Mouse germline cysts contain a fusome that mediates oocyte development

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ABSTRACT

Mouse female primordial germ cells undergo five synchronous, incomplete mitotic divisions, and send each resulting germline cyst into meiosis to fragment and produce 4-6 oocytes and 24-26 supportive nurse cells. How oocytes are specified, linked appropriately to nurse cells and acquire high quality organelles and cytoplasm remains unclear. We show that mouse cysts develop an asymmetric Golgi, ER and microtubule-associated structure, “Visham,” similar to oocyte-determining fusome in Drosophila cysts. Starting as the EMA granule, Visham distributes asymmetrically in cyst cells along with Pard3, and enriches in future oocytes. Transient spindle remnants containing stable acetylated microtubules link early cyst cells; a gap in these microtubules beginning at the 8-cell stage suggests cysts are programmed to fragment into six-cell groups. Visham also acts during meiosis to mediates Pard3-dependent polarity, Xbp1-dependent adaptive UPR, and organelle rejuvenation. In Dazl mutants, cysts still form, but normal Visham behavior, polarity, rejuvenation and oocyte production are blocked.

INTRODUCTION

Early female germ cell development occurs within interconnected cysts formed by consecutive incomplete divisions of a germline stem cell (GSC) or PGC in diverse animals. These include basal species like hydra (Littlefield, 1994), nematodes like C. elegans, insects like Drosophila, and vertebrates including fish, frogs, mice and humans (Gondos, 1973; King, 1970; Pepling and Spradling, 1998; Kloc et al. 2004a; Marlow and Mullins, 2008; review, Spradling, 2024). As they develop, cysts acquire polarity often associated with a microtubule-rich "fusome" (Giardina, 1901; Huynh and St Johnston, 2004; Spradling et al 2022). In Drosophila, the fusome arises from mitotic spindle remnants, contains ER cisternae and its polarity is essential for oocyte production. Later in meiotic prophase the fusome mediates centrioles, mitochondria, and germ plasm regulators such as oskar mRNA to move into the oocyte where they form the Balbiani body (Bb). Remarkably, mammalian oocytes also undergo these same events (review: Spradling et

al. 2022). Oocytes in many invertebrate and vertebrate species require Par genes to establish or maintain their polarity (review: Huynh and St Johnston, 2004).

In the mouse, germ cells are induced around embryonic day 6.5 (E6.5) (Lawson et al. 2002) and begin migrating toward the gonad as primordial germ cells (PGCs). En route, PGCs reprogram their epigenetic state and return to pluripotency (Seki et al. 2007; Saitou et al. 2011; Loda et al. 2022; Liu et al. 2025). However, after they enter the gonad at E10.5, PGCs turn on DDX4 and initiate cyst formation while reprogramming is still ongoing. During E11.5 they upregulate Dazl, an RNA-binding, post-transcriptional regulatory protein which is needed to produce and translate multiple target mRNAs (Zagore et al. 2018; Yang et al. 2020). Dazl plays an essential role promoting germ cell meiotic entry and oocyte production (Gill et al. 2011; Nicholls et al. 2019).

Mouse germline cysts arise from five rounds of mitotic divisions beginning at E10.5, so that each PGC generates almost 32 cells. However, the cysts break into an average of 4.8 smaller cysts by the time divisions are completed at E14.5 when cells enter meiosis (Lei and Spradling, 2013; Levy et al. 2024). During meiosis, each smaller cyst slowly loses component cells, as every 2 days about one cell on average becomes activated to act as a nurse cell. It transfers most of its cytoplasm into the residual cyst, shrinks to a small remnant and undergoes programmed cell death (Lei et al. 2016; Niu and Spradling, 2022; Ikami et al 2023). In pachytene, there is a substantial reorganization that brings mitochondria and ER sheets into close proximity by E18.5 (Pepling and Spradling, 2001). Cyst breakdown is complete by 5 days after birth (P5) with the formation of 4-6 primordial follicles, each containing a single oocyte in which organelles transferred from nurse cells are gathered into an aggregate known as the Balbiani body. Cyst polarity guides cyst fragmentation and oocyte specification, polarization and organelle acquisition, but a mouse analog of the fusome has not been identified.

Germline cysts also support germ cell rejuvenation, the universal process that allows damage to be removed each generation so that species can persist without known limit. Germline rejuvenation takes place

during meiosis in single celled eukaryotes (Unal et al. 2011; Suda et al. 2024; Xiao and Unal, 2025). Processes that rejuvenate organelles such as mitochondria, restore rDNA copy number, and reset epigenetic chromatin marks have also been documented in meiosis during animal gametogenesis (Cox and Spradling, 2003; Hill et al. 2014; Reik and Surani, 2015; Bohnert and Kenyon, 2017; Lieber et al. 2019, Pang et al. 2023; Yamashita, 2023). The Balbiani body, in newly formed oocytes, is associated with germ plasm formation and selection for mitochondrial function in many species (Heasman et al. 1984; Kloc et al. 2004b Marlow and Mullins, 2008; Spradling et al. 2022; Sekula et al. 2024).

Here we find that mouse PGCs and cyst cells elaborate a fusome-like structure we term "Visham" that is rich Golgi, endosomal vesicles and associates with ER. Visham distributes asymmetrically in association with stable microtubules that persist briefly for part of the cyst cell cycle. By the 8-cell stage, cyst cells reorganize into a rosette configuration that brings cells with the most bridges (potential oocytes) and associated Visham closer together. Developing cysts at this stage frequently fragment non-randomly at sites lacking microtubule connections into six-cell cysts, implying that mouse ovarian cyst structure is more highly controlled than previously realized. Beginning as PGCs and throughout cyst formation, EGAD-mediated UPR pathway proteins including Xbp1 are expressed, suggesting that the oocyte proteome quality begins to be enhanced beginning very early. After pachytene, centrosome/Golgi-rich elements move to the oocyte and mediate the acquisition of organelles by the Bb. In the absence of Dazl, cyst development and asymmetric Visham accumulation is disrupted, and no oocytes are produced.

RESULTS

PGCs contain a Golgi-rich structure known as the EMA granule

Early observations of a germ cell "epithelial membrane antigen (EMA)" (Hahnel and Eddy, 1986) identified an "EMA granule" in mouse primordial germ cells that resembles the Drosophila spectrosome, a fusome precursor (Lin et al. 1994; Pepling et al. 1998). To investigate this relationship further, we determined that the EMA granule appears in PGCs as early as E9.5 and continues to be expressed in later cysts (Figure 1A, Figure S1A). We used lineage labeling to mark cysts derived from single PGCs, and 3D surface rendering (Fig S1B-C) and volume quantification, to demonstrate a significantly uneven distribution of the EMA granule within cyst daughter cells (Fig 1B). This structural asymmetry prompted us to name the EMA-rich structure "Visham" (Sanskrit for asymmetric/uneven).

The EMA-rich material reorganized and moved closer together in female cysts by E13.5 (Figure 1C; Figure S1D-E), whereas in male cysts they remained mostly separate (Figure 1C' and graph). Cyst regions with enriched Visham staining contain an average of 3 Tex14-positive bridges, whereas unenriched zones had an average of just 1 (Figure 1D graph; Figure S1F). Thus, highly branched cells with more bridges, which are known to give rise to most oocytes (Lei and Spradling, 2013; Lei and Spradling, 2016; Ikami et al. 2023), contain more Visham. Similar clustering, known as rosette formation (Figure 1H), and increased fusome accumulation in pro-oocytes, occur as germline cysts grow in Drosophila and many species (see Hegner, 1914; Büning, 1994; Huynh and St Johnston, 2004). Rosette formation is thought to prepare for sharing between nurse cells and oocytes.

Visham is highly enriched in Golgi and vesicles

Electron microscopy (EM) of mouse germ cells documented EMA granules with prominent Golgi and endocytic vesicles in most E11.5 and E12.5 cyst germ cells (Figure 1E'; Figure S1K). Visham co-stained with the Golgi protein Gm130 (Figure 1F) and the recycling endosomal protein Rab11a1 (Figure 1F').

Visham often partly overlaps with ER (Figure S1I). EM sections of E14.5 cysts revealed clusters of Golgi within adjacent cyst cells near ring canals as expected for cysts after rosette formation (Figure 1E, 1E"). EMA-1 antisera react with multiple fucosylated glycolipids (Apostolopoulos et al. 2015; Figure S1H). EMA staining disappears from germ cells at E14.5 (Figure 1I), however, very similar (but non-germ cell-specific) staining continued with wheat germ agglutinin (WGA) at later stages (Figure 1G, G"; Figure S1G).

Visham overlaps centrosomes and moves on microtubules

A cell's microtubule cytoskeleton that emanates from its centrosomes, and from non-centrosomal microtubule organizing centers (ncMTOCs), frequently controls trafficking of Golgi elements and positions them near centrosomes. To investigate the relationship between microtubules and Visham, we stained E11.5 gonads with anti-acetylated tubulin (AcTub) and EMA. Visham usually overlapped the centrosomes in interphase germ cells (Figure 2A), indicating it occupies a peri-centriolar domain. In germ cells undergoing mitosis (Figure 2B), Visham associated with centrioles during interphase, but (typical for Golgi) dispersed as a spindle forms and for most of metaphase. In telophase, Visham vesicles gather at the spindle poles in both daughter cells and at the arrested cytokinesis furrow, which still retains the elongated spindle/midbody microtubules typical of cyst-forming cell cycles (Figure 2B). 3D summary movies of germ cells at these stages stained for AcTub and EMA are shown in Figure S2A.

These behaviors differ in detail from the pattern of Drosophila fusome segregation during cyst divisions (de Cuevas and Spradling, 1998). Visham was rarely seen at just one pole of a spindle like the fusome at very early stages of GSC and cytoblast division; some EMA-reactive material also appears at the cytokinesis furrow earlier than the fusome (Figure 2C; Figure S2C-D). Asymmetry in the Visham distribution arose only in late telophase. Clusters of Visham and centrosomes approached the arrested furrow (Figure 2D, S2C-D). At this point short microtubule arrays were visible (Figure 2D, S2C). Soon after, the amount of Visham in the two daughter cells becomes asymmetric, suggesting that differential activity or persistence of Visham transport late in cytokinesis generates asymmetry. Ovaries cultured

briefly in vitro support continued germ cell division and Visham asymmetry. Visham formation is severely affected in such cultures if MTs were disrupted by cold or by Ciliobrevin D treatment (Figure S2E graph).

Cyst fragmentation is non-random and correlates with microtubule gaps

Mouse cysts produce an average of 4-6 oocytes per PGC as a result of initial cyst breakage into multiple, independently developing subcysts that each produce one oocyte (Lei and Spradling, 2013; Niu and Spradling, 2022). Production of uniform-sized oocytes, as observed, would seem to require programmed breakage into uniform subcysts (Spradling et al. 2022), but some random breakage also occurs (Lei and Spradling, 2013; Ikami et al. 2023; Levy et al. 2024). To assess a role of microtubules and Visham in programming cyst fragmentation, we tracked AcTub and Visham in lineage-labeled E12.5 cysts. In about 10% of interphase cysts, presumably those in very late telophase or early in the subsequent cell cycle, persistent microtubule arrays were observed that connected the cells in pairs (Figure 2E). When we examined a total of about 50 large cysts, 15 larger cysts of 7-10 cells were found where the MTs in all cells could be analyzed completely. Unexpectedly, all showed a large discontinuity of 10μ or more in microtubule linkage between just two of the cystocytes (Figure 2E). Three examples are shown in which projections of 8-cell lineage labeled cysts are outlined (dashed lines). In the left column, Visham and MTs are shown (Fig. 2E-E”; Figure S2F’). In the middle column, microtubule bundles connect most cell cells, but are absent between two cells (straight dashed line). The absence of an MT connection predicts cyst breakage (Figure 2E"), where separation into a 2-cell and 6-cell cyst has already occurred.

The patterns of cyst breakage predicted by MT absence were non-random (Figure 2F). Production of a 6-cell cysts was strongly preferred based on the position of the gaps in 13/15 cysts analyzed. 8-cell or larger cysts all were predicted to generate a 6-cell cyst and a residual cell group. This pattern potentially explains six-cell mitoses frequently observed during E10.5-E14.5 (Pepling and Spradling, 1998), and the ~5-fold increase in the number of accumulated organelles in P4 oocytes compared to E14.5 germ cells (Lei and Spradling, 2016).

Visham associates with polarity and microtubule genes including Pard3

Microtubules play a critical role in ovarian cyst formation and polarity in multiple species. Some of the most important regulators of MTs and polarity are the Par proteins that function in epithelial cell polarity, embryonic development, asymmetric neuroblast division (Petronczki and Knoblich, 2001) and oocyte formation in C. elegans, Drosophila and vertebrates (Kemphues et al. 1988; Huynh and St Johnston, 2004; Moore and Zernicka-Goetz, 2005). Drosophila meiotic cysts express Par3/Baz and Par6, another apical Par complex protein, along with beta-catenin/Arm in a ring-like arrangement close to the ring canals (Cox et al. 2001; Huynh et al. 2001; Huynh and St Johnston, 2004). This relatively small membrane zone likely represents the apical domain of cyst germ cells.

