The late phase of rotation during stages 5-8 is not required for actin alignment but allows the fibrillar organization of the basement membrane (BM). Basal actin bundles lose their tissue-level alignment during stages 10B-11 but regain their proper orientation at around stage 12.

During stages 9-10, a group of 6–8 cells called border cells (BCs) migrate between NCs towards the oocyte to form the micropyle. Concurrent with this long-range movement, anterior FCs stretch along their A-P axes to adopt a squamous morphology over the NCs. The remaining FCs migrate posteriorly to cover the oocyte while adopting a columnar morphology. The increasing surface area of FCs during this period accommodates the internal expansion caused by oocyte growth which occupies half the total volume of the egg chamber at stage 10. Oscillating contractions of basal actin bundles (wavy arrow) begin at stage 9 near the egg chamber’s center and then over the oocyte at stage 10 to channel the oocyte growth and push NCs toward the anterior pole. Germ cell expansion and the active phase of egg chamber elongation completes after stage 10. **(C)** Closeup of a stage 7 egg chamber sectioned in a midsagittal plane. A-P and D-V axes are indicated. **(D)** Scheme of polarized columnar follicle cell epithelium (FCE) covering the oocyte of stage 10 egg chambers (boxed region). The apical surface of the FCE faces the oocyte while its basal membrane contacts the BM. The spatial distribution of cytoskeletal and membrane-associated proteins is shown. Transmembrane integrin receptors (cyan bars) at the basal side mediate the interaction of FCs with the BM. The DGC central components, Dg and Dys, localize to the basal FCE. Dg interacts with ECM proteins such as laminin and perlecan via its extracellular domain and links the BM to the actin cytoskeleton through its intracellular binding partner, Dys. β H-Spec is structurally related to Dys and is found on the apical side of the FCE. Linear actin bundles align parallel to one another at the basal side of individual FCs. **(E)** A stage 7 egg chamber sectioned in a transverse plane (dashed line in **B**) and illustrated in three dimensions. The collective migration of FCs along their surrounding stationary BM rotates the entire egg chamber, perpendicular to the elongation axis (black arrow). Linear BM fibrils (brown strands) are oriented in the same direction as actin bundles (solid red lines). In three dimensions, parallel actin bundles at FCs’ basal surfaces along with their fibrillar BM create a circumferential “molecular corset” around the egg’s exterior. The corset components can be viewed at the surface focal plane, closer to the cover slip. The “molecular corset” constrains the isometric germ cell growth (blue arrows) in an anisotropic fashion to further bias egg elongation along the A-P axis.

Figure 4: Components of the “molecular corset”.

(A) The basal epithelial surface of a stage 7 egg chamber shows parallel arrays of actin filaments in individual FCs and throughout the entire cell layer. **(B)** In the adjacent BM, the ECM proteins such as Laminin orient into fibril-like strands, perpendicular to the elongation axis. **(C)** The transmembrane protein, integrin, localizes to the extremities of cortical F-actin stress fibers, anchoring them to the ECM at focal adhesion sites. Scale bars 10 μ m.

CHAPTER II: RESULTS

Dys is required for proper wing and ovarian development

Table 1: List of Dys isoforms in *Drosophila*

Isoforms are derived from at least seven promoters and are sorted in the 5'-3' order of those promoter start sites, then by protein length. Predicted status of the protein in terms of the presence/absence of the amino-terminal ABD, whether mutated in *MB02524*, and whether GFP-tagged or bypassed in *Dys[GFP]*, is indicated. Alternative splicing occurs at certain internal coding exons, but this is not predicted to alter the effects of *MB02524* or *Dys[GFP]*. All isoforms would carry the Mab epitope.

Promoter	Isoform	A.a. length	ABD present?	<i>MB02524</i> effect	Tag in <i>Dys[GFP]</i> ?
Long forms:					
1	Dys-PF	3529	ABD	Mut	GFP
1	Dys-PI	3228	ABD	Mut	GFP
2	Dys-PH	3598	ABD	Mut	GFP
2	Dys-PA	3497	ABD	Mut	GFP
2	Dys-PK	3144	ABD	Mut	GFP
2	Dys-PC	3127	ABD	Mut	GFP
3	Dys-PG	3504	ABD	Mut	GFP
Short forms:					
4	Dys-PL	1323	No ABD	WT	GFP
5	Dys-PB	1669	No ABD	WT	GFP
6	Dys-PD	1854	No ABD	WT	GFP
7	Dys-PJ	1152	No ABD	WT	No GFP
7	Dys-PE	1051	No ABD	WT	No GFP

To investigate the role of Dys in *Drosophila* development, we first characterized the publicly available set of *Dys* alleles (listed in Table 2). The deficiency *Df(3R)Exel6184* breaks after the first alternative 5' exon, removing 99% of the Dys-PF/PI coding region, and 100% of the remaining isoforms (see Table 1). Thus, it is a molecular null allele. It also removes 9 downstream genes, and our copy of the chromosome is homozygous lethal (see also Christoforou et al. (2008)). A newer, previously uncharacterized *MiMIC* insertion allele *Dys[MI00812]* was also tested; it is a splice-capture construct and should place a stop codon just before the last spectrin repeat, terminating all isoforms. The allele *Dys[E17]* carries a point mutation creating a premature stop codon in the last spectrin repeat, in an exon common to all isoforms. Thus the latter two alleles are expected to be either complete protein nulls, or to make N-terminal fragments that cannot interact with Dg, the membrane receptor for Dys. As detailed below, all three of these alleles behaved as null or near null regarding the classic visible phenotype for Dys, the “detached” wing crossvein (Christoforou et al., 2008). Similar high rates of penetrance for “detached” were found in each heteroallelic combination

(detailed below). Thus, these alleles were used interchangeably, and since the mutations were made on different genetic backgrounds, the transheterozygotes are considered to remove only *Dys*.

As previously reported, *Dys* nulls produced short eggs (Fig 5A-A') and a defect in wing vein patterning, detachment of the posterior crossvein from longitudinal veins (Fig 5B-B'). Furthermore, loss of *Dys* perturbed the coordinated orientation of basal actin bundles across the FC layer (Fig 5C-C') and in the surrounding BM, drastically reduced the number and length of LanA fibrils (Fig 5D-D'). Notably, *Dys* null follicles showed a novel defect in their epithelia, with abundant cell-surface protrusions in the spreading FCs (Fig 5E-G'). Similar to lack of *Dys*, damaging NCs during dissection resulted in increased cytoskeletal activity (Villarreal and Edwards, unpub). Taken together, these results indicate that *Dys* is essential for the completion of posterior crossveins, egg elongation, basal F-actin organization, BM fibril deposition, and normal cytoskeletal activity. These data prompted examination of *Dys* subcellular organization in relation to the actin cytoskeleton during oogenesis.

***Dys* has a plane-polarized basal localization in the FCE, where it shows a dynamic, stage-dependent association with F-actin bundles**

To investigate the localization of *Dys* during egg chamber development, we first employed an endogenously-tagged *Dys* allele, *Dys*[GFP], to visualize *Dys*. *Dys*[GFP] signals were enriched at the basal FCE but absent from the apical membranes. Intriguingly, *Dys*[GFP] localization in follicular epithelia showed a striated plane-polarized pattern across the entire cell layer, perpendicular to the elongation axis (Fig 6A, B). *Dys*[GFP] striations were co-oriented with basal F-actin bundles, and appeared to have a stage-dependent interaction with F-actin. In stage 8 egg chambers, we observed that some *Dys*[GFP] signals were running alongside F-actin filaments, decorating them in a punctate pattern (Fig 6C"). In stage 10, a large population of *Dys*[GFP] signals appeared to drift away from nearby actin bundles (Fig 6H", 8B"). In addition, *Dys*[GFP] signals accumulated in trailing edge structures in the NCFCs which had minimal F-actin (Fig 6B, B", I-I").

Focal adhesions, containing integrins and Zyxin, complement the actin-to-ECM connective function of the DGC. Thus, in an attempt to simultaneously visualize focal adhesion sites and *Dys*[GFP] in the FC layer, we used a FC-specific driver (*c323-GAL4*) to coexpress RFP-tagged

Zyxin with the endogenously-tagged *Dys*[GFP] allele. We characterized this in stages 9-10 follicular epithelia as *c323-GAL4* expresses in most FCs from stage 8 onward, and Dys[GFP] signals were still abundant during these stages. RFP-Zyxin-labeled focal adhesion sites were tightly associated with F-actin stress fibers during these stages (Fig 8A', B', C'). In stages 9-10 egg chambers, two different subpopulation of Dys[GFP] signals were discernible using confocal microscopy with adaptive deconvolution. Specifically in stage 10, a main population of Dys[GFP] signals were off the F-actin filaments and their focal adhesions (Fig 8B"). In other cases, Dys[GFP] seemed to be well-associated with Zyxin even at spots where F-actin bundles were not present, with GFP and RFP signals being next to each other, in an alternating manner (Fig 8C").

In addition to the FC layer, Dys[GFP] localized in the germline as it was tightly associated with the membranes of NCs (Fig 6 D') and accumulated on the germ cell membranes surrounding actin ring canals (Fig 6E, F). At stage 11, when NCs simultaneously dump their cytoplasmic contents to the oocyte through ring canals, we did not detect any overlap between Dys[GFP] and NC cytoplasmic cables involved in NC dumping (Fig 6D").

Given the DGC is known to associate with muscle membranes outside oogenesis (Allen et al., 2016), we assessed the possible localization of Dys in the ovariole muscle sheath. Interestingly, Dys[GFP] signals were detected at the membranes of the muscle sheath but appeared much less abundant in muscle than in adjacent FCs (Fig 6G). Taken together, these data show that in oogenesis, Dys localizes basally in the FC layer and also associates with the germ cell and ovariole muscle sheath membranes. Furthermore, our data suggest that Dys[GFP] and F-actin have a dynamic relationship in the follicular epithelium, which seems to be stage-dependent.

Dys visualization with a mammalian Utrophin antibody reveals a complex polarized pattern for Dys isoforms at the basal side of follicular epithelium

Having documented the Dys[GFP] protein trap in the natural *Dys* locus, we next employed a monoclonal antibody against the mammalian Utrophin as a second method of Dys visualization. The reason that led us to further characterize Dys using this antibody was its possible broader detection of Dys pattern, in oogenesis. We considered that the GFP exon insertion in *Dys*[GFP] bypassed the shortest two fly Dys isoforms. Therefore, we used the Mab MANCHO17 that was

expected to label all Dys isoforms by binding to their conserved carboxy-terminal second coiled coil domain (Nguyen et al., 1992) (Fig 2). Strikingly, in the Dys[GFP] follicles stained with Dys Mab, anti-Dys signals showed a plane-polarized striated pattern across the entire basal follicular epithelium, very similar to Dys[GFP] signals (Fig 7A-B). However, although anti-Dys signals were cooriented with Dys[GFP] signals, perpendicular to the egg elongation axis, they revealed a broader pattern of Dys distribution than Dys[GFP] signals. In fact, our confocal imaging revealed an extended pattern of anti-Dys signals such that individual Dys Mab striations were only decorated with Dys[GFP] signals for part of their length (Fig 7A, B, F).

To confirm that the antibody functioned as expected, we utilized the FLP/FRT system to generate Dys-depleted clones in the *Dys[GFP]* ovaries where all cells expressed Dys[GFP] from its own promoter. Next, we stained Dys[GFP] egg chambers with anti-Dys to visualize RFP-marked *Dys-RNAi*(PTRiP.HMC03789, against all isoforms) clones, Dys[GFP], and anti-Dys signals together. Specifically in the RFP-marked clones, all detectable Dys[GFP] signal was eliminated (Fig 7C-E, top panels). Along with the basal striated pattern, Dys Mab showed a general non-structured cytoplasmic signal due to potential off-target antigens. Importantly, the structured portion of anti-Dys signals was eliminated in the Dys-depleted clones (Fig 7C-E, middle panels). This experiment confirmed that basal anti-Dys signals represented Dys protein, the *Dys[GFP]* line only tags Dys, and this *Dys-RNAi* construct removes all visible Dys.

