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Caspar specifies primordial germ cell count and identity in *Drosophila melanogaster*

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Abstract

Repurposing of pleiotropic factors during execution of diverse cellular processes has emerged as a regulatory paradigm. Embryonic development in metazoans is controlled by maternal factors deposited in the egg during oogenesis. Here, we explore maternal role(s) of Caspar (Casp), the *Drosophila* orthologue of human Fas-associated factor-1 (FAF1) originally implicated in host-defense as a negative regulator of NF-κB signaling. Maternal loss of either Casp or it's protein partner, Transitional endoplasmic reticulum 94 (TER94) leads to partial embryonic lethality correlated with aberrant centrosome behavior, cytoskeletal abnormalities, and defective gastrulation. Although ubiquitously distributed, both proteins are enriched in the primordial germ cells (PGCs), and in keeping with the centrosome problems, mutant embryos display a significant reduction in the PGC count. Moreover, the total number of pole buds is directly proportional to the level of Casp. Consistently, it's 'loss' and 'gain' results in respective reduction and increase in the Oskar protein levels, the master determinant of PGC fate. To elucidate this regulatory loop, we analyzed several known components of mid-blastula transition and identify the translational repressor Smaug, a zygotic regulator of germ cell specification, as a potential critical target. We present a detailed structure-function analysis of Casp aimed at understanding its novel involvement during PGC development.

eLife Assessment

This study investigates the role of Caspar (Casp), an orthologue of human Fas-associated factor-1, in regulating the number of primordial germ cells that form during Drosophila embryogenesis. The findings are **important** in that they reveal an additional pathway that contributes to germ cell specification and maintenance. The evidence supporting the conclusions is **solid**, as the authors identify Casp and its binding partner Transitional endoplasmic reticulum 94 (TER94) as factors that influence germ cell numbers.

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Introduction

As a model organism, *Drosophila melanogaster*, has been instrumental in establishing and advancing several developmental paradigms underlying embryonic development. *Drosophila* has also emerged as a relatively simple yet tremendously useful model, to analyze the underpinnings of the immune response (Medzhitov 2001; Lemaitre and Hoffmann 2007). Critically, despite the small size and modest cellular complexity, the insect immune system shares many fundamental traits with the higher vertebrates, including the humoral and innate arms and the dialogue between the two (Hultmark 1993; Adams *et al*. 2000). Furthermore, several factors that contribute to the proper functioning of the insect immune system are highly conserved and perform analogous functions across evolution (Hoffmann and Reichhart 2002; Lemaitre and Hoffmann 2007).

Interestingly, almost all the protein components of the insect immune system are highly pleiotropic and involve diverse activities during developmental progression. For example, the Toll class of proteins belonging to a larger family of pattern recognition receptors are essential while mounting robust immune response against the microorganismal invasion (Brennan and Anderson 2004; Wang and Ligoxygakis 2006). The same proteins are also deployed earlier during embryonic patterning and morphogenesis (Govind 1999; Hoffmann and Reichhart 2002; Valanne *et al*. 2011). Evidently, in several instances, different pathway components or modules used during early development are repurposed to mediate immunity both in the insect and the mammalian context (Belvin and Anderson 1996; Govind 1999; Roth 2023).

The involvement of early embryonic morphogen, Dorsal, during humoral response in flies is a canonical example of the context-specific and diverse functions of immune system components (Govind 1999; Roth 2023). Maternal loss of function of genes involved in Toll/Dorsal signaling affect Dorso-ventral patterning (Belvin and Anderson 1996). A nuclear-cytoplasmic gradient of Dorsal, set up by asymmetric Toll signalling establishes cell fate across the Dorsal-ventral axis in the syncytial embryo (Nusslein-Volhard 2022). Pathogenic invasion of insects induces both the humoral and cellular immune response (Belvin and Anderson 1996; Williams 2007). The humoral response consists of the production of antimicrobial peptides by the fat bodies, which serve as a first line of defence (Belvin and Anderson 1996; Imler and Hoffmann 2000). Two Rel family member proteins, Dorsal and Dif, homologous to the mammalian NF-kappa B, induce the expression of defense peptides (Belvin and Anderson 1996; Govind 1999; Buchon *et al*. 2014). NF-kappa B is essential for differentiating lymphocytes, which engineer the acute-phase response. Altogether, undertaking functional analysis of proteins, in a temporally distinct developmental context, has proven to be highly informative and insightful. Notably, however, thus far, such analysis has focused on only activator proteins. Here we have investigated the embryonic function of Caspar (Casp), a protein involved in inhibiting the immune response (Kim *et al*. 2006).