We investigated the expression of Pard3, the mouse ortholog of Par3/Baz to determine if a similar arrangement of the highly conserved Par proteins occurs in mouse germline cysts. Pard3 expression localized around Visham but extended beyond it, a pattern reminiscent of the larger ring of Baz in Drosophila cyst cells (Figure 3A, Figure S3A). At E13.5, cysts in rosette configuration show Pard3 and Visham enriched around a central cell (Figure 3B, dashed region) but not in E13.5 male cysts (Figure S3B'). This co-localization was validated in multiple lineage-labeled E13.5 cysts (Figure 3D; Figure S3D). Ring canals were concentrated in this zone (Figure 3C), hence mouse cysts have ring canals and an apical Par protein in the same small anterior location as in Drosophila, and that Visham occupies the corresponding zone as the Drosophila fusome. These findings suggest that mammals form and polarize oocytes using a conserved system found in diverse animals based on Par genes, cyst formation and a fusome.

Visham associates with Golgi genes involved in UPR beginning at the onset of cyst formation

We sequenced additional fetal germ cells (see Methods) and added them to data from Niu and Spradling (2022) to make an integrated dataset of more than 3,000 germ cells spanning E10.5 to P5 (Figure 3F-H; Figure S3E-I). Our gene expression studies showed that PGCs and early cysts actively express many genes that enhance proteome quality by monitoring, refolding or expelling misfolded or aggregated proteins

for degradation. The UPR pathway associated largely with the ER in yeast is regulated by Hac1, the ortholog of Xbp1. We observed high levels of Xbp1 (Figure 4E) and many of. its target genes (Figure 4I) that increase quality secretory protein production. Membrane proteins and lipids are also promoted by this pathway

In animals the UPR pathway has been expanded with several additional branches. We also observed expression Creb3l2 (ER/Golgi resident transcription factor), ATF4 (General UPR transcription factor activated by Golgi stress), Golph3 (Golgi phosphoprotein for membrane trafficking) and Arfgap(s) (involved in both Golgi to ER-and Golgi to endosome trafficking) during mouse cyst formation (Fig S3J). Xbp1 activates genes such as the chaperones (Hsp5a/Bip, Hsp90b1;DNAjc10; Lman2; Dnajb9), protein-disulfide isomerases (Erp44, Pdia3, Pdia4, Pdia6, Creld2), the ERAD regulator Syvn1/Hrd1 and the ERAD components (Sec61b; Ubxn4, Psmc6, Ube2j2, Get4, Ufd1). Thus, repair and expansion of ER, Golgi and secretory components begins immediately or even before migrating PGCs reach the gonad (Fig 3I).

Xbp1 also supports synthesis of ER membrane phospholipids via upregulation of targets such as Elovl1, Elovl5, and Elovl6, and Chka, Other highly expressed targets maintain ER and Golgi structure and are orthologs of Drosophila fusome proteins (Figure 3I'). Thus the adaptive UPR pathway and its branches are active during cyst formation, and later in prophase as well (Figure 3I-3I').

<u>UPR genes are active during cyst formation and depend on Dazl</u>.

To investigate the effects of Dazl on cyst formation we confirmed that Dazl$^{-/-}$ mice retard the PGC to germ cell transition (Figure S4C-E). Dazl$^{-/-}$ E12.5 cysts showed higher levels of the DNA methylase Dnmt3a expression compared to controls (Figure 4A; Figure S4A-B) suggesting that germ cell DNA demethylation has slowed. Ring canals highlighted by staining for Tex14 were only half the size of wild type canals at E13.5 or structurally abnormal showing that cyst formation was slowed and cytokinesis was sometimes abnormal(Figure 4B).

We also performed scRNA sequencing of Dazl female homozygous mutant gonads at E11.5 and E12.5 and reclustered them with wild type germ cells at these stages for comparison (Fig 4C). At E11.5 the merged germ cells cluster together suggesting that Dazl$^{-/-}$ has minimal effects on germ cells at E11.5, the stage it is first expressed (Fig 4C). However, E12.5 WT and Dazl$^{-/-}$ germ cells cluster separately (Fig 4C). Wild type and Dazl$^{-/-}$ expressed similar amounts of EGAD mediated UPR pathway genes at E11.5 germ cells (Figure S4F) whereas wild type germ cells expressed lower levels compared to Dazl$^{-/-}$ cells at E12.5 (Fig 4C'). Thus, UPR genes in Dazl$^{-/-}$ cells act similarly to pluripotency genes, and retain high expression levels later in cyst development than wild type.

The high expression of Xbp1 and many of its target genes in PGCs and early cyst cells argues that early germ cells are expanding ER and Golgi production, and increasing membrane protein quality. To validate that XBP1 is active in germ cells, we employed an IRE1-XBP1 ratiometric assay which utilizes a genetically encoded dual-fluorescent reporter system (see Methods, Figure S4I). First, we performed magnetic-activated cell sorting (MACS) to purify SSEA1 labelled germ cells from the E11.5 gonad (Figure S4G-H). We delivered the IRE1-XBP1 sensor to SSEA1+ve germ cells and SSEA1-ve control somatic cells by transfection. Both types of cells fluoresce in proportion to IRE1-splicing activity which measures Xbp1activation activity. The construct also constitutively expresses a fluorescent marker such that the color ratio will represent a quantitative measure of relative IRE1-XBP1 activity between cells and under different conditions. Our observations showed that IRE1-Xbp1 activity in E11.5 germ cells was significantly higher than in somatic cells (Figure 4D). Dazl mutant cells showed persistent higher levels of Xbp1 activity at E12.5 (Figure D', D").

The adaptive UPR pathway destroys misfolded proteins using proteasome activity, particularly the 20S proteasome (Vember and Brodsky, 2009; Figure 4H). We measured proteasome activity within E11.5 PGCs to investigate whether the UPR pathway was paired with increased proteasome activity indicating it served an adaptive cellular mechanism of cell rejuvenation in PGCs destined to produce oocytes. Using a

fluorogenic substrate to measure 20S Proteasome activity, we compared MACS sorted germ cells to somatic cells and found that germ cells have significantly higher proteasome activity than somatic cells at E11.5 (Fig 4E) and E12.5 (4E' Left). Proteosome activity was even higher in Dazl-/- germ cells (Figure 4E' Right, 4E").

We also studied the effects of Dazl mutation on the asymmetric enrichment of Visham in cells with multiple ring canals. Using both EMA and a Golgi marker, Gs28, we observed that germ cell Golgi begin to fragment in *Dazl*$^{-/-}$ mutant germ cells even by E12.5 (Fig 4F, dotted circles). High levels of Golgi stress documented by the increased IRE1-Xbp1 activity, and elevated proteolysis in these cells, may lead to such fragmentation. These problems were accompanied by a dramatic arrest in cyst polarity development. E13.5 in *Dazl*$^{-/-}$ germ cells showed virtually no differential expression of Pard3 or enrichment of Visham within particular cyst cells (Figure 4F'). Finally, in *Dazl*$^{-/-}$ P0 gonads, large cells destined by become oocytes do not appear (Figure S5E). The effects of Dazl-/- on germline cyst development and meiosis are summarized in Figure 4G. The observed loss of polarity caused by Dazl-/- mutation can explain the failure of oocyte production and sterility of Dazl homozygotes.

Visham participates in organelle rejuvenation during meiosis

In Drosophila, starting at pachytene the microtubule cytoskeleton changes its structure, and polarity (Cox and Sprading, 2006). The new polarity, with microtubules running along the fusome such that minus ends cluster in the oocyte, prepares selected nurse cell organelles for transport into the oocyte and the Bb of a new primordial follicle. Mouse nurse cell centrosomes, mitochondria, Golgi, ER and possibly other organelles also move from nurse cells to the oocyte between P0 and P4 in a microtubule-dependent process (Lei and Spradling, 2016; Niu and Spradling, 2022). Prior to or during these events, Drosophila mitochondria are selected and rejuvenated by programmed mitophagy to enhance functionality (Cox and Spradling, 2003; Lieber et al. 2019; Palozzi et al. 2022; Monteiro et al. 2023). It is not currently known if

selection and rejuvenation take place before movement to the oocyte, or if a significant amount also takes place en route.

Because the fusome is directly involved in organelle movement to the Drosophila Balbiani body we looked for changes in Visham and in gene expression that might relate to organelle rejuvenation and movement to the oocyte. Visham staining with WGA persists within meiotic germ cells at E17.5 (Figure 5A-A'; Figure S5A-C). Visham volume, remained in proportion with germ cell ring canals, as outer nurse cells (with one ring canal) transfer organelles and cytoplasm to cells with more ring canals located closer to oocytes (Figure 5A, graph). Pard3 content also showed such a proportion (Figure 5D). At E18.5, cyst development in the medulla toward wave 1 follicles, diverged from wave 2 follicles in the cortex that become quiescent primordial follicles (Figure 5B) (Yin and Spradling, 2025). Wave 1 oocytes grow substantially in volume, Visham content and Pard3 due to cytoplasmic transfer from surrounding nurse cells which decrease in size (Figure 5B-B', 5E-E'', Figure S5E-F, left). Oocyte enrichment of Visham and Pard3 was confirmed using lineage labelled cysts at P0 (Fig 5C and 5F). Such enrichment was significantly reduced in Dazl+/- heterozygotes (Figure 5G', Figure S5E-F, middle).

Wave 2 follicles which produce nearly all fertile oocytes, increase organelle content beginning at E18.5 and contain a finished Bb by P4 (Niu and Spradling, 2022). Initially, 4-5 transfered Golgi-centrosome pairs were observed at separate cytoplasmic locations in oocytes. These gradually come together and form an oval-shaped cytocentrum that nucleates a microtubule aster and organizes the Balbinai body. We carried out immunofluorescent staining at E18.5 and examined EMs that documented interactions between Golgi, ER and mitochondria. Figure 5H shows a wild type germ cell with a large crescent of perinuclear mitochondria (AT5a) adjacent to a large cluster of Visham (WGA). An EM provides a higher resolution view of a similar interaction (Fig 5H'). In contrast, relatively few mitochondria or Visham were stained in a Dazl+/- heterozygote (Figure 5H"). Mitochondria also interact extensively with ER at this time (Figure 5I;

Pepling et al. 2007). ER tracker and Mitotracker (Figure 5I') stained clusters throughout much of the germ cell cytoplasm.

DISCUSSION

Mouse germline cysts contain a fusome-like structure, Visham

Our results document a mammalian analog of the Drosophila fusome that displays conserved properties likely to underlie animal oogenesis. The fusome and Visham and both start as a small granular region within migrating PGCs before they arrive at the gonad. The granule transforms into an asymmetric structure during synchronous, incomplete divisions, involving stabilized spindle microtubules rich in AcTub and a program of arrested cytokinesis that produces distinctive ring canals. While stainable MTs persist for only part of the cell cycle, cyst and fusome polarity is maintained, as evidenced by preferential Visham accumulation in future oocytes, and rosette formation. Finally, the association of Visham with an apical Par complex located near the clustered ring canals, as originally described for the fusome in Drosophila cysts (Cox et al. 2001; Huynh et al. 2001), is particularly important. Apical-basal patterning of Drosophila oocytes differs from many epithelial cells (review Huynh and St Johnston, 2004), but Par genes are essentia for oocyte production and their action now appears to be conserved at least in part. Our results argue that apical-basal polarity formation within germline cysts utilizes a fusome to specify oocytes and to aid their patterning and rejuvenation.

Early germ cells in other female mammals likely also use closely related mechanisms, given the ancient origin of cyst formation in animal gametogenesis. Golgi/ER-associated membranes forming a spherical mass in germ cells were reported in classical studies of pig and mouse reproductive tissues (Anderson & Beams, 1960; Clark & Eddy, 1975; Ginsburg et al., 1990). Similar perinuclear structures associated with a Golgi complex in PGCs have been reported in other vertebrate species like chicken and quail (Fazel et al., 1987, 1990; Jung et al., 2005; Urven et al., 1988; Yoshinaga et al., 1991, 1992; Hen et al. 2014). The presence of a fusome in developing human germline cysts and its similarity to mouse Visham merits further investigation.

Mouse cysts develop multiple uniform oocytes using a novel specific-cleavage mechanism

Mouse PGCs usually generate 4-6 oocytes using a complicated mechanism involving synchronous incomplete cyst divisions, limited breakage into smaller cysts (<10% of cell-cell junctions) and continuous, slow nurse cell turnover (Lei and Spradling, 2013; Lei and Spradling, 2016; Niu and Spradling, 2022. Despite their complex pathway, mouse oocytes in newly formed primordial follicles appear quite uniform in size and are efficiently produced, suggesting that they develop from final cysts of similar size and nurse cell content. However, lineage analyses did not reveal a pathway that generated uniform cysts, but showed many cyst cells that had moved considerable distances apart at least transiently. Live imaging studies revealed active movement likely responsible for some cell displacement, but also cell detachment caused by random breakage (Levy et al. 2024).