Additionally, in the egg chambers imaged in cross section, anti-Dys signals recapitulated the localization of Dys[GFP] signals to the NC membranes and basal sides of follicular epithelia but with more cytoplasmic background (Fig 7G-G"). Altogether, these experiments suggest a complex plane-polarized distribution or orientation for Dys isoforms at the basal side of FCE where multiple copies of Dys are aligned, and possibly two or more isoforms are coordinated in this pattern.

Dys functions in follicle cells to orient F-actin stress fibers during oogenesis

Having characterized Dys organization pattern in different cell types and knowing its significance in egg chamber development, we wanted to determine the site of action for Dys in organizing the actin cytoskeleton and providing for proper egg shape. According to previous reports, *Dys* and *Dg* null mutant FC clones display misoriented basal F-actin while their neighboring WT clones

have normal F-actin orientation (Campos et al., 2020). To better document this phenomenon, we investigated the consequences of Dys depletion from FCs. To do so, we induced *Dys-RNAi* in FCs using two different drivers.

We first used *c204-GAL4* driver, with limited expression in the posterior FCs from stage 8 onward. Measuring the aspect ratio (length divided by width) of FC-depleted mature eggs, we found that *c204-GAL4 > Dys-RNAi* produced significantly shorter eggs than controls (not shown). Furthermore, we examined the effects of FC-specific Dys inhibition on F-actin stress fiber organization throughout stages 8-13. It is worth noting that the expression of *c204-GAL4* driver has just begun at stage 8 and so it is mainly effective past stage 9. We found that in stage 8 to 10A *c204-GAL4 > Dys-RNAi* egg chambers, actin filaments were slightly divergent and less parallel at the basal side of individual FCs. On a global tissue-level scale, although we noticed slight changes in their orientation from one cell to another, F-actin bundles appeared to be properly oriented perpendicular to the A-P axis (Fig 9A-D'). Recall that in *Dys* null mutants, basal F-actin organization was strongly disturbed in these stages (see Fig 5). Together, these findings indicate that during stages 8-10A, Dys functions in follicular epithelia to promote F-actin alignment within each cell, and also on a local scale between neighboring cells. Further, given the weak effect of *c204-GAL4* in stages 8-9 and the correct global orientation of stress fibers in Dys-depleted epithelia during these stages, only small amounts of Dys might be needed to properly orient F-actin fibers, perpendicular to the elongation axis.

In WT, concurrent with the beginning of NC dumping at stage 10B, stress fibers change their orientation such that they are less perpendicularly-oriented to the A-P axis. Given this fact, we assessed the stress fiber organization in *c204-GAL4 > Dys-RNAi* FCs at this stage and found that their circumferential orientation was perturbed, similar to but more pronounced than in control FCs (Fig 9E-E'). Later in oogenesis, at around stage 12 when dumping is completed, stress fibers return to their original orientation, and so we tested if these fibers regained their perpendicular orientation in Dys-depleted FCs. Our confocal imaging revealed that stress fibers failed to retrieve their original orientation in *c204-GAL4 > Dys-RNAi* FCs (Fig 9F-G'). In fact, in some FCs, these fibers were parallel to rather than perpendicular to the elongation axis. Notably, stress fiber reorientation in late oogenesis is reported to result from the disassembly of existing stress fibers and the synthesis of new fibers (Delon and Brown, 2009). In line with this concept, our findings suggest that in late oogenesis, Dys might facilitate the formation of new stress fibers while promoting their correct

perpendicular orientation. Overall, these findings indicate that in late oogenesis, *Dys* is required in FCs for proper orientation of basal F-actin stress fibers.

In a separate set of experiments, we used a second driver, *c355-GAL4*, which is expressed in the FCs over the oocyte (to see the expression pattern of this driver, refer to Appendix..) and also in the wing imaginal disk during larval stages. As expected, inhibition of *Dys* in the FCs using this driver yielded significantly shorter eggs (Fig 10J). In addition, we investigated the stress fiber orientation in *c355-GAL4 > Dys-RNAi* FCs at later stages. Similar to our observations with *c204-GAL4*, stress fibers did not show a proper orientation at stage 13 (Fig 10I-I'). Of note, we observed a stronger stress fiber misorientation at stage 10B with *c355-GAL4* driver as more FCs had misoriented stress fibers compared to control (Fig 10H-H'). Finally, although we used *c355-GAL4* to induce *Dys-RNAi* in FCs and study the possible consequences in egg morphology, we also noticed a wing vein patterning defect in *c355-GAL4 > Dys-RNAi* flies. Indeed, the posterior crossvein in their wings were detached from longitudinal veins (not shown), resembling the wing vein defect in *Dys* null mutant flies.

Collectively, these data point to important roles of *Dys* in follicular epithelium for proper egg elongation, maximum F-actin alignment on cellular and local scales in earlier oogenesis, and finally proper orientation of stress fibers in later oogenesis. Moreover, our results suggest that outside oogenesis, *Dys* might be required in epithelial cell layers that form *Drosophila* imaginal disks, to regulate the wing vein patterning.

***Dys* functions cell-autonomously to promote parallel alignment of stress fibers in individual FCs, and non-cell-autonomously for their global orientation across the FC layer**

The data above demonstrated that *Dys* functioned in FCs to promote parallel alignment of basal F-actin in individual cells and to allow the planar polarization of actin from cell to cell, producing an actin belt around the egg chamber. If *Dys* functions only to respond to mechanical or information cues, the function of *Dys* during these stages might be cell-autonomous. To test for a possible cell-autonomous defect in F-actin alignment, we generated FLP-out *Dys-RNAi*-expressing clones in the FC layer, via the same strategy that we employed in (Fig 7C-E). Although our results varied

between different clones, in stages 6-8, we observed a cell-autonomous defect in parallel alignment of F-actin bundles in *Dys*-depleted clones compared to the neighboring WT cells (Fig 11A-C"). Within each clone, F-actin bundles were more divergent and less parallel in each cell, and there was a slight change in their orientation from one cell to another (Fig 11A, B, C). According to our data, unlike *Dys* null epithelia (see Fig 5), the overall orientation of actin filaments in stage 6-10 *DysRNAi* clones remained intact (stage 6-8 clones are shown in 11A-C"). This indicates that *Dys* facilitates the global orientation of stress fibers, perpendicular to the elongation axis, in a non-cell-autonomous manner. Altogether, the results presented in the fourth and fifth sections of chapter II indicate: (i) *Dys* is required in the FCs for proper basal F-actin organization, (ii) *Dys* promotes parallel alignment of F-actin bundles on a cellular and local scale, in a cell-autonomous manner, and (iii) *Dys* acts non-cell-autonomously to facilitate the tissue-level perpendicular orientation of stress fibers.

***Dys* is not required in the germline nor in the ovariole muscle sheath to promote egg elongation**

Our findings above confirmed the follicular epithelium as the site of action for *Dys* to promote egg elongation and basal F-actin organization. However, one can assume there are many proteins that are likely to act in more than one tissue to regulate these processes during egg chamber development.

Based on our early observations indicative of excessive cytoskeletal activity in *Dys* mutant spreading cells, we hypothesized that loss of *Dys* caused these cells to respond to the presumably altered tension in the underlying *Dys* null NCs. Consequently, we tested if *Dys* was specifically required in germ cells for normal cytoskeleton activity and egg morphogenesis. We expressed *UAS-Dys-RNAi* with a germline-specific driver to knock down all isoforms in the germline only. We used the *MTD-GAL4* driver in which three GAL4 insertions together express GAL4 uniformly in the germarium and throughout oogenesis. Imaging egg chambers in their cross sections, we confirmed that *Dys-RNAi* specifically eliminated all the visible *Dys*[GFP] signals in the NCs but not in the somatic border cells (12A). F-actin staining of the basal surface of *Dys*[GFP] egg chambers with *Dys*-depleted germline revealed no apparent defect in egg elongation, nor in basal *Dys* and F-actin organization (12B-B"). These data indicate that inhibition of *Dys* in the germline does not recapitulate the cytoskeletal dysfunction associated with *Dys* null epithelia.

Finally, we set out to test the possible contributions of the muscle sheath Dys to egg chamber morphogenesis. Two reasons prompted us to test this possibility. First, our confocal imaging confirmed the localization of Dys to the membranes of muscle sheath, and the DGC is known to be required for muscle integrity in various biological contexts. Second, a prior study demonstrated a role for the binding partner of Dys, Dg, in the ovariole muscle sheath for proper egg shape (Andersen and Horne-Badovinac, 2016). For this experiment, we used a muscle-specific driver, *Mef2-GAL4*, to knock down Dys isoforms in the muscle tissue by RNAi. Expressing *Dys-RNAi* with this driver did not decrease the egg’s aspect ratio as confirmed by our statistical analyses (Fig 13G). In addition, basal F-actin organization in *Mef2-GAL4 > Dys-RNAi* follicles appeared normal through stage 8 of oogenesis yet a subtle decrease in basal F-actin alignment was observed in stage 8 (Fig 13A-D). We noticed that stage 9 and 10 *Mef2-GAL4 > Dys-RNAi* egg chambers had radially arrayed F-actin at the basal side of FC membranes (Fig 13E-F). This appeared similar to the defect seen in basal F-actin organization in *Dys* null mutant epithelia during these stages. These experiments suggest that suppressing Dys in the muscle affects actin organization when the oocyte is rapidly growing during stages 9-10 yet this apparent requirement becomes dispensable for proper egg elongation. However, further analyses of egg chambers in different stages using other muscle drivers will be required to confirm these results.

Dys isoforms have distinct roles in ovarian and wing development

Thus far, we have shown central roles of Dys in two developmental programs, egg chamber morphogenesis and wing vein patterning. We also found there may be a complex distribution pattern for Dys isoforms in the egg chamber’s follicular epithelium (Fig 7). One question that arose from these results is whether multiple Dys isoforms function differently to regulate these developmental processes.

To begin to address this question, we characterized the transgenic *Minos*-based *Mi{ET1}* insertion *Dys[MB02524]*. In *Dys[MB02524]*, a *Mi{ET1}* element disrupts the largest *Dys* exon, a 3465 nt exon that encodes Dys-PA a.a. 60-1214, including most of the actin binding domain and all of spectrin repeats 1-8 (Fig 2). *Mi{ET1}* is inserted at a.a. 444 of Dys-PA, and the mutated ORF terminates after six codons provided by *Mi{ET1}*. Thus, we conclude no full length, actin-binding

Dys isoforms can be made from this allele. Since *Mi{ET1}* lies 8 kb upstream of the next internal promoter (for Dys-PL), we expect that all of the short isoforms can be made without defect. We then tested combinations of this allele with three different *Dys* null alleles for their egg morphology and wing vein patterning. As expected from our earlier work, any combinations of the null alleles (*Dys*[*MI00812*], *Dys*[*E17*], and *Df(3R)Exel6184*) resulted in both detached posterior crossveins and short eggs. In contrast, combinations of these alleles with *Dys*[*MB02524*] triggered a strong detached crossvein phenotype but acted as WT for egg elongation (Fig 14).

In a separate experiment, we also combined *Dys* null alleles with the *Dys*[*GFP*] allele that we used earlier to visualize Dys. The egg’s aspect ratio (Fig 14C’) and wing veins (not shown) were normal in these combinations, providing yet more evidence that our *Dys*[*GFP*] allele was fully functional. Altogether, our findings indicate that lack of the longer Dys isoforms bearing actin-binding domains does not disrupt egg chamber elongation. Instead, the presence of shorter isoforms missing the sequences necessary for binding F-actin, is sufficient to promote egg morphogenesis. In the context of wing development, however, these shorter isoforms fail to rescue the detached crossvein phenotype. Taken together, our findings suggest that Dys isoforms have distinct developmental roles, with the same group of isoforms being required in one developmental context, and dispensable in another.