Casp is an intracellular negative regulator discovered in a genetic screen to identify suppressors of antibacterial immunity (Kim *et al*. 2006). Flies mutant for *casp* were identified due to their high rates of melanization, an innate immune response that leads to encapsulation of pathogens in the gut and fat body. Interestingly, *casp* mutants isolated in this study were resistant to Gram-negative bacterial infections due to elevated expression of the antimicrobial peptide (AMP) diptericin; consequently, infected flies survived longer than their wild-type counterparts. Strikingly, *casp* overexpression inhibited nuclear localization of Rel in response to infection in the fat body. Excess levels of Casp result in the cytoplasmic retention of Rel in its uncleaved, inactive form, presumably due to inhibition of the protease, Dredd (Kim *et al*. 2006).

Intriguingly, sequence analysis of Casp indicated a high degree of similarity to the mammalian Fas-associated factor 1 (FAF1) protein (Kim *et al*. 2006; Tendulkar *et al*. 2022) (**Fig.1A**). FAF1 is evolutionarily conserved and was initially discovered as an interactor of Fas, a pro-apoptotic member of the tumor necrosis factor receptor family (Chu *et al*. 1995). FAF1 also interacts with the components of the death-inducing signaling complex (DISC) such as the Fas-associated death domain (FADD) and Caspase-8 proteins (Ryu *et al*. 2003). These interactions are mediated by the death effector domains (DED) in FADD and Caspase-8 and the DED-interacting domain (DEDID) in FAF1 (Ryu *et al*. 2003). Both 'loss' and 'gain' of function experiments indicated that FAF1 is crucial in promoting cell death via transduction of the apoptotic signal (Ryu and Kim 2001; De Zio *et al*. 2008). FAF1, like its *Drosophila* ortholog, is a negative regulator of NFκB signalling (Min-Young *et al*. 2004; Park *et al*. 2007).

FAF1's myriad cellular functions can be attributed to its multiple protein-interaction domains that allow its participation in ubiquitin-related processes such as protein degradation (Song *et al*. 2005; Menges *et al*. 2009; Zhang *et al*. 2011). Consistently, FAF1 harbors a Ubiquitin-associated (UBA) domain, a UAS domain and a Ubiquitin-like regulatory X (UBX) domain (**Fig. 1A**). Furthermore, the N-terminal UBA domain recruits polyubiquitinated proteins, leading to their accumulation (Song *et al*. 2009). The C-terminal UBX domain, on the other hand, interacts with the molecular chaperone, valosin-containing protein (VCP/p97) bound to the Npl4-Ufd1 heterodimeric complex (Schuberth and Buchberger 2008; Kloppsteck *et al*. 2012; Ewens *et al*. 2014). FAF1 complexed with VCP-Npl4-Ufd1 and polyubiquitinated proteins is known to assist endoplasmic reticulum-associated degradation (ERAD) (Lee *et al*. 2013). The UAS domain, a domain of unknown function, is involved in interaction with long-chain fatty acids, which is thought to promote the polymerization of FAF1 (Kim *et al*. 2013).

While Casp has a well-established role in the immune response, modENCODE RNAseq and proteomics data suggest that *casp* is also highly expressed in the 0-3 hour old embryo (Brown *et al*. 2014; Casas-Vila *et al*. 2017). Consistently, a snapshot of *casp* staining in a high-throughput RNA *in-situ* experiment indicates ubiquitous expression of maternally deposited *casp* in the *Drosophila* embryo (Weiszmann *et al*. 2009). We thus wondered if Casp expression in early embryos is functionally relevant for proper developmental progression. To explore the possible developmental function of Casp, we first assessed if Casp function is needed for viability. Analysis of a hypomorphic allele of *casp* demonstrated that Casp is indeed required maternally for embryonic development. Consequently, roughly half of the embryos maternally compromised in *casp*, fail to undergo gastrulation. Furthermore, such embryos display developmental defects including aberrant cytoskeletal network starting from early blastoderm stages. Interestingly, Casp is expressed strongly in primordial germ cells (PGCs). Consistent with the enrichment, maternal reduction of *casp* significantly affects the total number of pole cells. Here, we present an analysis of *casp* function during early embryonic development and its role in the formation and/or specification of PGCs. We show that Casp activity regulates Oskar levels and centrosome function, two critical determinants of PGC fate in *Drosophila* embryo. Upon loss of *casp*, the total amount of Oskar and Smaug changed reciprocally to influence the PGC count. Ubiquitin-based protein degradation is critically involved during early embryonic events, including the maternal-zygotic transition. We present a model explaining the involvement of Casp and its protein partner, TER94, during germ cell development, considering their influence on the clearance of Smaug, a critical regulator of maternal to zygotic transition (MZT).