Our studies of Visham and stable microtubules support the view that programmed cyst breakage during mitotic divisions contributes strongly to oocyte uniformity. AcTub arrays in cysts of 7-10 cells frequently contained a single large gap lacking MTs between a group of 6-cells, and the other residual cells. Such gaps in a major cytoskeletal element of germ cells subjected to significant tissue movements might represent cleavage sites (Figure 2E"). Previous studies also strongly support the idea that 6-cell cysts play a special role. Synchronous mitoses in forming cysts are distinctive and easier to map unambiguously than the connectivity of lineage-labeled cells. 6-cell synchronous divisions are by far the most commonly observed cyst size not a power of 2 throughout E11.5 to E14.5 (Pepling and Spradling, 1998). Additionally, quantitation of organelles in developing germ cells throughout oogenesis showed that P4 oocytes contain approximately 5 times as many centrosomes, Golgi, and mitochondria and four times as much cytoplasm as E14.5 germ cells (Lei and Spradling, 2016). It is difficult to explain these results if random breakage due to excess random cell movement is a major factor determining cyst size and connectivity (Levy et al. 2024), although it undoubtedly occurs.

What might cause the preferential breakage of growing cysts in a 6:2 manner? One possibility is the uneven distribution of Visham in four cell cysts (Figure 1B"). The cell with the lowest amount might fail to sustain a microtubule connection at the next division, leading to 6:2 breakage. Involvement of the microtubule cytoskeleton in asymmetric divisions has been observed in many other situations (Fichelson & Huynh, 2007; Kaltschmidt & Brand, 2002; Sunchu & Cabernard, 2020; Meiring et al., 2020; Planelles-Herrero et al., 2022; Watson et al., 2023). Even if programmed breakage into 6-cell cysts predominates, other sources of variation likely also occur. Some 8-cell cysts persist and divide again, rarely completing intact 32-cell cysts (Pepling and Spradling, 1998). It is not known if these cells are cleavable into smaller cysts or not, or whether random breakage to a smaller size would make them cleavable. Nonetheless, given oocyte uniformity, it seems plausible that most functional oocytes derive from cysts with about 5 nurse cells.

Germ cell rejuvenation is highly active during cyst formation

Germ cells have long been known to propagate species without know limits over evolutionary time by rejuvenating gametes each generation in association with meiosis (Weissmann, 1892; Kirkwood, 1987). Recently, the detailed cellular and molecular genetic mechanisms that make this possible both in single-celled eukaryotes and in animals have generated increasing interest (Cox and Spradling, 2003; Unal et al., 2011; Bohnert and Kenyon, 2017; Lieber et al., 2019; Chen et al. 2020; Palozzi et al., 2022; Spradling et al., 2022; Yamashita, 2023; Xiao and Unal, 2025). By studying Visham, which is already present in migrating PGCs and E9.5, and by sequencing mouse germ cells from the earliest stages of ovarian development beginning at E10.5, we gained new insights into the timing and processes of germ cell rejuvenation during mouse oogenesis.

These studies made it clear that multiple processes of rejuvenation are highly active from the very earliest times of germ cell development (Figure 3I). In animals the UPR pathway has been modified by the addition of several additional pathways besides the Ire1-Xba1 branch, all of which were expressed in early

germ cells. Creb3l2 controls a transcription factor that increases production COPII vesicles that carry out ER to Golgi transport and maintain a balance between secretory supply and demand. Insufficient matching can lead to Golgi stress (Machemer, 2015), which can be managed by activating the ATF4 pathway branch.

Expression of Xbp1mRNA or even Xbp1 protein using a general antibody is not sufficient to show Xbp1 activity, since this is controlled by Ire1-mediated splicing to produce the active Xbp1s isoform. We were able to purify enough E11.5 and E12.5 germ cells to carry out biochemical assays and show that mouse female germ cells have substantial levels of Xbp1s activity at both times, higher than in somatic cells. The high expression of many known Xbp1 targets also documents the high activity of this pathway (Figure 3I,I'). Early germ cells also express genes suggesting that they are expanding membranes via lipid biosynthesis and secretory pathway activity. Consistent with active adaptive-UPR rejuvenation of their proteomes we documented substantial levels of active proteolysis in early germ cells at both E11.5 and E12.5

The observations reported here suggest that Golgi- and endosome-associated degradation (EGAD)—an emerging proteostatic mechanism plays a previously unappreciated role. EGAD complements the well-established ER-associated degradation (ERAD) pathway by facilitating the clearance of misfolded proteins via Golgi-endosome trafficking and proteolysis, rather than removal from the ER and degradation in the proteosome as in ERAD. We observed substantial amounts of ERAD based on the expression of Xbp1 and its many targets, and its documented activity and its endpoint 20S proteosome activity. It is hard to quantitatively compare the amount of EGAD activity, but the prominent role Golgi throughout the stages of mouse oogenesis and its location at the core of the Balbiani body suggests that EGAD either fulfills a unique role in oocyte production. or else is uniquely capable of operating at the highest level of proteostatic activity demanded by the much greater challenge of germline rejuvenation compared to any other tissue without generating damaging and self-limiting side effects.

Rejuvenation genes were also highly expressed later in meiosis during zygotene and pachytene, in diplotene when mitochondria/ER contact is extremely high (Figure 5I), as well as in primordial follicles (Figure 3I,I' "Dc"). The early appearance of Visham and its association with Golgi and ER-related processes that are central to adaptive-UPR, suggests that in animals, rejuvenation begins earlier in the germ cell cycle than it does in single celled eukaryotes. Animal germ cells return to a pleuripotent state prior to undergoing cyst formation. Both these events precede meiosis and represent an addition to the rejuvenation process (Spradling, 2024). Our findings of early rejuvenation align with those of Palozzi et al. (2022), who showed that mitochondrial rejuvenation during oogenesis uses a special germline mitophagy and begins at the onset of meiosis. In yeast, mouse and C. elegans oocytes, damaged materials are destroyed by lysosome-like degradation in mature oocytes and at meiosis II (Bohnert and Kenyon, 2017; Zaffagnini et al. 2024; Xiao and Unal, 2025). Thus, animal oocytes may engage in rejuvenation activities from before meiosis begins until it is completed.

Dazl regulates cyst formation, fusome polarization, and oocyte specification

Female gamete development in many organism is extensively controlled at the post-trancriptional level by RNA binding proteins (Mercer et al 2021; Conti and Kunitomi, 2024). In vertebrates, the RNA-binding protein Dazl is widely conserved and controls multiple aspects of early germ cell development by regulating a large number of target transcripts (Zagore et al. 2018). In mice, Dazl modulates the ongoing reprogramming of new arrived gonadal PGCs to pleuripotency beginning at E11.5 when it turns on (Gill et al. 2011; Nichols et al. 2019). Normally, Dazl continues to function at later stages of oocyte development (Conti and Kunitomi, 2024), but Dazl mutants fail to enter or progress in meiosis. In zebrafish, Dazl is required to establish germline stem cells and to properly form cysts via incomplete cytokinesis leading to their degeneration (Bertho et al. 2021).

Our studies defined a series of important new functions for Dazl during cyst formation. We confirmed that pluripotency reactivation is slowed in Dazl mutants since DNA demethylation was delayed,

and pluripotency genes failed to start downregulating at E12.5. Dazl mutant homozygotes failed to normally regulate UPR genes, leaving their mRNA levels elevated. This likely resulted from a failure to downregulate their Xbp1 activator, since both Xbp1 activity and 20S proteosome activity was also elevated in Dazl-/- germ cells. This overactivation of the UPR pathway appeared to cause Golgi fragmentation and These effects resembled the general slowing of normal developmental progression, as with pleuripotency downregulation. We also observed abnormal cyst formation with some small and defective ring canals as in zebrafish Dazl mutants, but cysts remained intact and continued to develop. This allowed us to document that Dazl is needed for cysts and for the fusome to become polarized. In Dazl mutants, cysts did not undergo rosette formation or concentrate Visham or Pard3 in cells with multiple ring canals. Oocytes apparently could not be specified and did not form. These results show that Dazl acts as a major regulator that is essential for the most important aspects of cyst formation and oocyte production.

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SUPPLEMENTAL INFORMATION

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https://drive.google.com/drive/folders/1XuOoesa_WKMJUaVTpOKGbWWhn_fJc56M

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DECLARATION OF INTERESTS

We declare no competing financial interests. One of us (ACS) is an adjunct professor (without compensation) at the Johns Hopkins University Department of Biology and Johns Hopkins University School of Medicine.

FIGURE LEGENDS

Figure 1. Mouse pre-meiotic PGCs contain a fusome-like structure, "Visham."

A. E9.5-E12.5 ovaries: EMA, DAPI. Box: EMA granule (triangle) in germ cells. B. E11.5 ovary: lineage-labeled clones (YFP), DDX4. B′. 2-cell cyst. EMA granules (boxed). Graph: EMA volume asymmetry N=16. B″. 4-cell cyst: EMA granules (triangles). Graph: EMA volume asymmetry N=18; Student's t-test, ***p<0.001. C. E13.5 ovary and testis (C'): GCNA, EMA. Visham: (dotted line); Graph: % cysts with rosette Visham (N=26; ***p<0.001). D. E13.5 YFP-marked cyst: EMA, TEX14. D' (boxed region). Graph: Ring canal number vs. Visham enrichment (≥10 μm³). (N=54; ***p<0.001). E-E″. EM images of rosette Visham spanning intercellular bridges: E14.5 (E, E''); EMA granule at E11.5 (E'). F. E11.5 ovary: GM130, EMA. F'. Rab11a1, EMA. G. E11.5 ovary: WGA, EMA; G'. E13.5 ovary: GCNA, WGA labels rosette Visham. (H) Schematic: Visham transformation from granular to rosette structure. (I) % germ cells with EMA structure during E9.5-E14.5 (N=15 per stage; ***p<0.001). Scale bars: 5μm (A, F′), 10μm (B-B″, C-C′, D′, F, G-G′), 20μm (D), 2μm (E).

Figure 2. Stabilized spindle microtubules mediate Visham asymmetry and cyst breakage.

A. Visham interphase pericentric localization. E11.5 germ cells: EMA, Pericentrin. A′. Summary. B. Cell cycle behavior of EMA during initial 2-cell cyst formation. B'. Summary diagrams. C-D. Asymmetric Visham segregation (E10.5). C. Symmetric telophase separation. D. Asymmetry at arrested cytokinesis. E-E''. Persistent spindle remnants presage cyst breakage. Three lineage-marked (YFP) E12.5 8-cell cysts (E-

E’’) in early interphase stained to reveal Visham and AcTub. MT channel alone shown in middle column. Diagrams at right suggest sites of breakage (dashed line). The E’’ cyst has already broken into 2-cell and 6-cell cyst derivatives. F. Cell number distribution of cyst breakage products predicted from 15 lineage-labeled cysts analyzed as in E (7-cell: 3; 8-cell: 8; 9-cell:1; 10-cell:3). Binomial test: 6-cell dominance in 13/15 cysts (****$p<0.0001$). G. Model of PGC cyst production and breakage into four 6-cell cysts and 4 2-cell cysts. Scale bars: 5μm (B), 10μm (A, C-E).

Figure 3. Visham associates with Pard3 and apical polarity

A-B. Pard3 overlaps Visham at E11.5-E12.5 (A, A'). and after rosette formation at E13.5 (B, B'). C-C'. Ring canals (RACGAP) localize within the Pard3+ apical domain. D. A lineage-labeled E13.5 cyst (YFP); channels below show overlap of Pard3 and Visham. (N=13; ***$p<0.001$). E. Xbp1 enrichment in EMA granule of E11.5 PGC. F-H. scRNAseq of E10.5-P5 germ cells. UMAP (F), UMI (G) NC =nurse cells. Summary (H): pre-meiotic (Pre-M), leptotene (Lp), zygotene (Zy), pachytene (Pa), diplotene (Dp), dictyate (Dc). (I-I′) Bar plots: Xbp1and target expression plots. I.' Targets orthologous to fusome components. Scale bars: 10μm (A-C), 20μm (D).

Figure 4. UPR genes are active during cyst formation and controlled by Dazl

(A) Dnmt3a and EMA levels at E12.5 are reduced in WT compared to Dazl$^{-/-}$ germ cells. (B) Ring canals ar smaller and defective in E13.5 Dazl$^{-/-}$cysts compared to WT. (N=44; **$p<0.05$). (C) Diverging expression o WT and Dazl$^{-/-}$ germ cells in UMAP plots. (C’) Xbp1, Xbp1 targets, and fusome orthologs in WT vs Dazl$^{-/-}$ germ cells. (D-D″) IRE1-Xbp1 assay comparing SSEA1+ germ vs SSEA1− somatic cells at E11.5 and WT vs Dazl$^{-/-}$ germ cells at E12.5. (E-E″) Proteasome activity comparing SSEA1+ germ vs SSEA1− somatic cells at E11.5 and WT vs Dazl$^{-/-}$ germ cells at E12.5. (F) Golgi fragmentation in E12.5 Dazl$^{-/-}$ germ cells. (F’) Failure of E13.5 Dazl$^{-/-}$ germ cells to form EMA rosettes or enrich Pard3. G. Dazl$^{-/-}$ effects. H. ERAD-UPR proteostasis. Scale bar: 10μm (except zoomed in 2μm).