Dys short isoforms are sufficient to orient F-actin stress fibers in late oogenesis

In order to determine how Dys short isoforms contribute to follicle elongation, we first assessed F-actin organization at the basal side of follicular epithelia from *Df(3R)Exel6184/Dys*[*MB02524*] females. Our prior experiments indicated that at around stage 9-10, inhibition of all Dys isoforms in the FCs caused a minor defect in the parallel alignment of basal actin filaments but did not affect their perpendicular orientation to the elongation axis. This inhibition, however, resulted in a major defect in stress fiber orientation starting at stage 12. Thus, we wanted to test for a possible rescue of these phenotypes in the presence of Dys short isoforms. We found that the maximum F-actin alignment was not achieved in stage 4-8 *Df(3R)Exel6184/Dys*[*MB02524*] follicles although stress fibers still had correct orientation, perpendicular to the A-P axis (Fig 15A’, B’, C’). The presence of short isoforms rescued the major defect in stress fiber orientation observed in stage 13 Dys-depleted FCs (Fig 15D-D”). These results indicate Dys short isoforms in particular, are not

required to establish/maintain the proper stress fiber orientation in early oogenesis. Nonetheless, Dys plays an important role in stress fiber orientation in late oogenesis which can be attributed to its short isoforms.

Dys short isoforms are important for BM fibril deposition

Given a polarized fibrillar BM is thought to be important for egg elongation, we next checked if Dys short isoforms promoted follicle elongation through their possible contributions to BM fibril deposition. To test this possibility, we used LanA-GFP to track the expression of a main ECM protein, Laminin A, which incorporates into BM fibrils. We then imaged the focal plane of LanA-GFP-tagged BM fibrils in *Df(3R)Exel6184/Dys[E17]* null and *Df(3R)Exel6184/Dys[MB02524]* follicles. Strikingly, Laminin A was organized into a regular pattern of long parallel strands along the basal surface of *Df(3R)Exel6184/Dys[MB02524]* follicular epithelia, similar to controls (Fig 16A'-A", B'-B", C'-C"). Indeed, our quantification of BM fibrils showed that both BM fibril length (Fig 16F) and number (Fig 16G) in these follicles were comparable to controls yet significantly higher than in *Df(3R)Exel6184/Dys[E17]* null follicles. This indicates that Dys short isoforms rescue the visible defects in the BM fibrillar organization associated with *Dys* null mutants. Therefore, their function in BM fibril deposition might contribute to their role in egg chamber elongation.

Dys and β H-Specterin overlap in their developmental functions

To this point, we have illustrated the subcellular organization of Dys protein in oogenesis and established its important developmental roles in organizing an effective “molecular corset” around the egg’s exterior to promote egg chamber morphogenesis.

Next, we set out to explore Dystrophin’s functional network during egg chamber development. To begin to dissect this network, we examined a structurally related member of the spectrin repeat superfamily, β H-Spec, and looked for shared developmental defects between *Dys* and β H-*spec* null mutants. We did not detect the typical phenotypes, characteristic to *Dys* nulls in β H-*spec* null follicles as their egg shape and F-actin organization appeared normal (Fig 17B, B'). To test if Dys and β H-Spec function redundantly in egg chamber development, we generated β H-*spec*, *Dys* double mutant flies.

The combination of these two alleles was highly lethal and surviving adults had severe ovary defects, with strongly disorganized basal F-actin, short eggs, and frequent egg chamber fusion (Fig 17D-E”). The strong phenotypes in double mutants and their high lethality compared to each single mutant suggests the two spectrin family members overlap in their developmental functions during oogenesis, and other unknown developmental contexts beyond egg chamber formation.

The results above prompted us to examine the localization of β H-Spec during egg chamber development. Remarkably, our imaging revealed that while considered to be apically localized, this protein also had a plane-polarized striated organization at the basal side of follicular epithelium (Fig 17F-G). This similar pattern for Dys and β H-Spec organization along with their strong loss-of-function genetic interaction reveals a novel functional network at the cell cortex involving the spectrins and Dys.

Finally, prior work in mice has provided evidence for a direct interaction between dystroglycan and the Hippo pathway effector, YAP (Morikawa et al., 2017). Moreover, PTPN14 (*Drosophila* *Pez* homolog) is shown to potently limit the transcription activity of YAP and thus behave as a tumor suppressor (Huang et al., 2013; Liu et al., 2013; Wilson et al., 2014). Other studies have documented a tumor suppressor behavior for dystroglycan in epithelial cells (Henry et al., 2001; Muschler et al., 2002). In *Drosophila*, β H-Spec acts as a tumor suppressor by regulating the Hippo signaling cascade (Deng et al., 2015), and on the other hand, requires Dg for its apical localization to the FCE (Deng et al., 2003; Schneider et al., 2006). These animal studies together and our findings above prompted us to check for the possible participation of the Hippo pathway in our proposed functional network during *Drosophila* egg chamber development.

To test for this possibility, we made FLP-out clones of FCs overexpressing WT *Pez* in β H-GFP follicles. We visualized these clones with antibody staining against *Pez*. We observed that ectopic *Pez* localized to the apical membranes of FCs, similar to endogenous *Pez* only more readily visible (Fig 17H, I). Strikingly, β H-GFP signals were greatly reduced in *Pez*-overexpressing clones compared to adjacent WT cells (Fig 17H’, I’). This experiment suggests the Hippo pathway regulator, *Pez*, controls β H-Spec during egg chamber development. Finally, we examined *Pez* mutant egg chambers and checked them for developmental defects associated with the DGC. We noticed that basal F-actin organization was perturbed in *Pez* follicles, similar to our observations with *Dys* nulls (Fig 17J-K’).

Overall, these data highlight a new functional network at the cell cortex engaging spectrins, the DGC central proteins, and the Hippo signaling pathway.

Figure 5: Visible phenotypes for *Dys* LOF mutants.

(A-A') Representative mature eggs from *Dys* null and control females. The horizontal and vertical lines correspond to the longest A-P axis and the widest D-V axis of the control egg chamber. The same lines are superimposed on the *Dys* null follicle. *Dys* null mature eggs have reduced length (green arrow) (A') and increased width (orange arrow) (A') compared to control, resulting in rounded rather than elongated eggs. Scale bars 50 μ m. (B-B') Representative images of WT (B) and *Dys* null (B') wings. In contrast to WT, mutants show a partial posterior crossvein, detached from the longitudinal veins (red arrow) (B'). (C-C') Stage 10 egg chambers stained with phalloidin for WT (C) and *Dys* null (C') genotypes to visualize F-actin. The basal F-actin organization is perturbed in mutant follicles with little coordination from cell to cell. (D-D') The basement membranes of Stage 7 egg chambers visualized with LanA[GFP]. The BM of WT follicles contains polarized fibrils (D). Loss of *Dys* negatively affects fibril incorporation into the BM, decreasing both the number and length of fibrils (D'). (E-G') F-actin staining of Stage 9-10B egg chambers for WT (bottom panels) and *Dys* nulls (top panels). Arrows in (E) and (F) point to excessive cell-surface protrusions in the spreading FCs at stage 9 and stage 10A egg chambers, respectively. (F-G') panels show three different confocal planes of F-actin signal; coded green, red, and blue from basal to apical. DNA stain is superimposed in red. Scale bars 10 μ m for (C-G').

Figure 6: Dys localizes in the follicular epithelium, germline, and ovariole muscle sheath, and does not necessarily overlap with all F-actin.

(A-A'') Basal view of follicular epithelium expressing endogenously-tagged *Dys-GFP* and stained with phalloidin. Basal F-actin is organized into parallel bundles, perpendicular to the A-P axis (A'). Dys is basally localized in FCs where it makes a plane-polarized striated pattern (A). (B-B'') Confocal images taken at the basal surface of stage 8 egg chambers. Dys aggregates in trailing edge structures (light orange arrows) (B) which are clearly distinct from nearby actin filaments (B''). (C-C'') A high-resolution close-up of a stage 8 egg chamber with WT FCs in the field of view. Dys[GFP] signals decorate adjacent F-actin filaments as punctae (C''). (D-D'') A cross section of stage 11 egg chamber with a few NCs in the field of view. Cytoplasmic actin cables (red arrows) (D) aid in NC dumping. Dys[GFP] lines NC membranes (red arrowheads) (D') yet does not overlap with actin cables (D''). Scale bars 10 μm for (A-D''). (E-F) A stage 9 egg chamber sectioned in a midsagittal plane. Dys flanks ring canals in the germline (cyan arrows) (E). Scale bar 50 μm . Close-up of ring canals shows the accumulation of Dys[GFP] on the membrane surrounding the actin ring (F). Scale bar 10 μm . (G) Outer surface of an ovariole with two back-to-back follicles. Dys[GFP] signals are visible in the membranes of muscle sheath (green arrows) but appear weaker than in FCs. Scale bar 20 μm . (H-H'') Closer view of the basal epithelial surface. The yellow arrow in (H'') points to a spot where Dys[GFP] signal clearly does not overlap with F-actin (stage 10). (I-I') Closeup of the spreading FCs. Orange arrows in (I) point to Dys accumulation in the trailing edge of spreading FCs. The horizontal arrow in (I') indicates the direction of spreading/migration (stage 8-9). Scale bar 10 μm .

Figure 7: Dys is both plane-polarized and molecularly oriented in the basal cortex.

(A-B) The signals from Dys[GFP] and Dys Mab MANCHO17 show different subsets of Dys organization; the GFP signal is localized within Dys Mab-labeled striations. (C-E) *Dys-RNAi* (PTRiP.HMC03789, to all isoforms) eliminates Dys[GFP] and the structured portion of the anti-Dys Mab signal. We made GAL4-expressing FLP-out clones (arrows) in follicular epithelia, driving *UAS-Dys-RNAi* along with the membrane marker *UAS-RFP-mCD8* (cyan). Specifically in the RFP-marked clones, all detectable GFP signal is eliminated. The Mab signal has both a general cytoplasmic component and a basal striated pattern. The striated pattern is eliminated by RNAi, confirming that it represents Dys protein; the cytoplasmic signal is not affected and therefore attributable to off-target antibody binding. All panels: approximate stage is indicated at the top. Scale bar, 10 μ m. (F) Enlargement of a Dys[GFP] follicle stained with Dys Mab. Dys[GFP] signals decorate only a portion of anti-Dys striations. (G-G'') Dys[GFP] Ovarioles viewed in a cross section and stained with Dys Mab. Anti-Dys signals match those from Dys[GFP] but have a more cytoplasmic 'background'. The yellow arrowhead refers to increasing Dys[GFP] signals in the NC membranes as oogenesis progresses through stage 10. Scale bar, 100 μ m.

Figure 8: RFP-Zyxin highly coincides with basal F-actin stress fibers, and in some cases associates with Dys[GFP] signals.

Images taken at the basal surface of stage 9-10 egg chambers. Scale bars, 10 μm . (A-C'') Focal adhesions are labeled by expressing *UAS-RFP-Zyxin* in FCs using the *c323-GAL4* driver. RFP-Zyxin tightly associates with F-actin fibers in all stages (A', B', C'). Yellow arrows in (A'', B'') point to spots where Dys[GFP] signals are distinct from stress fibers and their tagged focal adhesions. White arrows in (C'') show cases where Dys[GFP] associates with Zyxin even when F-actin bundles are not observed.

Figure 9: Dys functions in follicle cells to maximize basal F-actin alignment during earlier stages, and to properly orient stress fibers at later stages.

Scale bars 10 μm . (A-D') F-actin staining of basal epithelial surfaces from stage 7 to early stage 10. *c204-GAL4 > UAS-Dys-RNAi* was used to deplete all isoforms in FCs starting at stage 8. The RNAi construct is not induced in stage 7 follicular epithelia and so their basal F-actin organization is comparable to WT (A'). In Dys-depleted FCs, stress fibers show a correct global orientation, perpendicular to the A-P axis. Yet, their maximum alignment within each cell and between adjacent cells is not properly achieved (B', C', D'). (E-E') In stage 10B *Dys RNAi* follicles, stress fibers lose their correct orientation as they do in WT follicles. (F-G') Later in oogenesis, stress fibers return to their original orientation, beginning at stage 12. In FCs with suppressed Dys, these fibers fail to regain their proper orientation.

Figure 10: Depletion of Dys from follicle cells using the *c355-GAL4* driver.

(H-H') In stage 10B, both control and Dys-depleted follicular epithelia display misoriented stress fibers at their basal sides. However, this defect appears stronger in affected epithelia. (I-I') In stage 13, control egg chambers show well-organized basal stress fibers while in Dys-depleted FCs, the proper orientation of these fibers cannot be achieved. Scale bars 10 μ m. (J) Statistical comparison of aspect ratio for mature eggs from *c355-GAL4 > Dys-RNAi* and control females (n>130 eggs). The statistical test is ANOVA and compares the mean of the two control groups (red dashed line) with the mean of experimental group. Significance is ****p<2.2e-16.

Figure 11: Dys is required cell-autonomously in follicular epithelia for proper F-actin alignment.

Representative images of stage 6 and stage 8 follicles, in the focal plane of basal F-actin fibers. Scale bars 10 μm . FLP-out clones of Gal4-expressing FCs were generated to induce *UAS-Dys-RNAi* along with the membrane marker *UAS-RFP-CD8*. Induced clones are indicated by stars with dashed lines at the boundaries between affected clones and adjacent WT cells. (A-A'') In stage 6, stress fibers in the *Dys-RNAi* clone are fewer and less organized compared to the neighboring WT cells. (B) Blow-up of the stage 6 follicle in (A-A''). (C-C'') Stage 8 egg chamber with a Dys-depleted clone in the FC layer. Stress fibers are slightly reduced and less well-aligned than in their WT neighbors.

Figure 12: Germline Dys is not required for egg chamber development.

(A) Inhibition of Dys using the *MTD-GAL4* driver removes all the visible Dys[GFP] signals from germ cell membranes. Arrows point to somatic border cells (BCs) migrating toward the oocyte. Dys[GFP] signals are detected in BCs, confirming the RNAi construct function is specific to the germline. Scale bars 20 μm . (B-B'') Depleting Dys from the germline does not trigger defects in egg elongation nor in F-actin and Dys[GFP] organization in the follicular epithelium. Scale bars 10 μm .

Figure 13: Dys is not required in the muscle to promote egg elongation.

Basal view of stage 5 through stage 10 *Dys-RNAi>Mef2-GAL4* follicles. Scale bars $10\mu\text{m}$. (**A-D**) In stages 5-8, basal F-actin stress fibers have normal orientation, with a slight defect in their maximum alignment at stage 8. (**E-F**) In stages 9-10, the affected egg chambers appear to form radially arrayed basal F-actin, resembling the defect observed in *Dys* null epithelia during these stages. (**G**) Boxplot representation and quantification of the egg's aspect ratio for *Dys-RNAi>Mef2-GAL4* and controls ($n>140$ eggs). The statistical test is ANOVA which compares the mean of controls (dashed line) to *Dys-RNAi>Mef2-GAL4*. ns = not significant.

Figure 14: Distinct roles of *Dys* isoforms during development.

(A) Different combinations of *Dys* null alleles with each other result in partial absence of the wing posterior crossvein (bottom panels). A similar defect is observed in various combinations of null alleles with *Dys*[MB02524]. These combinations lack the longer *Dys* isoforms yet retain the shorter forms (top panels). (B) Characteristic images of mature eggs from combinations tested in (A). Contrary to null combinations (bottom panels), egg chambers properly elongate along their A-P axes in the presence of *Dys*[MB02524] (top panels). Scale bar 50 μ m. (C) Schematic illustrating how egg aspect ratio was measured. (C') Quantification of mature egg aspect ratio for *Dys* alleles tested. Aspect ratio is significantly reduced in *Dys* nulls. In contrast, MB02524 insertion in the *Dys*[E17]/*Dys*[MB02524] mutant shows no defects in egg elongation. *Dys*[GFP] allele is fully functional for egg elongation; *Dys*[GFP]/*Dys*[E17] and *Dys*[GFP]/*Df*(3R)Exel6184 eggs have correct shape. ANOVA compares the mean of WT (black dashed line) with transheterozygotes. Analyses performed in R; **** $p < 2.2e-16$; $n > 137$ per each genotype. (D) Percentage of penetrant females for wing defects observed in (A).

Figure 15: Dys short isoforms restore stress fiber misorientation in late oogenesis.

Confocal images through the focal plane of basal stress fibers in control and *Df(3R)Exel6184/Dys[MB02524]* egg chambers. Scale bars 10 μm . (**A-C**) Early in oogenesis, F-actin fibers are aligned parallel to one another in each FC, and are globally oriented perpendicular to the A-P axis, with their maximum orientation achieved at around stage 8. (**A'-C'**) In stage 4-8 *Df(3R)Exel6184/Dys[MB02524]* egg chambers, stress fibers have a correct orientation, but their maximum alignment within individual and between neighboring cells appears slightly defective. (**D-D''**) *Dys[MB02524]* rescues the defect in the late phase of stress fiber orientation seen when all Dys isoforms are absent (see Fig 9F', G').

Figure 16: Dys short isoforms restore BM fibril length and number.

Laser-scanning confocal images were taken in the focal plain of BM fibrils. LanA-GFP was used to visualize fibrils. Scale bars 10 μm . *Dys* null egg chambers show a defect in BM fibril deposition (**A-C**), with their fibrils being significantly shorter and fewer in number when compared to controls (**A''-C''**). Approximate stages are indicated in all panels. As egg chambers grow, BM fibrils decrease in density while retaining their length. As a result, fibrils are less abundant in stage 10 control egg chambers (**C''**), yet hardly detectable in *Dys* null chambers at the same stage (**C**). In all stages shown, *Dys[MB02524]* restores both BM fibril length and number (**A'-C'**). Enlargements of BM fibrils are shown in (**D-E''**). (**F**) Quantification of BM fibril length in control, *Df(3R)Exel6184/Dys[E17]*, and *Df(3R)Exel6184/Dys[MB02524]* egg chambers at different developmental stages. Data distribution and quartiles are visualized by violin and boxplot. Statistical comparisons of representative groups are performed via unpaired t-test in R. ns = not significant. (**G**) Quantification of BM fibril number in control, *Df(3R)Exel6184/Dys[E17]*, and *Df(3R)Exel6184/Dys[MB02524]* egg chambers at stage 8 and stage 10. The statistical test is unpaired t-test, comparing the mean of representative groups. ns = not significant.

Figure 17: Dys and β H-Spec overlap in their developmental roles, and Hippo pathway regulator Pez controls β H-Spec and its LOF perturbs F-actin organization during oogenesis.

(A-E'') Comparison of *Dys* and β H-*spec* single mutants with β H-*spec*, *Dys* double mutant ovaries: β H-*spec* mutants have grossly normal egg chambers and well-aligned F-actin stress fibers in the basal follicular epithelium (B-B'). *Dys* mutants have mostly disorganized basal F-actin and short, rounded egg chambers/eggs (C-C'). Surviving β H-*spec*, *Dys* double mutants eclose with a severe defect in basal F-actin organization in their follicles, short eggs, and fused cysts (red arrow) (D-D'). Representative images of egg chamber fusion defect in the double mutants are shown in (E'-E''). Scale bars 50 μ m for (E-E''). (F-G) illustrate localization of β H-Spec to apical epithelial membranes (yellow arrow), and its organization into a striated plane-polarized pattern at the basal side of follicular epithelium. Scale bar 50 μ m for (F) and 10 μ m for (G). (H-I'') show confocal images of β H-GFP follicles with FLP-out clones of FCs overexpressing WT Pez. Anti-Pez immunostaining was performed to visualize clones. Similar to endogenous Pez, the ectopic expression of Pez is detected at the apical membrane of FCs only more readily observable. Overexpression of Pez decreases apical β H-GFP signals, specific to clones (stage 9, magenta arrows in H-H'). (I-I'') show Z-stack projection through the apical surface of the FC layer at stage 10; β H-GFP makes a dense meshwork as expected, but not where Pez aggregates are present (red arrows). Scale bars 10 μ m for (I-I''). (J-K'') demonstrate basal F-actin misorganization in *Pez* null epithelia, mirroring the defect observed in *Dys* mutants. Scale bars 10 μ m for (J-K'')

CHAPTER III: DISCUSSION

Our study has revealed unexpected complexity in the function and potentially in the subcellular organization of the various isoforms of the Dys protein. Dys has seven documented promoters, and each leads to a unique N-terminus for the Dys protein. We divide the isoforms into “long” (over 3000 a.a) or “short” (under 2000 a.a.) for discussion purposes (see Table 1). All long forms have the ABD and the amino-terminal set of spectrin repeats, are disrupted by the *MB02524* insert, and are internally tagged by GFP in the Dys[GFP] protein trap. All short forms lack the ABD and amino-terminal set of spectrin repeats, and are wild type in *MB02524* (specifically, their promoters are at least 8 kb downstream of the insert). Three short forms, Dys-PL, Dys-PB, and Dys-PD, carry GFP in the protein trap line, but the Dys-PE/PJ isoforms from the last promoter bypass the GFP exon and are not tagged. All isoforms include the carboxy-terminal DG binding domain and the Mab epitope. Long forms should be able to engage actin, DG, and potentially other partners in a large, flexible, elastic complex with “shock absorber” and likely scaffolding and signaling roles. The short forms should lack the “shock absorber” role and be confined to the scaffolding and signaling roles specific to the carboxy-terminal region, but may have isoform-specific functions that arise from their unique amino-termini.

A novel pattern of distribution and distinct functions of Dys isoforms in egg chamber and wing development

The major finding of this work is that Dys long forms are dispensable for the main tissue-level phenotypes of *Dys*, misoriented basal FC actin cables and short eggs. This result is based on a single allele, *Dys*[*MB02524*], but this allele must disrupt the long forms based on its insertion of a stop codon into the ORF. In agreement with this, it has a fully penetrant wing phenotype (detached posterior crossvein), which is highly characteristic of Dys LOF (Christoforou et al., 2008). Because this allele separates the two main developmental defects of *Dys* (those of ovaries and wing discs), it reveals that the locus has a complex structure-function relationship. Clarifying this relationship will be critical for understanding the effects of DMD treatments that manipulate the Dys protein in humans.

We found evidence that there is another isoform-based division of labor in the *Dys* locus. The GFP protein trap tags all but the promoter 7 proteins Dys-PE/PJ, while the Dys Mab labels all forms. The GFP signal is not identical to the Mab signal; rather the GFP signal occupies a sub-region of each Mab-positive striation. If the result was the reverse, we might speculate that the Mab epitope is present but blocked in certain regions. The more limited GFP signal is harder to account for. The GFP domain may be pulled into an unfolded state by mechanical forces on Dys, and this would quench the GFP signal in those locations (suggested by R. Fehon). However, this is unlikely because when use a GFP antibody against the GFP domain in that construct, we observe a similar pattern of distribution for Dys (see Figure 21). Alternatively, the Dys-PE/PJ proteins could account for all of the signal that is Mab-positive and GFP-negative; this would suggest Dys-PE/PJ play a special functional role, perhaps as the basis for the egg chamber defects. It will be important to specifically tag and also knock out Dys-PE/PJ. A third possibility is that, since the GFP and Mab sites are 870 a.a. apart in the Dys sequence, we are resolving a specific orientation of individual Dys proteins in the basal cortex. Perhaps the GFP site lies preferentially in one part of each striation, and the C-termini are spread out along the striations. Based on our literature searches, this would be a novel organization for a spectrin family member. We note that these possibilities are not mutually exclusive: the GFP forms could be oriented and also segregated from Dys-PE/PJ.