Figure 5. Visham and Pard3 Associate with ER and mitochondria prior to Balbiani body formation

A-A′. E17.5 ovary stained for WGA, GCNA, and TEX14 ; Graph: Visham volume versus ring canal numbe (N=65; ANOVA, *p<0.1, ***p<0.001). B-B′. E18.5 ovary shows Visham enrichment in large medullary oocytes vs smaller nurse cells; graph compares Visham volume versus Germ cell nucleus diameter (N=54; **p<0.01). line: medulla/cortex boundary; dotted circle: large medullary oocytes; white dotted area: small nurse cells. C. Single-cell lineage-labeled E18.5 ovary shows Visham volume difference according to germ cell nucleus size (N=10; ****p<0.0001). D. Pard3 enrichment along Visham proportional to number of ring canals. E-E’. Pard3 enrichment within newly arising medullary Oocytes (Big cells, GCNA) along with Visham (WGA). F. Lineage-labeled cells (YFP): Pard3 enrichment versus germ cell (GCNA) nucleus size. G-G′. Dazl+/- E18.5 ovary- Visham (WGA) and Pard3 enrichment failure in medullary Oocytes (GCNA). H-H″. Organelle enrichment analysis: E18.5 (WT, H and Dazl+/- ovary, H”) stained for WGA, mitochondrial marker ATP5a and GCNA. H’ -EM image of Golgi-rich Visham (arrow) surrounded by mitochondria. I-I′. ER-mitochondria association in E18.5 ovary: I-EM image of ER tubules (arrow) wrapping mitochondria and I’- GCNA, ER and Mitochondria tracker staining. Scale bars: 20μm (A-F, G-G′, H, I′), 5μm (B′, E′), 0.5μm (EM images H′, I).

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STAR* METHODS

Key Resource table

Reagent or Resource	Source	Identifier
Antibody/Lectin/Stains		
EMA	DSHB	EMA-1 AB_531885
GCNA	Abcam	ab82527
DDX4	Abcam/R&D systems	ab13840 & ab27591/ AF2030
SSEA1	DSHB	MC-480
WGA	Thermo Fisher	W7024
Gm-130	ThermoFisher/Novus Biologicals	610822/NBP2-53420
Rab9	Thermo Fisher	MA5-31997
Rab11a1	Thermo Fisher	700184
Tex14	Proteintech	18351-AP
GFP/YFP	Aves Labs	GFP-1020
Dazl	BioRad Labs/GeneTex	MCA2336/GTX89448
Lysotracker deep	Thermo Fisher	L7528
Pard3	Novus Biologicals	NBP1-88861
LAMP1	Cell Signaling Technology	9091T
Xbp1	Abcam	ab37152
Gs28	BD biosciences	61184
AAL	Vector Labs Inc	FL-1391-1
LCA	Vector Labs Inc	FL-1041-5
Sec63	Thermo Fisher	PA5-100180
Calnexin	Abcam	ab219644
Gs28	Proteintech	16106-1-AP
Acetyl-α-Tubulin (Lys40)	Cell Signaling. Tech	5335S
Alpha Tubulin (acetyl K40)	Abcam	ab289875
Pericentrin	Abcam	ab4448/ab28144
Anti-GCNA1 antibody	Abcam	ab82527
Rac GAP1 Antibody (A-6)	Santa Cruz Biotechnology, Inc	sc-271110
Dnmt3a	Cell Signaling. Tech	3598T
ATP5A	Abcam	ab14748
Mitotracker /Deep	Thermo Fischer	M7514/ M22426
ER-Tracker™ /	Thermo Fischer	E34251/ E34250
Quinacrine dihydrochloride	Sigma chemical	Q3251
Chemicals		

Paraformaldehyde	Electron Microscopy Sci.	15714
Tamoxifen	Sigma Chemical	T5648
Corn Oil	Sigma Chemical	C8267
Tween 20	Sigma Chemical	P1379
Triton X	Sigma Chemical	X100
Mounting media	Vector Labs Inc.	H-1000
Trypsin-EDTA (0.25%)	Fisher	25200056
Fetal Bovine serum	Sigma Chemical	F2442
Bovine serum albumin	Sigma Chemical	A4503
SSEA1 (CD15) microbeads	Miltenyi Biotech	130-094-530
DMEM/F-12	Fischer	11320-033
Celiobrevin D	Millipore	250401
Critical Commercial kits		
Kappa fast hot start ready-mix	KAPPA Biosystems	KK5608
KAPPA express extract	KAPPA Biosystems	KR0383-v4.16
10X Genomics kit		
IRE1-Xbp1 Assay	Montana Molecular	U0921G
Proteasome Activity	Amplite	13456
Superscript IV cells direct cDNA synthesis kit	Fischer	11750510
Experimental models		
C57BL/6J	Jackson Laboratory	000664
CAG-Cre-ER	Jackson Laboratory	004682
R26R-EYFP	Jackson Laboratory	006148
Dazl1L $^{-/+}$	Jackson Laboratory	035880
Others		
MACS MS separation columns	Miltenyi Biotech	130-042-201
Cell culture inserts	Millipore	PICM01250
Cell strainer	Sigma	CLS431752
Software and Algorithms		
FIJI	FIJI	Version: 2.16.0/1.54p
IMARIS	Oxford Instruments (formerly Bitplane)	Version: 10.2
SEURAT	Satija Lab	Version: 5.1.0
CELL RANGER PIPELINE	10X genomics	Version: 6.0.1/8.0.1
GRAPHPAD PRISM	GraphPad Software	Version: 10.5.0

PRIMER DETAILS

Primer Name	Sequence	Amplicon Size and Reference
Uba1_Forward	5□-TGGTCTGGACCCAAACGCTGTCCACA-3□	Male- 198 bp Female- 217 bp *McFarlane et al., 2013 (https://doi.org/10.1159/0003486*7
Uba1_Reverse	5□-GGCAGCAGCCATCACATAATCCAGATG-3□,	
Sly_Forward	5'-GATGATTTGAGTGGAAATGTGAGGTA-3'	Male- 280 bp Female- 685 and 48 bp *McFarlane et al., 2013 (https://doi.org/10.1159/0003486*7
Sly_Reverse	5'-CTTATGTTTATAGGCATGCACCATGTA-3'	
Zfy_Forward	5'-GACTAGACATGTCTTAACATCTGTCC-3'	Male- 180 bp *McFarlane et al., 2013 (https://doi.org/10.1159/0003486*7
Zfy_Reverse	5'-CCTATTGCATGGACTGCAGCTTATG-3'	
DDX4 Forward	5'-GAGATTGCCTTCAGTACCTATGTG-3'	105 bp *Gao et al., 2011 (https://doi.org/10.1095/biolrepro 10.084590)*
DDX4 Reverse	5'-GTGCTTGCCCTGGTAATTCT-3'	
GAPDH Forward	5'-GGTGAAGCAGGCATCTGAGGG-3'	246 bp *Chunmei et al., 2011 (https://doi.org/10.1093/glycob/cv 52)*
GAPDH Reverse	5'-GGTGGGTGGTCCAGGGTT-3'	
Dazl Common	5'-GAC ATT ACT AAG AAA ACA GCA GTG G-3'	Mutant=~300 bp, Heterozygote=~300 bp and 192 b Wild type=192 bp *JAX Protocol (Strain #035880, Primer# 56340, 56341 and 56342)*
Dazl WT reverse	5'-TTC TGC ACA TCC ACG TCA TT-3'	
Dazl Mut Reverse	5'-ATC CCT CCC TTT AGG GCT CA-3'	

RESOURCE AVAILABILITY

Lead contact Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Allan C. Spradling (spradling@carnegiescience.edu).

Materials availability

This study did not generate new unique reagents.

Data availability

Raw sequencing data has been deposited at Gene Expression Omnibus (GEO)

database (accession: GSE303512). Any additional information required to reanalyze

the data reported in this paper is available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse strains used-C57BL/6, CAG-cre/ERT2 mice, R26R-EYFP (as described in Lei and Spradling, 2016) and Dazl1L$^{+/-}$ mice (Strain #035880 from JACKSON laboratories). Genotyping was performed according to protocols provided by the JAX Genotyping was performed according to protocols from the JAX Mice database. Animals were provided with a proper light-dark cycle, temperature, humidity, food and water. Sexually mature females and males (6-8 weeks old) were kept for mating. Mating was confirmed through the observation of a vaginal plug. As per standard protocol, we designate the midday of the corresponding day as E0.5, which marks the beginning of embryonic development. Fetal gonads were dissected during E10.5-E18.5 as needed. The day pups are born is designated as P0 and were dissected for some experiments. The Institutional Animal care and use committee of Carnegie Institution of Washington approved procedures for animal handling and experimentation.

METHOD DETAILS

Single germ cell lineage labeling

The protocol used for single germ cell lineage labeling was performed as described previously (Lei and Spradling, 2016). Briefly, to obtain fetuses for lineage marking, adult female R26R-EYFP mice were mated with male CAG-cre/ERT2 mice. Tamoxifen was dissolved in corn oil (Sigma) and a single dose was injected intraperitoneally at 0.2 mg per 40 g body weight into pregnant female R26R-EYFP mice at E10.5. Fetal gonads/Pups were dissected as needed in between E11.5-E18.5/P0. To analyze the lineage-labeled germ cells, fixed and frozen whole gonads were subjected to whole mount staining for chicken-GFP, the number of lineage-labeled

germ cells of each ovary was determined by examining optically sectioned Z-stack images generated by confocal imaging of the entire ovary.

Immunostaining

Dissected fetal gonads/ovaries were washed in Phosphate buffer saline (PBS) and fixed immediately in cold 4% paraformaldehyde overnight at 4^0 C. Following the fixation, the gonads were washed three times with $PBST_2$, which is a mixture of phosphate-buffe saline (PBS) with 0.1 % Tween 20 (Sigma) and 0.5 % Triton X (Sigma). Each wash cycle lasted for 30 minutes. Next, gonads were Incubated with primary antibodies mixed with Blocking solution ($PBST_2$+ 10 % Normal donkey serum) overnight at room temperature. Following day, gonads were incubated with fluorescein-conjugated secondary antibodies (Donkey Anti-Rabbit/mouse/rat Alexa-fluor 488/568/647, Invitrogen) overnight at room temperature. Next day, gonads were washed with PBST and stained with DAPI (Sigma) to visualize nuclei. Gonads were mounted on slides with a mounting medium (Vector Labs) and analyzed using confocal microscopy (STELLARIS 8 DIVE Multiphoton Microscope, Leica). Germ cell numbers are quantified in each ovary by manually counting EMA/DDX4 stained germ cells in multiple 0.45 μm optical sections from different ovaries.

3D reconstruction

Three-dimensional model images and movies were generated by Imaris software (Bitplane).For z-stack images, different visualization options in the Surpass mode of Imaris helped to gain visualization control of the objects. To perform surface rendering and volume quantification using Imaris software, we imported your 3D image dataset for EMA/WGA staining. We started with a “Volume” rendering for channels corresponding to EMA/WGA staining and then we adjusted the contrast, brightness and transparency in the “Display

Adjustment” window. Next, we used the Surfaces module to create surface renderings by selecting the appropriate fluorescence channel, applied manual thresholding and adjusted smoothing parameters to define the structure accurately. Once the surface is rende, we navigated to the statistics tab to access volume measurements. Further visualization of the results was performed in 3D or orthogonal views to confirm accuracy, and adjusted rendering properties such as color or transparency as needed. To create spots for easy counting of labelled cells, we used the "Spots" icon-option in the "Creation" menu and started the spot creation wizard. We set the approximate spot size as per the requirement. Next, we selected the "Manual Creation" option to place spots individually. Each click placed a single spot at the corresponding position.

Sexing and Genotyping

Tail samples from mouse were collected and subjected to lysis and DNA extraction using Proteinase K based lysis buffer treatment at 55^0C overnight followed by 95^0C incubation for 10 minutes. The samples were then centrifuged at 14000 rpm for 5 min to pellet the debris. Extracted genomic DNA from supernatant was used directly and amplified with the Uba1, Sly and Zfy primer pairs for sex determination (McFarlane et al., 2013, Table S2). PCR reactions were performed using KAPA Fast Hotstart ReadyMix with dye (Kappa Biosystems, #KK5608) using the following PCR parameters: initial denaturation at 95 ° C for 5 min, 35 cycles with 94 °C for 30 s, 60° C and 72° C for 30 s, followed by final elongation at 72° C for 5 min. PCR products were analyzed with a DNA ladder (100 bp) on 2% agarose gels and visualized with ethidium bromide under UV-illumination. The male and female amplicon can be distinctly visualized due to different amplicon sizes of PCR products (See Primer details).

Electron microscopy

Whole ovaries were dissected in PBS and fixed in 4% Paraformaldehyde overnight. After three 3-min washes in cacodylate buffer, ovaries were postfixed in 1% OsO4, 0.5% K3Fe {CN)6, in cacodylate buffer for 1 hour and were rinsed twice for 5 min in cacodylate buffer and once for 5 min with 0.05 M maleate (pH 6.0). The ovaries were stained in 0.5% uranyl acetate overnight at 4 degrees rinsed in water and dehydrated through an ethanol series. Following two 10-rain washes with propylene oxide, the ovaries were infiltrated with resin. The resin-embedded specimen was polymerized by incubation at 45^0 C and 70^0 C for 12 hours each. Silver-gold sections were cut, stained with lead citrate, and observed in the electron microscope.