The basal localization of Dys in the follicular epithelium has been previously documented (Schneider et al., 2006). However, this study is the first to provide detailed localization data for Dys protein. The Dys[GFP] striations do not tell us the orientation of entire protein, since GFP is labeling only a point on each protein and the rest is invisible. This makes the presence of striations intriguing: the pattern could mean Dys is arranged in a staggered manner, forming filaments along each striation, or that Dys proteins lie side-by-side in register, with the amino- and/or carboxy-termini off the striations. If the carboxy-terminus projected away from the Dys[GFP] striations, the Mab signal would be offset from the GFP signal, but we do not observe that. If the amino-terminus projected away from the Dys[GFP] striations, we would expect to see actin filaments at a distance from each striation, and we do observe this, yet in a stage-specific manner (below). If Dys makes filaments or other repeating structures, it will be important to know what Dys-Dys or Dys-partner interactions are organizing those complexes.

Since Dys proteins (long forms) bind actin, and Dys and actin are both striated and plane polarized in the FCs, we expected Dys[GFP] and F-actin signals to be largely coincident throughout oogenesis. However, we see a dynamic relationship between F-actin and Dys across stages. For instance, before stage 9-10, Dys[GFP] signals decorate basal F-actin bundles and appear as punctae running along actin filaments. At around stage 9-10, however, we observe a large population of Dys[GFP] signals that lie distant from the main F-actin bundles, forming Dys[GFP] speckles that are not clearly associated with actin cables and their RFP-Zyxin-tagged focal adhesions. A second set of Dys[GFP] signals associate with RFP-Zyxin even when F-actin bundles are not visible. Considering that many copies of non-bundled F-actin are uniformly distributed in the cortex, it might be possible that Dys binds to a subpopulation of cortical F-actin that we cannot resolve with our confocal imaging. These findings show that care needs to be taken when interpreting the Dys-actin relationship results. We consider the possibility that the ABD and GFP sites could be resolved in our Lightning Deconvolution imaging method. Given these sites are roughly 100 nm (3 pixels) apart in space, it would not account for larger distances that we often observe between Dys[GFP] signals and the ABD. The presence of Dys[GFP] signals in a distance from the actin filaments could mean that Dys is oriented in a nonrandom manner, with the carboxy-terminal regions grouped distantly from the ABDs. Together, our results suggest that in oogenesis, Dys associates with F-actin in a dynamic, stage-specific manner.

Specific distribution patterns of Dys isoforms in the ovary might reflect their distinct cellular functions in egg chamber development. For example, loss of Dys does not block egg chamber rotation within the BM (Campos et al., 2020). This suggests that in *Dys* nulls, FCs do not lose their ability to link their cytoskeleton to the ECM, a major mediator of follicle rotation (Haigo and Bilder, 2011; de la Loza et al., 2017). The transmembrane protein integrin is shown to play important roles in cell-ECM interactions to facilitate egg chamber rotation and activate different intracellular signaling pathways (de la Loza et al., 2017). Notably, integrin maintains its proper localization at the termini of basal F-actin stress fibers in *Dys* null follicles (Campos et al., 2020). Nonetheless, lack of Dys significantly reduces BM fibrils containing laminin, a well-known extracellular ligand for integrin. Here, we provide evidence that the presence of Dys short isoforms is sufficient to rescue the BM fibril deposition defect observed in *Dys* null egg chambers. Given these findings and striated distribution of anti-Dys Mab signals, it might be possible that short isoforms spread out evenly

along the basal epithelial surface to signal from focal adhesions to mediate BM fibril deposition and orientation. Although these results do not provide clarity on the molecular mechanism by which Dys short isoforms function in BM fibril organization, they underscore their important functions in this process.

In addition to their roles in BM organization, our data suggest Dys short isoforms also contribute to the proper orientation of basal F-actin fibers during late oogenesis. Thus, in *Drosophila* egg chamber development, Dys short isoforms promote follicle elongation through their early function in BM fibril deposition and their later function in basal F-actin organization. In contrast, Dys longer isoforms seem to be dispensable for egg elongation, BM fibril deposition, and stress fiber orientation. Our observations with fly wings, however, suggest an important role for these longer isoforms in wing vein patterning. It has been previously shown that Dys regulates the intercellular signaling pathways involved in wing crossvein formation (Christoforou et al., 2008). Here, we show the presence of short isoforms per se is not sufficient to promote crossvein formation. These isoforms contain the necessary sequences required for their interactions with the DG complex and various signaling proteins (Fig 2). Therefore, they are expected to carry out signaling functions associated with the DGC. However, they fail to rescue the detached crossvein phenotype observed in *Dys* null mutants. This indicates that in the context of wing development, short isoforms are not specifically selected to function in this process. Instead, longer isoforms and their actin-to-membrane linking ability seem to be critical for the efficacy of signal transduction involved in crossvein specification.

A closer investigation of individual Dys isoforms and their distinct behaviors and distribution pattern will help us better understand smaller Dystrophins and how they regulate tissue shape. For example, the presence of unique exons at the amino-termini of short isoforms including Dys-PB/Dp186, Dys-PD/Dp205, and Dys-PE/PJ (see Fig 2) makes it possible to specifically knock them down by RNAi and assess their distinct roles in oogenesis. Furthermore, advances in CRISPR technologies allow us the opportunity to edit the endogenous *Dys* locus and add visible tags at distinct positions and to individual isoforms. For instance, it will be important to tag the amino-termini of long isoforms where they bind F-actin and check if these tags colocalize with any subpopulation of F-actin (see the strategy in the first section of Appendix B). If so, it would indicate these isoforms fulfill their typical actin-to-membrane linking function, yet this function might not be not required for proper egg chamber morphogenesis. A similar strategy can be employed to

insert a tag in-frame with the carboxy-terminal end of Dys isoforms and validate the distribution pattern detected by anti-Dys Mab (Fig 7). Such approaches could provide valuable information on the orientation and spatial distribution of multiple Dys isoforms in developing tissues.

A role for Dys in regulation of cytoskeletal activity during egg chamber development

Cells exert mechanical forces to their surrounding ECM and their neighboring cells (Kurazumi et al., 2011; Vining and Mooney, 2017). The mechanical coupling of cells with each other and their environment guides various aspects of tissue development, mediated by actomyosin contractility and cytoskeletal assembly (Vining and Mooney, 2017). It is now becoming more clear that mechanical loads regulate several cell functions including cell proliferation, growth, and differentiation to maintain tissue homeostasis (Yang et al., 2004; Sun et al., 2016). There is also evidence that mechanical forces control other cellular functions such as collagen synthesis, an important component of the ECM (Bourdón-Santoyo et al., 2014). Previous findings in mammalian cell culture suggest elevated matrix content during development (e.g. fibril deposition into the existing BM) increases the mechanical coupling between the surrounding environment and cell intrinsic physical forces, an important aspect of mechanosensing (Vining and Mooney, 2017). In *Drosophila*, it is thought that BM fibrils make a stiffer BM around the egg’s middle region, creating a gradient of ECM stiffness which resists isometric germ cell expansion in an anisotropic fashion, leading to egg elongation along the A-P axis (Cetera et al., 2014; de la Loza et al., 2017).

Our study in *Drosophila* egg chambers implicates Dys as a potential regulator of mechanotransduction between neighboring cells and their physical environment. Indeed, our results show *Dys* null epithelial cells display signs of increased cytoskeletal activity midway through oogenesis where FCs stretch out over the NCs. During this period, FCs undergo a series of morphological changes, germ cells are increasing in size, and the entire egg chamber unit is actively elongating along its A-P axis. Therefore, it might be a possibility that loss of Dys alters the way FCs normally behave to handle the mechanical stimuli triggered by these morphological changes. Recall that in *Dys* nulls, FCs seem to be able to adhere to the ECM through focal adhesions (Campos et al., 2020), yet their BMs have a defective fibrillar organization. ECM is shown to change its composition and organization over time during oogenesis (Isabella and Horne-Badovinac, 2015, 2016). Thus, it could

provide a mechanical rheostat for cells and transduce general mechanical signals into specific cell responses based on the ligands present in the BM (Vining and Mooney, 2017). Therefore, decreased BM fibrils in *Dys* null follicles might alter the normal behavior of spreading FCs in response to the mechanical stimuli they are subject to during mid-oogenesis. Consequently, they manifest this aberrant response by increasing their cytoskeletal activity, making excessive cell-surface projections.

According to our observations, WT egg chambers can display a similar phenotype when chambers become damaged during dissection, and presumably lose tension (Martinelli et al., 2013). Given these findings, we initially hypothesized that either damage or loss of *Dys* might alter the mechanical properties of germ cells which in turn signal “distress” to the overlaying FCs to start a wounding response. This would suggest that in WT, FCs respond to appropriate tension to suppress actin polymerization and instead set up circumferential actin bundles. In contrast, in *Dys* null or damaged chambers, NC cortical tension is lost/altered, leading to an aberrant response in the spreading FCs, forming radially arrayed actin and profuse cell surface protrusions. However, this hypothesis was not supported by our data showing the germline-specific depletion of *Dys* does not disturb F-actin organization. Therefore, *Dys* suppression in the germline alone is not sufficient to trigger the aberrant response in FCs. This supports the idea that it is the altered tension in the FC layer itself and/or altered physical cues in their environment that could be causing this phenotype. A more detailed analysis of *Dys* null chambers and assessing their mechanical properties will be required to confirm these findings. For example, it will be interesting to determine if/how *Dys* acts as a mechanotransducer to relay BM stiffness to actin. Exploring these events could also provide an alternative mechanism to describe altered cell behavior in DMD. For instance, a loss of cortical tension in the muscle may bias the muscle stem cells toward a motile phenotype, and away from a proliferative phenotype, depleting the supply of new muscle cells.

The tissue-specific requirement for *Dys* in egg chamber morphogenesis

Our research findings indicate that *Dys* is required for egg chamber morphogenesis in some tissues, but dispensable in others. During oogenesis, F-actin fibers align parallel to one another within each cell, and are coordinated from cell to cell. This alignment increases as egg chambers proceed through stage 8, when they acquire their maximum alignment. These basally-aligned actin

bundles are globally oriented perpendicular to the egg's elongation (A-P) axis throughout oogenesis, except in stages 10B-11 when their proper orientation is lost, concurrent with NC dumping (Campos et al., 2020). Starting at around stage 12, F-actin stress fibers return to their original orientation. Here, we have shown Dys functions in FCs to promote basal F-actin alignment and proper stress fiber orientation in earlier and later oogenesis, respectively. In addition, depletion of Dys from the FCs produces short eggs, indicating its requirement in the follicular epithelium for proper egg elongation.

In the germline, Dys associates with the membranes of NCs and concentrates around the ring canals. However, its germline localization is dispensable for egg chamber development. This is surprising since Dys increasingly accumulates in NCs in preparation for dumping, presumably to strengthen their membranes. A full 3D analysis of germline-depleted chambers at stage 11 will be required to detect any potential mild phenotypes in their NC membranes. Finally, ovarioles are surrounded by a tube of muscle sheath whose circular fibers are mainly oriented perpendicular to the egg's elongation axis, similar to actin filaments in FCs and BM fibrils. The latter two are thought to resist the isotropic germ cell expansion in an anisotropic fashion to channel egg elongation through its A-P axis (Andersen and Horne-Badovinac, 2016). Although the ovariole muscle sheath is not in direct contact with the BMs of follicles, it might exert exogenous forces to the "molecular corset" and contribute to its resistance to the germ cell growth. Prior research suggest Dystroglycan is required in the ovariole muscle sheath for proper egg shape (Andersen and Horne-Badovinac, 2016). Knowing Dystroglycan and Dys are central components of the DGC and given our evidence for the localization of Dys to the ovariole muscle sheath membranes, it might be possible that muscle sheath Dys plays a role in egg chamber morphogenesis. We have found that depletion of Dys from the muscle tissue does not affect egg elongation but results in radially arrayed basal F-actin during stages 9-10. What causes this phenotype and what makes it dispensable for egg elongation, remains to be determined. Of note, the muscle driver used in this study expresses in most if not all muscles. In the future it will be useful and informative to employ an alternative strategy to deplete Dys from the ovariole muscle sheath only and test how it affects egg chamber morphogenesis. The timing of the requirement (at stage 9 or earlier in oogenesis) could be investigated by using temperature to vary GAL4 expression levels. It will also be interesting to investigate if/how Dys suppression in the

muscle sheath alters the mechanical environment of follicles, and how this relays to the “molecular corset”.

A novel functional network in cortical organization involving spectrins, Dys, and Hippo pathway

Here, we have shown a strong loss-of-function genetic interaction between Dys and a structurally related member of the spectrin repeat superfamily, β H-Spec. Our analyses of the β H-spec, *Dys* double mutant flies reveal severe defects in their ovaries, with short eggs, disorganized basal F-actin, and frequent egg chamber fusion. In order to provide clarity on what causes egg chamber fusion in the double mutants, it will be necessary to examine their germaria, where egg chambers are initially assembled.

The germarium is located at the anterior end of each ovariole, and is divided into several regions. At the anterior tip of region 1, two or three germline stem cells (GSCs) divide asymmetrically to produce a daughter stem cell and a cystoblast. Four incomplete divisions in each cystoblast generate a rounded cyst of 16 interconnected germline cells in region 2a. Halfway down the germarium at region 2a/2b boundary, a single cyst flattens to form a lens-shaped disk which spans across the whole width of germarium, perpendicular to the A-P axis. The flattening of the cyst rearranges the germ cells to position the selected oocyte in the center of the disk in contact with the FCs that migrate posteriorly to encapsulate one cyst at a time (González-Reyes and St Johnston, 1998; McCaffrey et al., 2006). These FCs are generated via two follicle stem cells (FSCs) on opposite sides of the region 2a/2b boundary whose division is thought to be induced by the cyst contact (Nystul and Spradling, 2010). Interestingly, the flattening of the cyst at region 2a/2b boundary is shown to be dependent on the homotypic cadherin-dependent adhesion between the germ cells. In fact, at 2a/2b boundary, the *DE*-cadherin transiently accumulates around the ring canals which interconnect the germ cells within a cyst, reflecting its concentration at sites of adhesions between the germ cells as they spread to form a lens-shaped disk (González-Reyes and St Johnston, 1998). On the other hand, an unusual heterotypic cadherin-dependent adhesion anchors the selected oocyte to the posterior FCs, ensuring the oocyte rather than the less adhesive NCs protrudes posteriorly into the FC layer to adopt the posterior position as the cyst rearranges back to the spherical shape

during the transition to region 3, where it buds off to exit the germarium (González-Reyes and St Johnston, 1998). A detailed examination of germaria in the $\beta H\text{-}spec$; Dys double mutants will shed light on the aspect(s) of egg chamber assembly that might be affected in these mutants.

The presence of side-by-side cysts with two posterior oocytes and extra NCs encased by a single layer of FCs (see Fig 17 E'') raises the following possibilities : (i) In $\beta H\text{-}spec$; Dys mutant germaria, the cysts might fail to flatten at the region 2a/2b boundary, and thus two rounded cysts can be simultaneously placed side-by-side at this region. At the same time, the FSCs in these mutants act as WT and produce a limited number of FCs, sufficient for encapsulating one cyst at a time. (ii) Dys and $\beta H\text{-}Spec$ might be independently required for the homotypic cadherin-mediated adhesion between the germ cells to flatten the cyst across the whole width of germarium. Of note, we have found that Dys [GFP] signals accumulate around the ring canals in egg chambers (see Fig 6 E-F). (iii) Despite having a rounded rather than flattened morphology at the 2a/2b boundary, the oocytes in the rounded cysts could still adopt and sustain the posterior position as they protrude into the FC layer in transit from the region 2a/2b boundary to region 3. (iv) Acquiring and maintaining the posterior position by the side-by-side oocytes suggests the specific cadherin-mediated adhesion between the selected oocytes and the posterior FCs might not be perturbed in the double mutants, leading the more adhesive oocytes rather than the NCs to protrude posteriorly. (v) In the case of triplet cysts with one posterior oocyte and two anterior side-by-side oocytes (see Fig 17 E'), it might be possible that in one of the rounded cysts, the selected oocyte adopted a posterior position and could outcompete the other two oocytes which presumably failed to position their oocytes correctly to adhere to the posterior FCs.

Notably, FCs migrate to surround the posterior of the cyst before they surround the anterior (González-Reyes and St Johnston, 1998). Thus, one could envision that as FCs reach to the anterior of the two outcompeted oocytes, they adhere more strongly to the anterior mispositioned oocytes due to their adhesive nature, and the stronger adhesion between these anterior FCs and the oocytes sustain their anterior position as the triplet reaches the region 3. This possibility would support the hypothesis that it is the adhesion between the germ cells themselves and not the adhesion between FCs and germ cells that might be affected in the double mutants.

Prior works show a similar paired cyst phenotype in ovaries lacking the p21-activated kinase (dPak) in somatic cells, with the side-by-side cysts packaged together in a single FCE. Contrarily, in

dPak nulls, the paired cysts are individually encapsulated with their own complete FCE due to the increased FSC activity in these mutants (Vlachos et al., 2015). In cultured cells association of PAK with PIX allows it to localize to focal adhesions (FAs) (Manser et al., 1997, 1998; Zhao et al., 2000). In *Drosophila*, *dpak*, *dpix*, and *git* mutant egg chambers do not proceed to form mature eggs, fail to elongate properly, lose FCE monolayering, show altered myosin activity, and have individually packaged paired cysts (Dent et al., 2019; Vlachos and Harden, 2011; Vlachos et al., 2015; Conder et al., 2007). Intriguingly, dPak, dPix, and Git are enriched at the basal side of FCE with the later 2 showing a plane-polarized pattern across the entire basal FCE (Dent et al., 2019; Conder et al., 2007). This specific pattern of dPix-Git complex in the FCs is reminiscent of our data showing a plane-polarized striated pattern for Dys and β H-Spec at the basal membrane of FCE. Furthermore, in *Drosophila*, β H-Spec levels in the FCs are shown to be regulated by dPak, and this regulation is thought to be required for correct epithelial apical-basal polarity (Conder et al., 2007). Given these findings, it will be interesting in the future to determine if the DGC and FAs compensate for each other in terms of sensing and responding to the extracellular-mediated signals during development. For instance, the presence of DGC and FAs in the muscle seems to be critical for muscle integrity (Masuda et al., 2009; Flük et al., 1999; Graham et al., 2015). However, in other developmental contexts, it is not clear if the DGC and FAs compensate for each other or they have distinct roles.

Previous studies showed that in *Dg* or *Dys* nulls alone, stress fibers have correct localization of integrin to their FAs (Campos et al., 2020). However, these mutants produce short eggs and have misaligned basal stress fibers, suggesting FAs and the DGC might play distinct roles in oogenesis. In contrast, the single *β H-spec* mutant egg chambers are grossly normal for egg elongation and have well-organized basal F-actin. This suggests that the DGC might compensate for the presumably altered function of the integrin-Git-dPix-Pak module due to loss of β H-Spec during oogenesis and beyond. Severe additive defects in egg chamber/egg morphology and high lethality in the *β H-spec*; *Dys* double mutants further reinforce these possibilities.

Here, we have demonstrated a genetic redundancy between β H-Spec and Dys, and a similar plane-polarized organization pattern for these proteins in oogenesis. The work of others using *Drosophila* egg chambers shows *Dg* and *Dys* are interdependent for their localization in the basal membrane of FCE, and on the other hand, *Dg* is required for the apical localization of β H-Spec in the FCs (Deng et al., 2003; Schneider et al., 2006). Interestingly, in epithelial cells, reduced

expression of Dg is associated with increased invasiveness of cancer cells, pointing to its “tumor suppressor” behaviour (Henry et al., 2001; Muschler et al., 2002). Notably, a recent work finds dystroglycan 1 (Dag1) directly binds to the Hippo pathway effector, Yap, to inhibit cardiomyocyte proliferation in mice (Morikawa et al., 2017).

As a transcription factor, YAP mediates the expression of many growth-promoting or anti-apoptotic genes. The transcriptional activity of YAP is, however, subject to negative regulation through its phosphorylation and cytoplasmic sequestration by the Hippo signaling cascade (Ramos and Camargo, 2012; Huang et al., 2013; Liu et al., 2013). In addition, the non-receptor protein tyrosine phosphatase type 14 (PTPN14) binds to YAP to inhibit its transcriptional activity and thus acts as a tumor suppressor whose decreased activity has been linked to increased metastatic potential in cancer cells (Huang et al., 2013; Liu et al., 2013; Wilson et al., 2014). Furthermore, the *Drosophila* PTPN14 homolog, Pez, is a strong proliferation suppressor required for regulation of intestinal stem cells (Edwards et al., 2001; Poernbacher et al., 2012). Strikingly, in *Drosophila*, β H-Spec is shown to act as a tumor suppressor by regulating Hippo signaling through modulating cortical actomyosin activity (Deng et al., 2015). More recently, it was shown that the *Drosophila* YAP ortholog, Yorkie, also functions at the cell cortex to activate myosin contractility which might in turn amplify the effects of mechanical tension on Yorkie activity (Xu et al., 2018). Here, we show that similar to β H-Spec, Pez, is an apical membrane-bound protein whose increased expression dissolves the apical β H-Spec network. In addition, we find that Pez loss of function disrupts stress fiber orientation at the basal FCE, similar to the phenotype observed in *Dys* and *Dg* mutants.

Taken together, our work and other studies outline a novel functional network in cortical organization involving the spectrins, DGC, and Hippo signaling pathway. These proteins are positioned to integrate mechanical stress and extracellular matrix information with growth, proliferation, shape, and patterning of epithelia. *Drosophila* genetic and cell biological tools will facilitate future works defining this network’s organization and functions in multiple developmental contexts.

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APPENDIX A: MATERIALS AND METHODS

Drosophila stocks and genetics

Key genotype information for each experiment is provided in (Table 3). For most experiments, crosses were raised at 25°C and newly-eclosed experimental females were cultured on fresh yeast for 1.5–3 days in the presence of males, at the same temperature. In the case of *βH-spec*, *Dys* double mutant generation, crosses were reared at RT and eclosed females of appropriate genotype were shifted to 25°C and yeast-fed for 2 days before ovary dissection. *Dys* transheterozygous null mutants were generated by 3 independent crosses using a set of publicly available *Dys* alleles (See Table 2). To study *Dys* short isoforms in oogenesis, the *Dys*[*MB02524*] allele was used in combination with *Dys* null alleles. *βH-spec* nulls were generated by crossing the *kst*[*MI13613*] allele to the *Df*(*3L*)*Exel6093* deficiency. *βH-spec*, *Dys* double mutants were generated by crossing *Df*(*3L*)*Exel6093*, *Dys*[*MI812*]/*TM6Tb* flies to *kst*[*MI13613*], *Df*(*3R*)*Exel6184*/*TM6Tb* flies. The FC-specific drivers were *c323-GAL4*, *c204-GAL4*, and *c355-GAL4*. The germline-specific and muscle-specific drivers were *MTD-GAL4* and *Mef2-GAL4*, respectively. *UAS-Dys-RNAi* (PTRiP.JF01118) and *UAS-Dys-RNAi* (PTRiP.HMC03789) were used to knock down *Dys*. Stocks used in this study were obtained from the Bloomington Drosophila Stock Center ((NIH P40OD018537), Indiana Univ.) unless otherwise stated. For a detailed stock information, see Table 2. For key information on the experimental genotypes, see Table 3.