Single cell RNA sequencing of Wildtype E10.5, E11.5 and E15.5 gonad

Embryonic ovaries were dissected such that individual fetal gonad at E10.5 (18 gonads from 3 females), E11.5 (12 female gonad from 3 females) and E15.5 (10 gonad from 2 females) were collected. For E10.5 and E11.5: corresponding tail were labelled for identification and placed in 1× PBS on ice. We performed quick Quinacrine and DAPI staining (~15 min) of corresponding E10.5 and E11.5 fetal tail samples to eliminate the male gonad samples -with positive Quinacrine staining of tail samples. We then pooled the dissected female gonad sample and dissociated it into single cells using 0.25% Trypsin at 37^0 C for 5-7 min with two pipet trituration in between. Fetal bovine serum (10 %) was then used to neutralize trypsin. Dissociated cells were passed through 100-µm strainer. The cell suspension was centrifuged at 300 g for 5 minutes and cell pellet was resuspended in freshly prepared and filter-sterilized 0.04% BSA. Viability of cells was assessed via trypan staining and >90 percent viable samples were selected and loaded (~10000 live cells for E11.5 and E15.5, ~20000 cells for E10.5) onto the 10X Genomics Chromium Single Cell system using the for E11.5 and E15.5-v3 and for E10.5 v4 chemistry as per the manufacturer's instruction. Single-cell RNA capture and library

preparations were performed, and standard data was data processing was performed using Cell ranger pipeline (6.0.1 - for E11.5 and E15.5 and 8.0.1-for E10.5) and later data was visualized and analyzed by Seurat v5.1.0.

Single cell RNA sequencing of Dazl1L mutant gonad

Dazl+/- female and males were kept for mating and plugged females were marked as E0.5. The gonad from E11.5, E12.5 fetuses were dissected, fetuses were collected in and kept in cold PBS in 12-well plate such that each well with individual fetus is marked for identification. Fetal tails were collected, labelled and subjected to kapa express extract kit (Catalog #KR0383-v4.16) mediated fast DNA extraction according to manufacturer's protocol. Briefly, one step lysis and DNA extraction system was set up in 100 µl volume by adding 10 µl express extract buffer, 2 µl of express extract enzyme and 88 µl of PCR grade water in each tail sample followed by 75^0 C incubation in heating block for 15 minutes for lysis. The samples were then incubated at 95^0C in heating block for 5 minutes for DNA extraction. The sample was then centrifuged briefly to pellet the debris, and supernatant was used directly for PCR as mentioned in methods section of RNA isolation, cDNA synthesis and PCR. The standard genotyping JAX protocol for strain 035880 is refer to identify homozygous / heterozygous and wild type fetuses. Simultanoeusly male/female sexing was also performed (primer details in Table S2). After correct identification, homozygous fetuses-from E11.5 (6 gonad from 3 females) and E12.5 (6 gonad from two females)- were trypsinized to prepare live single cells which were then subjected to 10X Genomics Chromium (v3 chemistry) and data was processed using cell ranger pipeline 6.0.1 and analyzed using seurat v5.1.0 in same manner as mentioned previously for E11.5 gonad.

Cell Identification and Clustering Analysis

Single-cell RNA sequencing (scRNA-seq) data were analyzed using the Seurat package (v5.1.0, https://satijalab.org). Count data generated by the Cell Ranger pipeline (6.0.1/8.0.1) were imported into R using the Read10X function and converted into a Seurat object with the CreateSeuratObject function. R packages were used to filter out the low-quality cells, and the following criteria were used to filter cells:

a) **for E10.5 WT gonad**: nFeature_RNA > 500 & nFeature_RNA<8500 & nCount_RNA > 500 & percent.mt<5

b) **for E11.5 WT gonad**: nFeature_RNA > 100 & nFeature_RNA<11000 & nCount_RNA > 300 & percent.mt<10

c) for E15.5 WT ovary: nFeature_RNA > 500 & nFeature_RNA<9000 & nCount_RNA > 500 & percent.mt<10.

The filte count matrices were Log-normalized using the "NormalizeData" function and scaled with the "ScaleData" function to prepare for dimensionality uction. "Principal Component Analysis (PCA)" was then applied to uce dimensionality, followed by using top 15 dimensions and default resolution to cluster cells based on gene expression profiles using the "FindNeighbors" and "FindClusters" functions. Cell populations were visualized using the umap method, facilitating the identification of distinct cell types.

Bioinformatic segregation of female germ cells

To segregate E10.5 and E11.5 XX-specific germ cells with complete surety we performed bioinformatic segregation of female germ cells to address the limitations of primitive Quinacrine staining. The expression of the X-linked gene Xist, Ddx3x, Utx etc., were used as a marker. Cells with significant Xist or other X-linked gene expression expression were labeled as "XXonly" using the "Idents" and "WhichCells" R functions. A new Seurat object containing only XX-specific cells was created using the subset function. Germ cells within this subset were identified by examining the expression of germ cell-specific marker genes, and a further subset of E10.5

and E11.5 female germ cells was created using the subset function to focus on germ cells specifically.

Wildtype ger cells-merged dataset creation and Validation

ScRNA-seq datasets from E11.5 to P5 (retrieved from the GEO database, GSE136441 and merged object submiited to Github) were visualized using Seurat. E11.5 cells were discarded and new merged dataset from E10.5-P5 was created, subsets of germ cell clusters from E10.5, E11.5, and E15.5 were integrated using Seurat's “Merge” function. The resulting merged dataset was then processed by normalizing and scaling the data, followed by the identification of variable genes. Th ebatch correction was performed using and pre-harmony and post-harmony data was assessed by visualizing LISI (Local Inverse Simpson’s Inde) score confirming successful batch mixing. Cell clusters were determined using Seurat's sha nearest neighbor (SNN) algorithm with PCA uction using top 15 dimesnions and 2.8 resolution. To visualize these clusters, dimensionality uction was applied (umap) with following criteria: RunUMAP(new_merge, dims=1:15, n.neighbors=40, min.dist=0.6, spread=1) enabling the identification of distinct cell populations.

Cell cycle stages of germ cells across the different developmental stages (E10.5-P5) were validated by visualizing meiosis stage specific expression pattern via feature plot. This visualization technique allows for the capture of subtle trends and helps in comparing expression peaks, providing insights into the transition from mitosis to meiosis across the different developmental stages. Additionally, a violin plot was employed to visualize the variability in average raw UMI levels across germ cells. This approach allowed for an effective comparison of gene expression profiles, highlighting the differences in gene expression patterns between different stages.

Dazl mutant merged dataset

For E11.5 and E12.5 Dazl$^{-/-}$ dataset, cells were filte based on following criteria:

a) **for E11.5 Dazl -/- ovary** : nFeature_RNA > 500 & nFeature_RNA<9000 & nCount_RNA > 500 & percent.mt<5

b) **for E12.5 Dazl-/- ovary** nFeature_RNA > 500 & nFeature_RNA<10000 & nCount_RNA > 500 & percent.mt<5

Top 15 dimensions were used at default resolution to cluster the cells which were then visualized by umap. To create merged dataset from subset containing only germ cells were created and merged using "merge" function as described previously. Three dataset were created -for E11.5 wildtype and Dazl$^{-/-}$ dataset, for E12.5 and Dazl-/- dataset and one dataset was created where both E11.5 and 12.5 WT and Dazl-/- were merged. Batch correction was applied using harmony. Merged dataset was normalized, scaled and top 15 dimensions at resolution of 0.4 was used for visualization using umap.

In vitro gonad culture: microtubule inhibitor treatment and organelle tracker assay

To perform in vitro culture of mouse fetal ovaries on membrane inserts with or without inhibitors, we prepared sterile culture medium, constituting DMEM/F-12 supplemented with 10% fetal bovine serum and penicillin-streptomycin. Inhibitor stock solutions were prepared in DMSO and diluted at desi concentration in culture media (Ciliobrevin D from Millipore, 25 μM for 6 hours). Control medium containing the vehicle DMSO was prepared simultaneously. Fetal ovaries were dissected from E11.5 embryos under a stereomicroscope in sterile PBS, we have kept the surrounding mesonephric tissue intact and attached to the gonad to ensure proper development. We have also collected corresponding fetus tail of each fetal gonad to perform genetic sex determination. We have placed membrane inserts into a 24-well plate and added

~500 µL of culture medium (with or without inhibitors) to the lower chamber and ensu no contact between medium and the insert surface. Next, we gently placed isolated ovaries onto the membrane surface using sterile forceps. The plate is then incubated in a CO□ incubator at 37°C for ~6 hours. After the designated culture period, confirmed ovaries (post genetic-sex analysis), were carefully removed from the membrane inserts and washed three times with basal media to remove any residual inhibitors. Next, we collected ovaries and fixed in 4% PFA for downstream immunolocalization studies. To inhibit the microtubule growth using cold treatment, we transfer dissected gonad to a chilled culture medium, maintained at 0-4°C. The tissue is fully immersed in the pre-cooled medium and incubated on ice for ~60 minutes. This low-temperature exposure destabilizes microtubules by disrupting tubulin dynamics, effectively depolymerizing existing microtubules and inhibiting further polymerization. After the incubation, the tissue is washed with fresh ice-cold medium and fixed in 4% PFA. To stain specifically for organelles: Fetal ovaries were dissected and cultu in vitro in DMEM/F12 medium supplemented with organelle-specific fluorescent dyes. LysoTracker™ Deep (Thermo Fisher, L7528, 1 mM stock) or MitoTracker™ (Thermo Fisher, M7514) or MitoTracker™ Deep (Thermo Fisher, M22426), and ER-Tracker™ (Thermo Fisher, E34251) or ER-Tracker™ (Thermo Fisher, E34250) were each added at a final concentration according to the manufacturer's recommendations. The ovaries were incubated in this dye-containing medium at 37□°C with 5% CO□ for 12 hours. Following incubation, tissues were collected and fixed in 4% paraformaldehyde (PFA) for subsequent immunostaining.

Magnetic activated cell sorting (MACS)

Embryonic ovaries were pooled and treated with Trypsin then were neutralized followed by centrifugation as mentioned before to in ScRNA Sequencing method section to create

dissociated single cells. The resulting cell pellet was re-suspended in ~80 microliters MACS buffer i.e. PBS with 0.5% BSA and 2 mM ethylene diaminetetraacetic acid (EDTA). ~20 microliters Anti-SSEA1 (CD15) microbeads (MiltenyiBiotec Inc., #130-094-530) were added to the cell suspension followed by incubated for 20 min on ice. After adding 1 ml MACS Buffer to the suspension, the SSEA1 bound cells were pelleted by centrifugation at 300 g for 10 min at 4°C. SSEA1+ve and SSEA1-ve cells were then separated by applying cell suspension to MS columns according to the manufacturer's instruction (MiltenyiBiotec Inc.). The SSEA1+ve cells were retained by the column and were filte thrice to obtain pure germ cell population. The cells were counted, and viability was assessed via trypan staining and using automated cell counter (Fischer Scientific). For validation of MACS protocol the cells were fixed in 4 % PFA to perform Immunostaining of DDX4 and were also sto in trizol to perform RNA synthesis and PCR for germ cell specific gene expression.

IRE1-Xbp1 Assay

The IRE1-XBP1 ratiometric assay (from Montana Molecular #U0921G) is a genetically encoded biosensor system utilizes a BacMam Vector carrying dual-fluorescence biosensor. For each assay reaction 50 µl transduction mix consisting of XBP1-IRE1 sensor (15 µl) plus sodium butyrate (0.6 µl per well) was prepared in basal media. To standardize the working conditions, manufacturer provided thapsigargin (1 µm per well) was used as positive control and untransduced cells were used as negative control. For each experiment equal amounts of SSEA1-ve and SSEA1 +ve cells were added to a 96-well plate in duplicates. ~50 microliters of transduction mix was added to each reaction well. The plate was incubated for 45 minutes at room temperature and then transfer to 5% CO2 and 37°C for 24 hrs. The enzymatic activity was then measured at 37°C by monitoring XBP1-IRE-1 sensor's fluorescent intensity (top read) at

Ex/Em=488/525 nm and basal constitutive fluorescent intensity at 565/620 using microplate reader (BioTek Synergy H1). IRE1-Xbp1 assay comparing MACS sorted SSEA1□ germ vs SSEA1□ somatic cells at E11.5 was performed across 6 experiments (~32 mice, ≥5 mice and >=20 ovaries per experiment). IRE1-Xbp1 assay comparing SSEA1□ vs SSEA1□ E12.5 cells in WT or Dazl mutant mouse across 3 experiments (~40 mice, ≥5 mice and >=25 ovaries per experiments)

20S Proteasome activity assay

To perform proteasome activity assay (Amplite #13456) we followed manufacturer's instructions. We first standardized the working conditions using trypsin enzyme as technical positive control. Trypsin is loaded on different well in different concentrations in duplicates. Blank medium along with assay buffer were added in separate wells to act as Negative control. For each experiment equal amounts of SSEA1-ve and SSEA1 +ve cells were added to a 96-well plate in duplicates. ~50 microliters of working solution consisting of fluorogenic substrate in assay buffer (prepared according to manufacturer's instructions) were added and plate was incubated for 2 hours at 37°C. The enzymatic activity was then measured at 37°C by monitoring fluorescent intensity (top read) at Ex/Em=490/525 nm (Cut off =515 nm) using microplate reader (BioTek Synergy H1). Proteasome activity in MACS-sorted SSEA1□ vs SSEA1□ cells were assessed using 3 biological assays with ~35–60 E11.5 ovary per assay. Proteasome activity ratio of MACS-sorted SSEA1□ and SSEA1□ cells compared at E12.5 with ~25–28 E12.5 ovaries were used per assay.