Immunohistochemistry

Ovaries were dissected in phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde for 15-20 min. Tissues were rinsed 3-4 times in 1X PBS and incubated in last wash for at least 15 min. Tissue permeabilization was performed with PBT (PBS + 0.2% TritonX-100). Samples were then incubated in staining solution (typically phalloidin & Alexa-Wheat Germ Agglutinin (Alexa-WGA)) either overnight at 4°C or 5 hrs at RT. Stained tissues were rinsed and stored in PBT until mounted in Vectashield. For *Dys* Mab antibody staining, following ovary dissection, fixation, and permeabilization, egg chambers were blocked in PBT + 4% goat serum for 1 hr. Primary and secondary antibody incubations were either overnight at 4°C or 5 hrs at RT in PBTG (PBT + 1%

goat serum). Fluorophores were included at the same step when secondary antibody staining was performed. Ovaries were washed in PBTG 3-4 times for a total of 45 min after primary antibody incubation and at least 1 hr after secondary antibody incubation, and kept in PBTG until mounted in Vectashield. The Dys Mab primary dilution was performed according to the recommended concentration range for Immunohistochemistry (2-5 $\mu\text{g}/\text{ml}$) in Developmental Studies Hybridoma Bank (DSHB). The primary antibody and dye stains are listed in Table 2.

In this study, tissues were examined using a Leica SP2 or Leica SP8 White Light Laser confocal microscopy system, using sequential scanning to avoid detecting emissions in the wrong channel. The Lightning Deconvolution imaging method was used for Figures 8 and 11.

Generation of FLP-out clonal tissue

Following Singari et al (2014), flies of the genotype P[hsFLP]12, P[GAL4-Act5C (FRT.CD2).P]; responder were reared at RT for 1-2 days, and heat shocked in a 37°C water bath for 1hr. Heat shock induction allowed the expression of GAL4 from Act5C promoter in random cells. Heat shocked flies were raised at 25°C on standard media and a plenty of fresh yeast for 2.5 days, and the ovaries were dissected, fixed, and stained as described above.

Egg aspect ratio measurement

Flies were raised at 25°C for 3-5 days, ovaries were dissected in 1X PBS, fixed in 2% paraformaldehyde, then rinsed with and mounted in PBS. Vacuum grease was used to create a small gap between the coverslip and the tissue to prevent smashing eggs and affecting their dimensions. Mature egg chambers were imaged with a camera mounted to a dissecting microscope. Using ImageJ, egg length and width were measured along its longest A-P axis and widest D-V axis, respectively. Dorsal appendages were excluded from calculations. Aspect ratio was determined by dividing length by width for several egg chambers ($n>130$) from at least 10 different females, per each genotype. Data were visualized by boxplots using the ggboxplot() function in the ggplot2 package of R. Statistical tests were ANOVA and significance were **** $p<22.e-16$ for (Fig 10 J) and ns=not significant= 0.8 for (Fig 13 G).

BM fibril length and number measurement

Flies were fed with standard fly food and fresh yeast and incubated at 25°C for 2 days before ovary dissection. Fibrils were visualized by LanA-GFP and laser-scanning confocal imaging in their focal planes. Fibril length and number was measured as in Isabella and Horne-Badovinac (2015). Fibrils were isolated in Fiji software by adjusting intensity threshold to remove approximately 95% of dimmest pixels. The “Analyze Particles” tool was used to exclude objects with an area of $<0.38 \mu\text{m}^2$ and circularity >0.35 from measurements. Fibril length (feret’s diameter) was calculated for each fibril using the “Analyze Particles” tool. Data distribution and quartiles were depicted by violin and boxplots using `ggviolin()` function in the `ggplot2` package of R. Data points were added to plots by `geom_point()` function in the same package. Statistical test was unpaired t-test, comparing the mean of two independent groups. Significance was **** $p = 4.5\text{e-}14$. Fibril numbers (number of fibrils per μm^2) were calculated by selecting the “Area” measurement parameter and calculating Area% (fibril count per area) using the “Analyze Particles” tool. Data were represented by bar plots using the `geom_bar` function in the `ggplot2` package of R. Unpaired t-test was performed to compare the mean of 2 independent groups.

Table 2: Key Resources

<i>Panel A: Drosophila Stocks:</i>			
Name	Source	Identifier	Additional information
<i>w¹¹¹⁸</i>	S.Benzer	<i>w¹¹¹⁸</i>	
<i>Dys</i> [MB02524]	BDSC	BDSC-24182	<i>w¹¹¹⁸</i> ; <i>MiGFP</i> [E.3αP3]= <i>ET1Dys</i> [MB02524]
<i>Dys</i> [E17]	BDSC	BDSC-63047	<i>w</i> [*]; <i>st</i> [1] <i>cu</i> [1] <i>e</i> [1] <i>Dys</i> [E17]/ <i>TM6B</i> , <i>Tb</i> [1]
<i>Dys</i> [MI00812]	BDSC	BDSC-37109	<i>y</i> [1] <i>w</i> [*]; <i>Miy</i> [+ <i>mDint2</i>]= <i>MICDys</i> [MI00812]
<i>Df</i> (3R) <i>Erex6184</i>	BDSC	BDSC-7663	<i>w¹¹¹⁸</i> ; <i>Df</i> (3R) <i>Erex6184</i> , <i>Pw</i> [+ <i>mC</i>]= <i>XP-UExel6184</i> / <i>TM6B</i> , <i>Tb</i> [1]
<i>Dys</i> [GFP]	BDSC	BDSC-59782	<i>y</i> [1] <i>w</i> [*]; <i>MiPT-GFSTF.0Dys</i> [MI01893- <i>GFSTF.0</i>]/ <i>TM3</i> , <i>Sb</i> [1] <i>Ser</i> [1]
<i>Df</i> (3L) <i>Erex6093</i>	BDSC	BDSC-7572	<i>w¹¹¹⁸</i> ; <i>Df</i> (3L) <i>Erex6093</i> , <i>Pw</i> [+ <i>mC</i>]= <i>XP-UExel6093</i> / <i>TM6B</i> , <i>Tb</i> [1]
<i>kst</i> [MI13613]	BDSC	BDSC-59172	<i>y</i> [1] <i>w</i> [*]; <i>Miy</i> [+ <i>mDint2</i>]= <i>MICkst</i> [MI13613]/ <i>TM3</i> , <i>Sb</i> [1] <i>Ser</i> [1]
<i>βH</i> [GFP]	BDSC	BDSC-60193	<i>y</i> [1] <i>w</i> [*]; <i>MiPT-GFSTF.1kst</i> [MI03134- <i>GFSTF.1</i>]
<i>UAS-Zyr.ChRFP</i>	BDSC	BDSC-28875	<i>w</i> [*]; <i>Pw</i> [+ <i>mC</i>]= <i>UAS-Zyr.ChRFP2</i>
<i>PTRαP.JF01118</i>	BDSC	BDSC-31553	<i>y</i> [1] <i>v</i> [1]; <i>Py</i> [+ <i>t7.7</i>] <i>v</i> [+ <i>t1.8</i>]= <i>TRαP.JF01118attP2</i>
<i>PTRαP.HMC03789</i>	BDSC	BDSC-55641	<i>y</i> [1] <i>sc</i> [*] <i>v</i> [1] <i>sev</i> [21]; <i>Py</i> [+ <i>t7.7</i>] <i>v</i> [+ <i>t1.8</i>]= <i>TRαP.HMC03789attP40</i>
<i>c204-GAL4</i>	BDSC	BDSC-3751	<i>w¹¹¹⁸</i> ; <i>Pw</i> [+ <i>mW.hs</i>]= <i>GawBc204</i> / <i>TM3</i> , <i>Ser</i> [1]
<i>C355-GAL4</i>	BDSC	BDSC-3750	<i>Pw</i> [+ <i>mW.hs</i>]= <i>GawBc355</i> , <i>w</i> [1118]
<i>MTD-GAL4</i>	BDSC	BDSC-31777	<i>textitPw</i> [+ <i>mC</i>]= <i>otu-GAL4</i> :: <i>VP16.R1</i> , <i>w</i> [*]; <i>Pw</i> [+ <i>mC</i>]= <i>GAL4-nos.NGT40</i> ;
<i>LanA</i> [GFP]	VDRC	VDRC-318155	<i>Pw</i> [+ <i>mC</i>]= <i>GAL4</i> :: <i>VP16-nos.UTRCG6325</i> [MVD1]
<i>Df</i> (2L) <i>Erex9038</i>	BDSC	BDSC-7800	<i>PBac</i> [<i>TRG00574.sfGFP-TVPTBFVK00033</i>] <i>w¹¹¹⁸</i> ; <i>Df</i> (2L) <i>Erex9038</i> , <i>Pw</i> [+ <i>mC</i>]= <i>XP-UExel9038</i> / <i>CyO</i>
<i>Panel B: Primary Antibody and dye stains:</i>			
Name	Source	Identifier	Additional information
MANCHO17(4C8) utrophin	DSHB	RRID:AB_2618134	Type: primary, clonality: monoclonal, target: Dys-trophin
Wheat Germ Agglutinin (WGA)	Thermo Fisher Scientific	W32466	Alexa Fluor 647 conjugate
Phalloidin	Thermo Fisher Scientific	A30104	Alexa Fluor Plus 405
<i>Panel C: Software/Application/Tool:</i>			
Fiji(ImageJ)			https://fiji.sc/
R			https://www.r-project.org/
BioRender			https://biorender.com/