RNA isolation and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Briefly, tissue samples were homogenized in TRIzol, or cells were incubated in TRIzol for 5 min at RT, followed by phase separation with chloroform (200 µl/ml TRIzol, shake for 10-15 seconds and keep at RT for 5 min) and centrifugation at 12,000 × g for 15 minutes at 4°C. The aqueous phase was collected, and RNA was precipitated with equal amount of isopropanol (10 min RT then same centrifugation as last step), washed with 75% ethanol, centrifuged, removed the supernatant ethanol and air-dried. Resuspended pellet in RNase-free water. RNA quality and concentration were determined spectrophotometrically. For cDNA synthesis we used Superscript IV cells Direct cDNA synthesis kit (Invitrogen, #11750150) with and modified manufacturer's protocol. We skipped the lysis step and directly added DNASe I to our sample (0.5 µl each) followed by 10 minutes incubation on ice. Then we added Stop solution (3 µl each) and kept the mixture at RT for 2 minutes. Then we set up RT reaction as per manufacturer's instruction by adding 8 µl of RT mix to each sample and setting up the following reaction in PCR thermocycler: 25^0 C for 10 min, 55^0 C for 10 min, 85 ^{0} C for 5 min and hold at 4 ^{0} C. cDNA was used directly for PCR as mentioned in Sexing and genotyping using DDX4 and GAPDH Primers (See Primer details)**.**

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of germ cell numbers

PFA fixed ovaries were subjected to whole mount immunostaining as described previously. Z-stack images for whole ovaries were analyzed using FIJI. Germ cell numbers are quantified in each ovary by manually counting EMA/DDX4 stained germ cells in multiple 0.45 µm optical sections from different ovaries using FIJI.

3D volume Quantification

Whole mount immunostaining was performed and the z-stack images were converted to 3D image to analyze using Imaris as described previosuly. After using the Surfaces module to create surface rendering and applying manual thresholding and smoothing to define the structure accurately, we navigated to the statistics tab to access volume measurements. Further visualization of the results was performed in 3D or orthogonal views to confirm accuracy, and adjusted rendering properties such as color or transparency as needed. On an average the average values from three measurements performed were conside and plotted as graph using Graphpad prism.

Area of staining quantified using FIJI

Images were analyzed using FIJI to calculate area of staining by selecting region of interest and using measure command from Analyze tab. The results were plotted as number of pixels to depict the area corresponding to stained regions.

Statistics

Data are presented as mean±SEM (Standard error of mean). Data were analyzed by t-test/one-way Anova. P-value less than 0.05 is denoted by one star. P-value less than 0.01 is denoted by two stars. P-value less than 0.001 is denoted with three stars.

SUPPLEMENTARY REFERENCES

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Gao, L., Kim, Y., Kim, B., Lofgren, S. M., Schultz-Norton, J. R., Nardulli, A. M., Heckert, L. L., & Jorgensen, J. S. (2011). Two Regions Within the Proximal Steroidogenic Factor 1 Promoter Drive Somatic Cell-Specific Activity in Developing Gonads of the Female Mouse. *Biology of Reproduction*, *84*(3), 422-434. https://doi.org/10.1095/BIOLREPROD.110.084590

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Supplementary Video Link and legends:
Here is the link to Videos use in supplementary figures:
https://drive.google.com/drive/folders/1XuOoesa_WKMJUaVTpOKGbWWhn_fJc56M

Supplementary Video legends

Video S1A: E13.5 ovary stained GCNA in Green and EMA in Red

Video S1B: E12.5 ovary stained with YFP in Green, EMA in Red, DAPI in Blue

Video S1D1: E13.5 ovary stained for GCNA in Green, DAPI in Blue and EMA in Red

Video S1D2: E13.5 ovary, same region as S3D1 stained for GCNA in Green, and EMA in Red

Video S2A1-S2A5: E11.5 gonadal tissue stained for EMA in Red, Ac. Tub. In Green and DAPI in Blue

Video S2F: 3D modelling of Visham in E12.5 lineage labelled 8-cell cyst: YFP in Green, EMA in White and Ac. Tub in Red. Green spheres are spots generated using Imaris software to count lineage labelled cells and Grey surface rendering was performed to visualize the EMA volume.

Video S3A: E13.5 Ovary stained for PARD3 in Red, EMA in Green and DAPI in Blue.

Video S3B1: E13.5 Ovary stained for GCNA in Blue, PARD3 in Red and EMA in Green

Video S3B1: E13.5 Testis stained for GCNA in Blue, PARD3 in Red and EMA in Green

Video S5C: 3D surface rendering of E17.5 ovary: WGA in Red enriched within region spanning ring canal stained by Tex14 in Yellow and GCNA stained germ cells are shown in Green.

Video S5D: E18.5 Ovary stained of WGA in Red, GCNA in Green and DAPI in Blue

Video S5 E1-E3: E18.5 Wildtype (E1), E18.5 Dazl +/- (E2) and E18.5 Dazl -/- (E3) stained for GCNA in Green and WGA in Red

Video S5 F1-F3: P0 Wildtype (F1), Dazl +/- (F2) and Dazl -/- (F3) stained for GCNA in Green and PARD3 in Red

SUPPLEMENTARY FIGURE LEGENDS

Fig. S1: **EMA/Lectin-stained aggregate (Visham) distribution in pre-meiotic PGCs** (A) Immunostaining of E9.5-E12.5 ovary for germ cell specific marker EMA (Red) and DAPI (Blue) and for E13.5 ovary (EMA in Red, GCNA in green, Z-stack video see Video S1A). (B) Z-stack video of E12.5 ovary stained for EMA (Red), DAPI (Blue) and lineage labelling shown via YFP (Green) (See Video S1B) (C) Volume rendering of EMA aggregate (White arrows) within lineage labelled cyst (Green, YFP) using Imaris software. (D-D') Videos of at E13.5 ovary stained for EMA (Red), GCNA (Green) and DAPI (Blue) (see Video S1D1 and S1D2) (E) Random 3-D sampling using Imaris for E13.5 ovary showing branched Visham structure within GCNA labelled female germ cells. (F) Images of different E13.5 ovary stained for EMA (Red), GCNA (Green), Tex14 (Yellow) and DAPI (Blue). Central region with enriched Visham is often associated with higher number of ring canals. (G) E11.5 ovary stained for WGA (Red) and YFP (Green) as lineage labelling marker. Dotted line marks the WGA aggregate within germ cells. (G') E13.5 ovary stained for WGA (Red), GCNA (Green) and Tex14 (yellow) forming branched WGA-stained Visham structure (dotted lines). **(**H and H') E11.5 gonad stained for general fucosylation specific lectins, AAL/LCA (Green), DAPI (Blue) and Visham marker EMA (Red). (I and I') Localization of ER markers in vicinity to Visham is validated by staining ER-specific Sec63 or Calnexin (Green), DAPI (Blue) and EMA (Red). (K) Electron microscopy of E11.5 gonad depicting Golgi clusters (Dotted red lines, labelled as Visham) near intercellular bridges (IB marked by red solid arrows). Scale bar. 100 μm (A), 20 μm (B), 10 μm (in D-H).

Fig. S2. **Validating microtubule dependent Visham formation and its distribution during cyst fragmentation.** (A) Z-stack videos for images used in figure 3A (See Videos from S2A1 to S2A5). (B) E11.5-E12.5 ovary stained for EMA (Red), Pericentrin (PCNT, Green), Ac. Tub (Grey) and DAPI (Blue). Arrows mark duplicated centrosome during Interphase. Dotted circles mark centrosomes. Smooth line marks the DAPI stained region during Anaphase depicting clear separation of Nuclei. (C) E10.5 gonad stained for EMA (Red) and Ac. Tub. (Green) Dotted line marks the newly formed fusome as spindle remnant. (D) Visham asymmetry arises during cytokinesis depicted in E11.5 ovary stained for EMA (Red) (E) In-vitro cultured untreated gonad and treated gonad (microtubule inhibitor Ciliobrevin D and cold treatment) stained for Ac. Tub

(Green), EMA (Red) and DAPI (Blue). Quantification showing % germ cells with Visham in Untreated versus Celioberivin D/Cold treated E11.5 gonad. (F) Lineage labelled 4-cell cyst stained at E12.5 for YFP (Green) , EMA (Red) and Ac. Tub (Grey). (F') 3D modelling of lineage labelled cyst at E12.5. Video depicts animation video generated using Imaris software to create uniform spheres in green to position the labelled 8-cells within cyst and surface rendering of Visham in White within lineage labelled germ cells (See Video S2F). Scale bar: 5 μm (A-B), 10 μm (C-F).

Fig. S3: Pard3 gene expression in E12.5-E13.5 gonad (A) Z-stack video of E12.5 gonad stained for EMA (Green), PARD3 (Red) and DAPI (Blue) (See Video S3A). (B) Z-stack video of E13.5 ovary **(**♀**,** See Video S3B1**)** and E13.5 testis **(**♂**,** See Video S3B2**)** stained for EMA (Green), PARD3 (Red) and GCNA (Blue). (C) E13.5 ovaries stained for GCNA (Blue), EMA (Green) and PARD3 (Red). (D) Lineage labelled E13.5 ovary stained for YFP (Green), GCNA (Blue), PARD3 (Red) and EMA (Grey). (E) Quinacrine (Male chromosome specific stain, Red) and DAPI (Green) staining of male vs female fetal tail samples (F) Feature plot and UMAP plot depicting Xist expression within E10.5 and E11.5 gonad followed by bioinformatic segregation of female gonadal germ cells (XX only) to avoid Quinacrine false-negative by segregating the cells expressing significant amount of XX specific genes (eg: Xist, Ddx3x, Utx) depicted in UMAP plot as XX only cells. (G) Cluster of germ cells from E12, E14, E16, E18, P1 and P5 of previously published data (NCBI: Niu and Spradling, 2020) were used. E10, E11 and E15 (10X genomics) ScRNA seq was performed and germ cells isolated bioinformatically to create E10.5-P5 merged germ cell data to comprehensively look at gene expression across developmental stages. The number of cells used to create merged dataset is shown. (H) Feature plot validating all the cells express germ cell specific markers (Dppa3/DDx4) (I) Pre-meiotic/Meiotic gene expression analysis depicted by feature plot for various developmental stages within merged dataset. (J) Stack violin plot depicting Golgi-UPR pathway associated gene expression pattern across E10.5 to P5 meiotic germ cell and nurse cell developmental stages. Scale bar= 20 μm (A,B-B, D) and 10 μm (C), 100 μm (E).

Fig. S4: Validation of female Dazl mutant phenotype, MACS and Activity assays. (A) Expected absence of Dazl protein in Dazl mutant gonad is shown by staining of E18.5 WT and Dazl-/- gonad for Dazl (Red), GCNA (Green) and DAPI (Grey). (B) E12.5 WT and Dazl$^{-/-}$ gonad

stained for DNMT3a (Red), EMA (Green) and DAPI (Grey) (C) Fetal tail genotyping for ScRNA sequencing: Gel electrophoresis showing the standard *Dazl* genotyping PCR assay by Jackson (Stock No: 035880 Protocol 40585) with expected results: Wild type (192 bp), Heterozygous mutant (300 and 192 bp) and Homozygous mutant (300 bp). (D) Feature plot depicting positive Xist Expression in ScRNA seq data of E11.5 and E12.5 Dazl mutant fetal gonad, thus concluding them as female samples. (E) Stackviolin Plot depicting pluripotency gene expression pattern in E11.5-E12.5 WT vs Dazl-/- gonad. (F) Stackviolin Plot depicting Xbp1 targets gene expression pattern in E1.5 WT vs Dazl-/- gonad (G) Staining of both unbound SSEA1(-ve) and the bound fraction consisting of SSEA1(+ve) cells for DDX4 (Magenta) and DAPI (Grey). (H) Gel electrophoresis showing the PCR amplification of housekeeping gene GAPDH and Germ cell specific marker DDX4 in SSEA1(-ve) and SSEA1 (+ve) germ cells from E11.5 and E12.5 ovary. The amplicon size is indicated on left. (I) Proof of functioning of Xbp1 assay is shown by staining of both mixture of SSEA1(-ve) and SSEA1(+ve) cells for GCNA (Green), DAPI (Grey) and Xbp1(Red) (J) Proof of function of proteasome activity assay using Trypsin as technical positive control. The proteasome activity assay was shown for two different assays (I and II) with technical duplicates at three different Trypsin concentrations. Mean fluorescent intensity in arbitrary units is shown obtained using plate reader. Scale bar: 20 μm (A), 10 μm (B), 100 μm (G and I).

Fig. S5: Visham enrichment within destined medullary Oocytes in WT vs Dazl mutant. (A-B) Zoomed out E17.5 ovary covering large span of tissue stained with DAPI (Blue), WGA (Red), GCNA (Green) and Tex14 (Yellow). White dotted line is zoomed in and shown separately in main figure (Refer to main Fig 5A and A'). (C) Video created using Imari software showing E17.5 ovary stained for GCNA (Green), Tex14 (Yellow) and WGA (Red) followed by surface rendering of WGA aggregate (continuous one) associated with ring canals. (See Video S5C) (D) Video of E18.5 female gonad stained with DAPI (Blue), GCNA (Green) and WGA (Red) depicts the cortex and medullary region where medullary region showing distinct enriched WGA aggregate compared to surrounding small nurse cells. (See Video S5D) (D') One of the z-stack form E18.5 ovary Video in E stained with DAPI, GCNA and WGA depicts the medullary big Oocyte-like cells showing distinct enriched WGA aggregate compared to surrounding small nurse cells. E) Z-stack video of E18.5 WT, Dazl +/- and Dazl-/- gonad stained for GCNA

(Green) and WGA (Red). (See Videos S5E1 to S5E3). (F) Z-stack video of P0 WT, Dazl +/- and Dazl-/- gonad stained for GCNA(Green) and PARD3 (Red). (See S5 F1 to F3) (G) E18.5 WT and Dazl +/- gonad stained for Mitotracker (Red) and GCNA (Green). Scale bar: 20 μm (A-B and G), 50 μm (D-E), 100 μm (F).

Fig 1

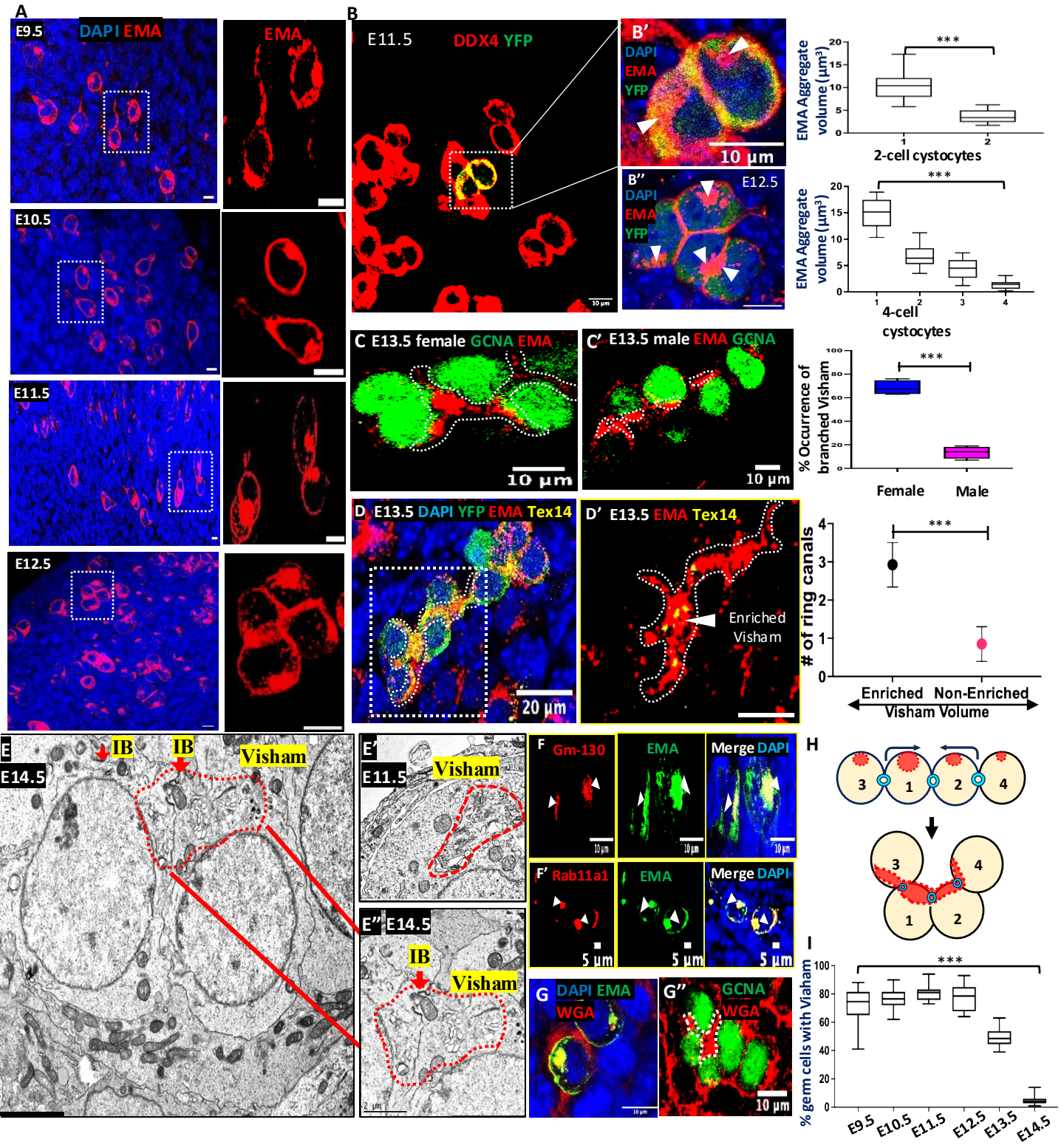
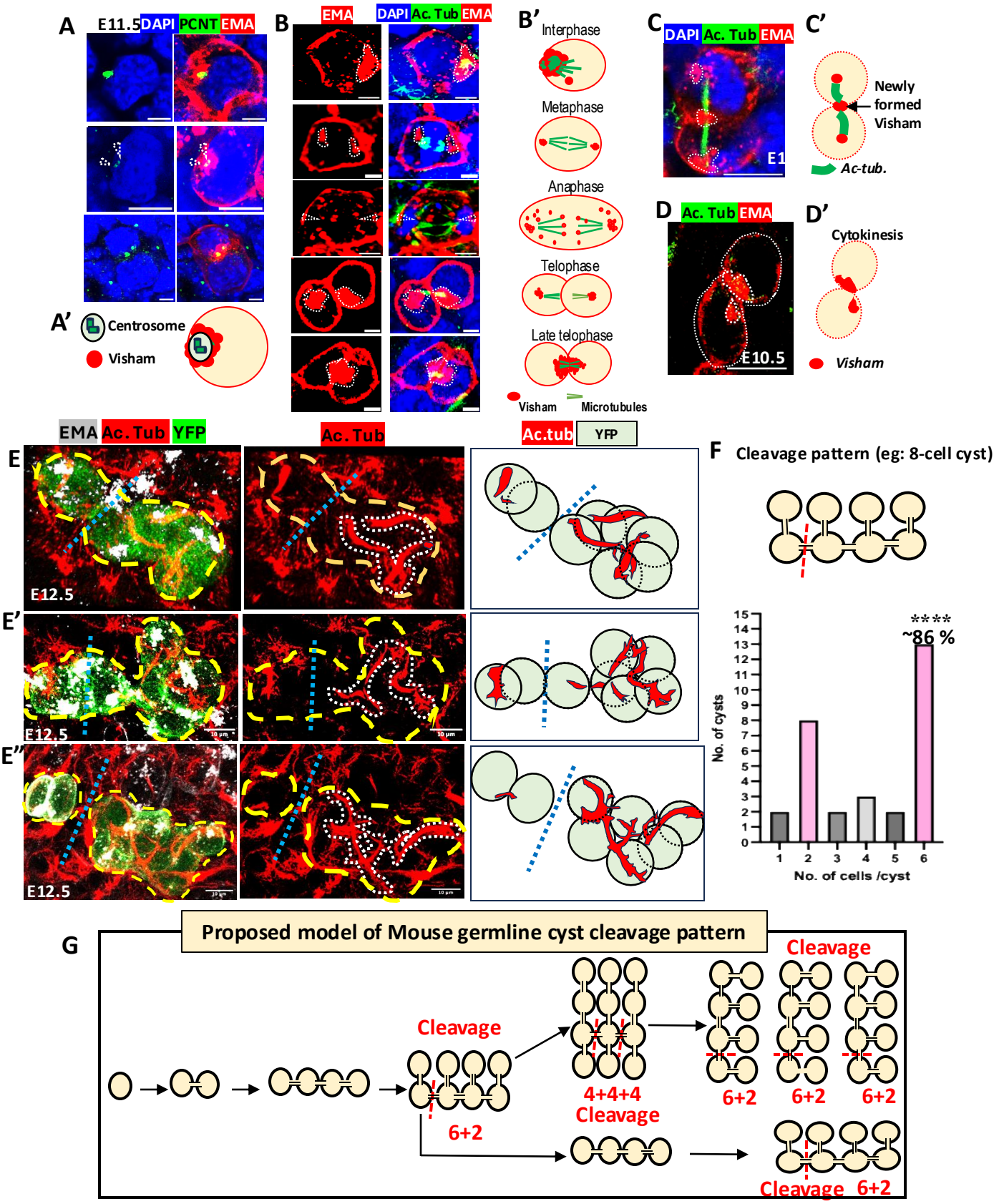

A
E9.5
DAPI EMA
EMA
E10.5
E11.5
E12.5
B
E11.5
DDX4 YFP
B'
DAPI
EMA
YFP
10 µm
B''
E12.5
C E13.5 female GCNA EMA
C' E13.5 male EMA GCNA
10 µm
D E13.5 DAPI YFP EMA Tex14
20 µm
D' E13.5 EMA Tex14
Enriched Visham
E
E14.5
IB
Visham
E'
E11.5
E''
E14.5
F Gm-130
EMA
Merge DAPI
F' Rab11a1
5 µm
G DAPI EMA WGA
G'' GCNA WGA
EMA Aggregate volume (µm³)
***
2-cell cystocytes
4-cell cystocytes
% Occurrence of branched Visham
Female
Male
# of ring canals
Enriched
Non-Enriched
Visham Volume
H
I
% germ cells with Viaham
E9.5
E10.5
E11.5
E12.5
E13.5
E14.5

Fig 2


A
E11.5
DAPI
PCNT
EMA
A'
Centrosome
Visham
B
EMA
DAPI
Ac. Tub
EMA
B'
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Metaphase
Anaphase
Telophase
Late telophase
Visham
Microtubules
C
DAPI
Ac. Tub
EMA
E1
C'
Newly formed Visham
Ac-tub.
D
Ac. Tub
EMA
E10.5
D'
Cytokinesis
Visham
EMA
Ac. Tub
YFP
E
E12.5
E'
E12.5
E''
E12.5
Ac. Tub
Ac.tub
YFP
F
Cleavage pattern (eg: 8-cell cyst)
No. of cysts
No. of cells /cyst
****
~86 %
G
Proposed model of Mouse germline cyst cleavage pattern
Cleavage
6+2
4+4+4
Cleavage
6+2
Cleavage
6+2
6+2
Cleavage
6+2