Table 3: Experimental genotypes

Figure #	Key genotype information	Genotype/Abbreviation in figures	Additional information/Notes
5 (A', B', C', D', E)	<i>Df(3R)Ere6184/Dys[E17]</i>	<i>Df Ere6184/Dys[E17]</i>	Dys null mutant
5 (A)	<i>Dys[E17]/+</i>	Control	Control for 5 (A')
5 (D)	<i>Df(3R)Ere6184, LanA[GFP]/TM3Sb</i>	Control	Siblings for 5 (D')
5 (D')	<i>Df(3R)Ere6184, LanA[GFP]/Dys[E17]</i>	<i>Df Ere6184/Dys[E17]</i>	BM fibril visualization in Dys null follicles
5 (F, G)	<i>Dys[M1812]/Dys[E17]</i>	<i>Dys[M1812]/Dys[E17]</i>	Dys null mutant
6 (A-B'', D-I')	<i>Dys[GFP]</i>	<i>Dys[GFP]</i>	Dys[GFP] protein trap; endogenous expression
8 (A-C'')	<i>c323-GAL4/+; UAS-RFP-Zyx/+; Dys[GFP]/+</i>	<i>Dys[GFP], Zyxin</i>	RFP-Zyxin labels focal adhesions
7 (C-F)	<i>P[hsFLP]12, P[GAL4-Act5C (FRT.CD2).P]/+;</i> <i>DysRNAi/+; Dys[GFP]; UAS-RFP-CD8/Dys[GFP]</i>		UAS-DysRNAi PTRiP.HMC03789, to all Dys isoforms
7 (G-G'')	<i>Dys[GFP]</i>	<i>Dys[GFP]</i>	Dys[GFP] protein trap; endogenous expression
9 (A, B, C, D, E, F, G)	<i>Cyo/+; Dys[GFP]/c204-GAL4</i>	Control	Siblings for 9 (A', B', C', D', E', F', G')
9 (A', B', C', D', E', F', G')	<i>UAS-Dys-RNAi/+; Dys[GFP]/c204-GAL4</i>	<i>Dys RNAi</i>	UAS-DysRNAi PTRiP.HMC03789, to all Dys isoforms
10 (H, I)	<i>C355/+; Cyo/+; Dys[GFP]/+</i>	Control	Siblings for 10 (H', I')
10 (H', I')	<i>C355/+; UAS-Dys-RNAi/+; Dys[GFP]/+</i>	<i>Dys RNAi</i>	UAS-DysRNAi PTRiP.HMC03789, to all Dys isoforms
11 (A-C''')	<i>P[hsFLP]12, P[GAL4-Act5C (FRT.CD2).P]/+;</i> <i>Dys[GFP]; UAS-RFP-CD8/UAS-Dys-RNAi</i>	<i>Dys[GFP], Dys RNAi clone</i>	UAS-DysRNAi PTRiP.JF01118, to all Dys isoforms
13 (A-F)	<i>UAS-Dys-RNAi/+; Dys[GFP]/Mej2-GAL4</i>	UAS-DysRNAi PTRiP.HMC03789, to all Dys isoforms	Siblings for 15 (A', B', C', D'')
15 (A, D)	<i>Df(3R)Ere6184/TM3Sb</i>	Control	Siblings for 15 (A', B', C', D'')
15 (B, C, D')	<i>Dys[MB02524]/TM3Sb</i>	Control	Mutant transheterozygote; short isoforms remain intact
15 (A', B', C', D'')	<i>Df(3R)Ere6184/Dys[MB02524]</i>	<i>Df Ere6184/Dys[MB02524]</i>	BM fibril visualization in Dys null follicles
16 (A, B, C, D'', E'')	<i>Df(3R)Ere6184, LanA[GFP]/Dys[E17]</i>	<i>Df Ere6184/Dys[E17]</i>	BM fibril visualization in the presence of Dys short isoforms
16 (A', B', C', D', E')	<i>Df(3R)Ere6184, LanA[GFP]/Dys[MB02524]</i>	<i>Df Ere6184/Dys[MB02524]</i>	Siblings for 16 (A', B', C', D', E')
16 (A'', B'', C'', D, E)	<i>Df(3R)Ere6184, LanA[GFP]/TM3Sb</i>	Control	betaH-spec null mutant
17 (B', B')	<i>kst[MI13613]/Df(3L)Ere6093</i>	<i>betaH-spec</i>	Dys null mutant; control for betaH-spec, Dys double mutant
17 (C, C')	<i>Df(3R)Ere6184/Df(3L)Ere6093, Dys[M1812]</i>	<i>Dys</i>	betaH-spec, Dys double mutant
17 (D, D', E', E'')	<i>Df(3L)Ere6093, Dys[M1812]/kst[MI13613], Df(3R)Ere6184</i>	<i>betaH-spec, Dys</i>	
17 (F-G)	<i>kst[MI03134-GFSTF.1]/Df(3L)Ere6093</i>	<i>betaH-GFP</i>	

APPENDIX B: SUPPORTING INFORMATION

Labeling the termini of Dys proteins in vivo

Given the potential complexity in the subcellular organization of various Dys isoforms (see Figure 7 and the first section of chapter III), we set out to label the amino-termini of Dys long isoforms to better understand the orientation and distribution of Dys proteins in oogenesis. In this section, I will briefly outline our tagging strategy and the steps that have been successfully completed.

In order to incorporate a fluorescent tag into the Dys isoforms, we followed a previously described, efficient protocol to edit the *Drosophila* genome (Gratz et al., 2015) using the following components (see the strategic planning flowchart in Figure 18):

A) Transgenic fly lines that express Cas9 under the control of the germline *vasa* promotor are generated and available at the Bloomington *Drosophila* Stock Center. The Cas9 enzyme introduces double-strand breaks (DSBs) in the target DNA, in this case the coding region for the amino-terminal end of Dys long isoforms (Gratz et al., 2015; Bier et al., 2018).

B) Guide RNA (gRNA) plasmid which comprises pU6-BbsI-gRNA vector and a pair of short complementary oligonucleotides for each specific target site. We isolated genomic DNA from our *Dys[GFP]* fly strain and sequenced the genomic locus to check for SNPs within the target region. Our injection strain will be generated by crossing the *Dys[GFP]* allele to a *vasa-Cas9* fly strain. Our sequencing results confirmed there are no SNPs in our target. We selected two target sites using the fly “CRISPR Optimal Target Finder” tool (Gratz et al., 2015). One target site is at the beginning of Dys exon 2 which is used by Dys long isoforms and the other is in the downstream intron (see Figure 19). We successfully cloned the two gRNA sequences into two separate pU6-BbsI plasmids using Inverse PCR. We divided each gRNA sequence in half and added sequence (or reverse complement) to the end of each respective primer containing complimentary sequence to the pU6-BbsI-gRNA vector (this strategy for gRNA construct design was suggested by Melissa Harrison, University of Wisconsin, School of Medicine and Public Health). This results in the expression of gRNA under the control of small RNA promoter of *Drosophila* U6 gene, guiding Cas9 to the targeted locus (Gratz et al., 2015).

C) A double-strand DNA (dsDNA) donor repair template comprising target site-specific homology arms flanking the fluorescent tag sequence (mScarlet in our case) (see donor construct design in Figure 19). Since the homology arms are homologous to the sequences adjacent to the cleavage site, the homology-directed repair (HDR) employs the m-Scarlet-containing template to repair the DSB, resulting in the insertion of tag into the targeted locus. We purchased the commercially available pHD-DsRed vector which includes a removable DsRed eye marker for rapid identification of desired flies and contains multiple cloning sites (MCS) for inserting the locus-specific dsDNA donor. We successfully cloned the mScarlet sequence and the left homology arm (LHA) into the pHD-DsRed backbone using the Gibson Assembly cloning method (suggested by Melissa Harrison). The next step will be to clone the right homolgy arm (RHA) into our current donor construct. The following step will be to inject our two gRNA constructs and the fully-assembled donor construct into our desired injection fly strains, using standard *Drosophila* injection techniques. Finally, we will perform PCR using primers specific to the targeted locus to validate the precise insertion of the protein tag into the desired location. Moreover, we will examine the egg morphology and crossvein pattern in the flies in which the modification has been made to verify that the insertion of the protein tag into the targeted *Dys* locus does not interfere with the normal function of Dystrophin.

Tagging the amino-termini of Dys proteins in vivo will allow us the opportunity to understand which isoforms contribute to the antibody pattern in Figure 7, if they have distinct patterns, and if they are oriented (when viewed in combination with Dys[GFP]). For lines that are informative in this regard, we will also tag the carboxy-terminal end with a blue fluorescent protein. If, for example, the long isoforms are aligned laterally with each other, we may observe a striped pattern of red, green, and blue signals using confocal microscopy (see Figure 20). In addition, since our RFP tag (m-Scarlet) labels the amino-terminal end of Dys, where it binds to F-actin, staining for the F-actin using the egg chambers with the RFP-labeled isoforms, might show an overlap between the actin bundles and the amino-terminal end of Dys. This would suggest that long Dys isoforms extend from the membrane to cortical actin.

Figure 18: Strategic planning flowchart.

The options outlined in the tagging strategy in this study are indicated. See text (the first section of Appendix B) for a more detailed discussion of each choice point.

Figure 19: Donor construct design.

To tag the amino-termini of Dys long forms, the commercially-available pHD-DsRed vector is used. Target site 1 is selected close to the tag (m-Scarlet) insertion site and target site 2 is in the nearby intron where the DsRed marker will be placed. Homology arms include sequences immediately flanking the cleavage sites. The left homology arm contains the in-frame tag and sequences between the tag and the DsRed marker. Upon Cas9 cleavage and HDR, the untagged region is replaced with a tagged region and a visible 3xP3-DsRed marker. Using Cre recombinase, the DsRed marker can be removed leaving only the tagged coding sequence and a single LoxP site (black diamond). This strategy is modified from (Gratz et al., 2015)

Figure 20: Plan for labeling the termini of Dys proteins in vivo.

(A) The RFP tag (m-Scarlet in this study) is to be inserted by CRISPR into the amino-terminal (NT) end of Dys long isoforms. The GFP exon (green) is inserted into spectrin repeat 22 (SR22), tagging the long isoforms. The RFP tag (m-Scarlet) will be independently inserted into this allele so that orientation of the long forms can be detected. (B) Triple tagging of Dys. A BFP tag is to be inserted into the carboxy-terminal (CT) end of the double-labeled *Dys* allele, as well as untagged forms (not shown). This would further define any patterns observed with the other tagged forms, and also validate the anti-Dys Mab (see Figure 7).

Supplementary figures

Figure 21: Plane-polarized striated pattern of Dys[GFP] in follicular epithelia.

Confocal images taken in the focal plane of Dys[GFP] signals at the basal side of the follicle cell layer (stage 7 egg chambers). Scale bars, 10 μm . (A) Dys[GFP] signals show a plane-polarized striated pattern in basal epithelial membranes, perpendicular to the elongation axis of the egg chamber. (B) An anti-GFP antibody against the GFP domain in the *Dys[GFP]* allele shows a similar organization pattern for Dys, confirming Dys[GFP] signals are not affected by potential mechanical forces on Dys. (C) The overlay of the green and red channels show the colocalization of Dys[GFP] and anti-GFP signals.

Figure 22: Dys is required in follicular epithelia to promote egg elongation.

(A-A') *Mef2-GAL4* is used to induce *Dys-RNAi* in muscle. Depletion of Dys from the muscle tissue does not affect egg shape as depleted eggs' dimensions are comparable to controls (For statistical analyses, see Figure 13). (B-B') *C355-GAL4* driver is used to deplete Dys from follicular epithelia. This inhibition results in short eggs, with affected eggs having a significantly lower aspect ratio (length divided by width) compared to controls. The horizontal line corresponds to the longest A-P axis of the egg in control. The same line is superimposed on the Dys-depleted egg. The orange arrow indicate that the length of the affected egg is shorter than in control. (For statistical analyses, see Figure 10).

Figure 23: Expression pattern of *c355-GAL4* in follicle cells.

(A) Representative Image of ovarioles taken in a focal plane, closer to the basal surface. Expression of a cell membrane marker using *c355-GAL4* show its expression pattern in oogenesis. This driver is expressed in the posterior follicle cells over the oocyte. Its expression has just begun at around stage 8 and only clonally then. Its expression becomes stronger and is detected in most posterior follicle cells as egg chambers proceed through stage 10. (B) An ovariole imaged in a cross section. The *c355-GAL4* driver is expressed in the posterior follicle cells covering the oocyte. (C) Representative images of stage 12-13 egg chambers. *c355-GAL4* expression is detected in follicle cells in late oogenesis.

Figure 24: Dys long isoforms are not required for egg elongation.

Representative images of mature eggs for various combinations of *Dys* alleles. (A-C') Different combinations of *Dys* null alleles produce rounded eggs with shorter A-P axes and wider D-V axes compared to controls. For detailed description of *Dys* null alleles, see text (the first section of chapter II). For statistical analyses, see Figure 14. (D, D') In the combination of *Dys*[MB2524] allele with a deficiency allele, *Dys* long isoforms are perturbed but short forms remain intact. This combination does not affect egg shape, indicating *Dys* long isoforms are dispensable for egg elongation. For detailed description of the *Dys*[2524] allele, see text (the seventh section of chapter II). For statistical analyses, see Figure 14. (D, D') Combination of the *Dys*[GFP] allele with the deficiency allele does not trigger the egg elongation defect, providing evidence that the *Dys*[GFP] allele is fully functional for egg elongation. For statistical analyses, see Figure 14.