Fig 3

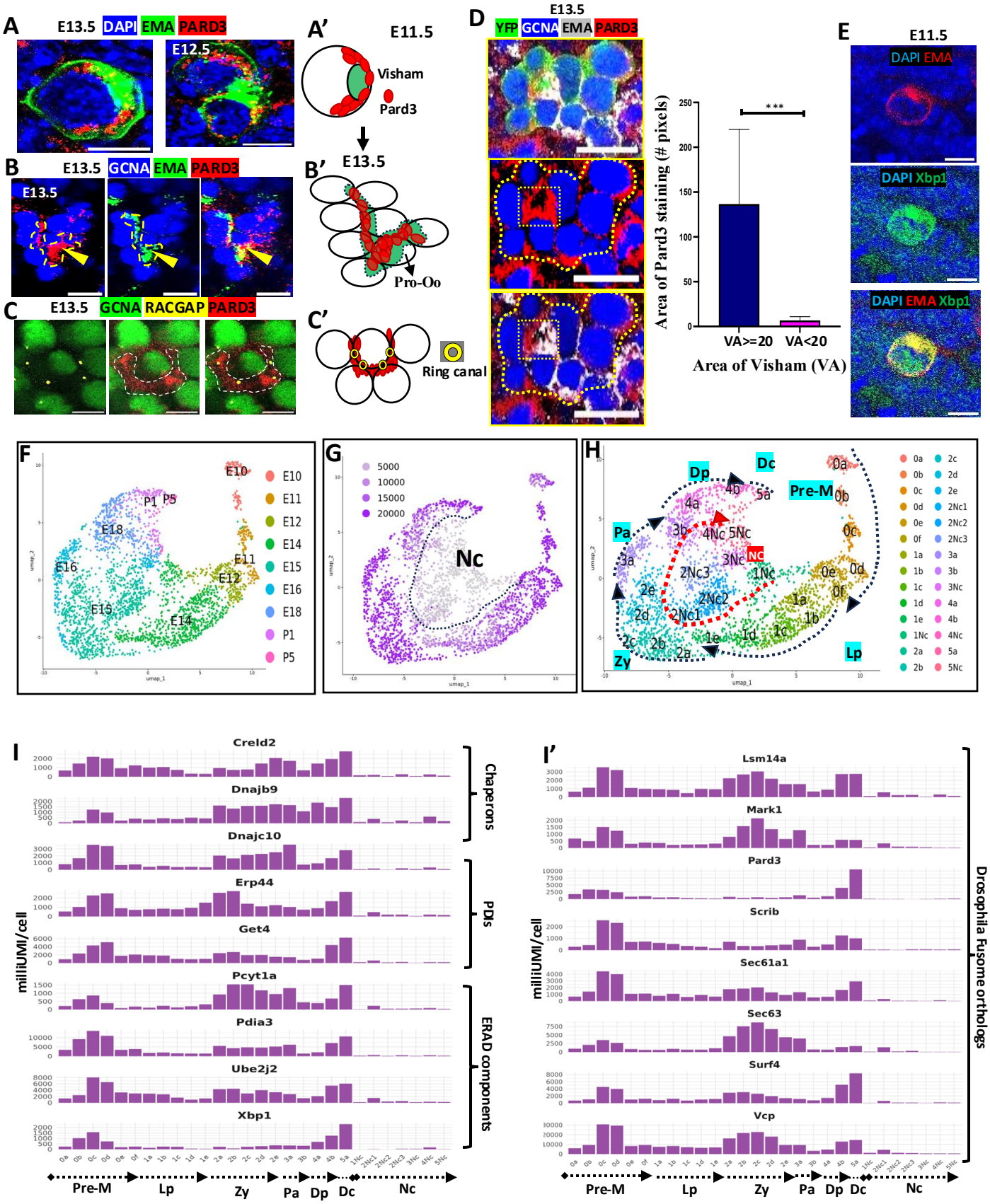

A
E13.5 DAPI EMA PARD3
E12.5
A'
E11.5
Visham
Pard3
B
E13.5 GCNA EMA PARD3
E13.5
B'
E13.5
Pro-Oo
C
E13.5 GCNA RACGAP PARD3
C'
Ring canal
D
E13.5
YFP GCNA EMA PARD3
Area of Pard3 staining (# pixels)
***
VA>=20
VA<20
Area of Visham (VA)
E
E11.5
DAPI EMA
DAPI Xbp1
DAPI EMA Xbp1
F
E10
E11
E12
E14
E15
E16
E18
P1
P5
G
5000
10000
15000
20000
Nc
H
Dp
Dc
Pre-M
Pa
Nc
Zy
Lp
I
Creld2
Dnajb9
Dnajc10
Erp44
Get4
Pcyt1a
Pdia3
Ube2j2
Xbp1
Chaperons
PDIs
ERAD components
milliUMI/cell
Pre-M
Lp
Zy
Pa
Dp
Dc
Nc
I'
Lsm14a
Mark1
Pard3
Scrib
Sec61a1
Sec63
Surf4
Vcp
Drosophila Fusome orthologs

Fig 4.

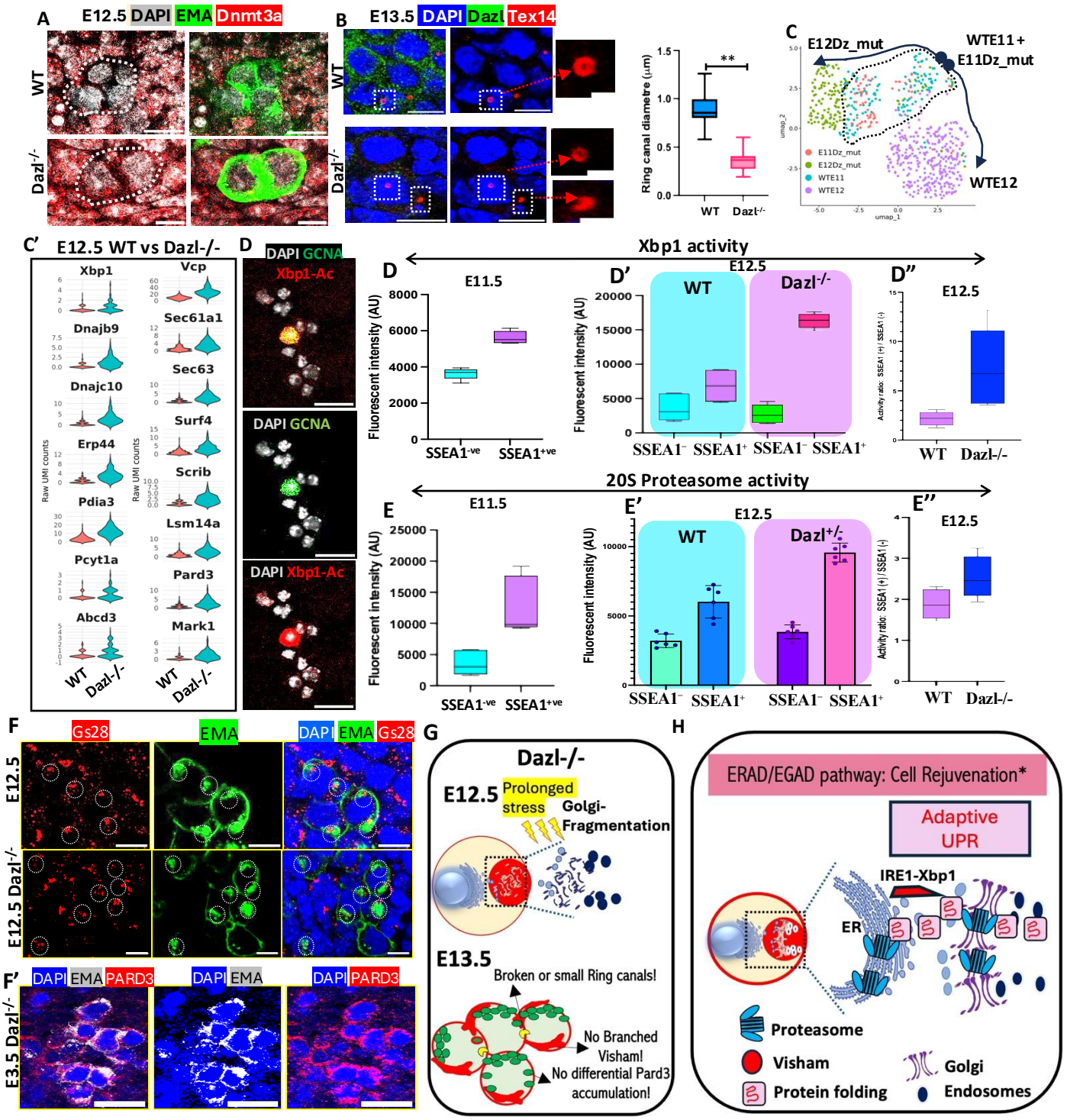
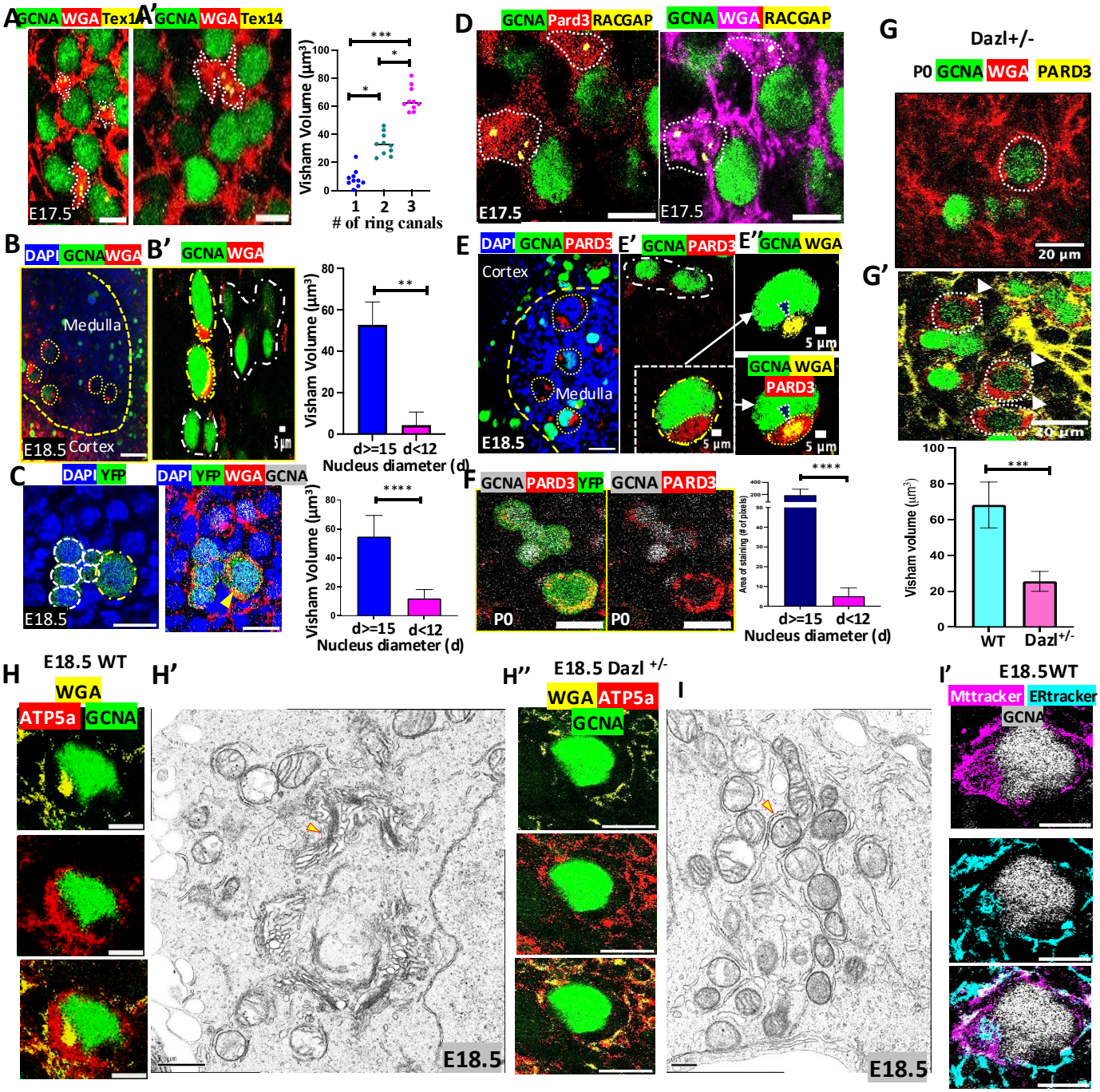

A
E12.5 DAPI EMA Dnmt3a
WT
Dazl-/-
B
E13.5 DAPI Dazl Tex14
WT
Dazl-/-
Ring canal diametre (µm)
**
WT
Dazl-/-
C
E12Dz_mut
WTE11 + E11Dz_mut
WTE12
E11Dz_mut
E12Dz_mut
WTE11
WTE12
umap_1
umap_2
C'
E12.5 WT vs Dazl-/-
Xbp1
Vcp
Dnajb9
Sec61a1
Sec63
Dnajc10
Surf4
Erp44
Scrib
Pdia3
Lsm14a
Pcyt1a
Pard3
Abcd3
Mark1
Raw UMI counts
WT
Dazl-/-
D
DAPI GCNA
Xbp1-Ac
DAPI GCNA
DAPI Xbp1-Ac
Xbp1 activity
D
E11.5
Fluorescent intensity (AU)
SSEA1-ve
SSEA1+ve
D'
E12.5
WT
Dazl-/-
SSEA1-
SSEA1+
D''
E12.5
Activity ratio: SSEA1 (+) / SSEA1 (-)
WT
Dazl-/-
20S Proteasome activity
E
E11.5
Fluorescent intensity (AU)
SSEA1-ve
SSEA1+ve
E'
E12.5
WT
Dazl+/-
SSEA1-
SSEA1+
E''
E12.5
Activity ratio: SSEA1 (+) / SSEA1 (-)
WT
Dazl-/-
F
Gs28
EMA
DAPI EMA Gs28
E12.5
E12.5 Dazl-/-
F'
DAPI EMA PARD3
DAPI EMA
DAPI PARD3
E3.5 Dazl-/-
G
Dazl-/-
E12.5
Prolonged stress
Golgi-Fragmentation
E13.5
Broken or small Ring canals!
No Branched Visham!
No differential Pard3 accumulation!
H
ERAD/EGAD pathway: Cell Rejuvenation*
Adaptive UPR
IRE1-Xbp1
ER
Proteasome
Visham
Protein folding
Golgi
Endosomes

Fig 5


A
GCNA WGA Tex14
E17.5
A'
GCNA WGA Tex14
Visham Volume (µm³)
# of ring canals
B
DAPI GCNA WGA
Medulla
Cortex
E18.5
B'
GCNA WGA
5 µm
Visham Volume (µm³)
d>=15 d<12
Nucleus diameter (d)
C
DAPI YFP
E18.5
DAPI YFP WGA GCNA
Visham Volume (µm³)
d>=15 d<12
Nucleus diameter (d)
D
GCNA Pard3 RACGAP
E17.5
GCNA WGA RACGAP
E17.5
E
DAPI GCNA PARD3
Cortex
Medulla
E18.5
E'
GCNA PARD3
5 µm
E''
GCNA WGA
GCNA WGA PARD3
5 µm
5 µm
F
GCNA PARD3 YFP
P0
GCNA PARD3
P0
Area of staining (# of pixels)
d>=15 d<12
Nucleus diameter (d)
G
Dazl+/-
P0 GCNA WGA PARD3
20 µm
G'
20 µm
Visham volume (µm³)
WT
Dazl+/-
H
E18.5 WT
WGA
ATP5a GCNA
H'
E18.5
H''
E18.5 Dazl +/-
WGA ATP5a
GCNA
I
E18.5
I'
E18.5WT
Mttracker ERtracker
GCNA