Results

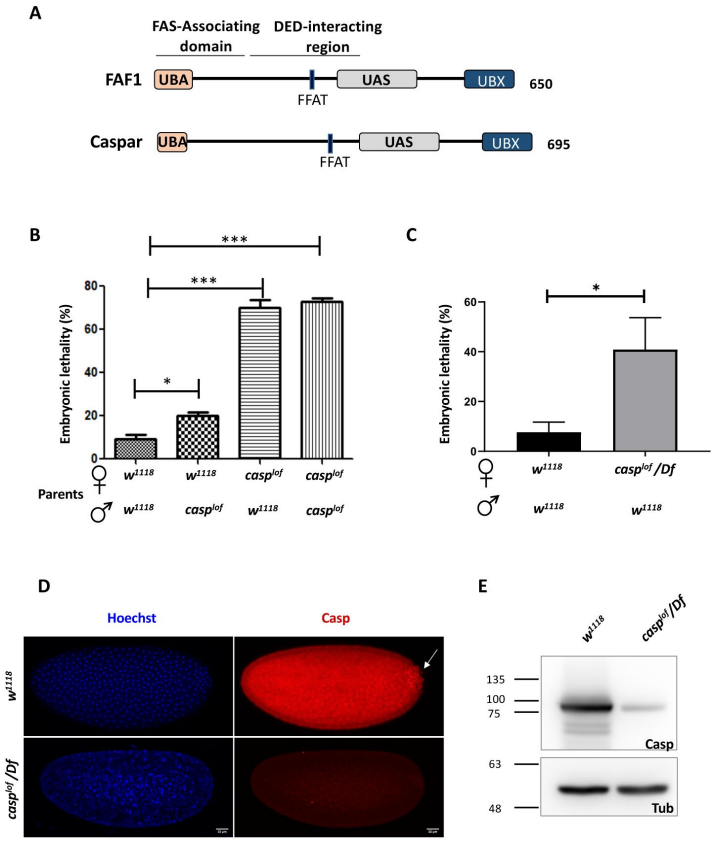

Fig. 1.

casp is a maternal effect gene.

(A) Comparison between human FAF1 and Casp shows conserved protein domains, which are described in the text. (**B**) Embryos laid by homozygous *casp*^{lof}females show ~70% lethality, irrespective of the paternal genotype (*w*^{1118} or *casp*^{lof}), suggesting a strictly maternal function of *casp* (**C**) The use of a Deficiency in the *casp* locus validates the lethal phenotype associated with the mutation, which drops to ~40%. In panels B and C, the parental genotype is listed on the X-axis, with percent embryonic lethality plotted as a bar graph. N = 3, ordinary one-way ANOVA/ unpaired t-test, (***) *P* <0.001, (*) *P* <0.05. (**D**) Immunofluorescence images of 0-3 h embryos derived from *w*^{1118} and *casp*^{lof}*/Df* females, stained with Hoechst and Casp (**E**) Casp protein levels were assessed in 0-3 embryos laid by *w*^{1118} and *Casp*^{lof}*/Df* animals, evaluated via western blotting. Tubulin is used as a loading control.

Casp^{c04227} is a loss of function allele of casp

To better understand embryonic function of Casp we decided to first characterize a *casp* allele, w^{1118}*; pBac(PB)casp*^{c04227} (Bloomington stock number:11373) (Supplementary Fig. 1A; labelled as S1A). This allele is induced by a piggyBac insertion in the 5' regulatory region of *CG8400/FBgn0034068/Casp* locus which is situated at 2R, region 52D14-15 (Thibault *et al*. 2004). As summarized in **Fig. 1**, our data indicate that *casp*^{c04227} is a strong hypomorphic allele and is referred to as a 'loss of function' allele (*casp*^{lof}) here onwards.

~70% embryos laid by the homozygous *casp*^{lof} females failed to hatch into larvae (**Fig. 1B**). Moreover, the number of embryos that failed to hatch did not change substantially when *casp*^{lof} virgin females were mated either with w^{1118} males or *casp*^{lof}males. That the extent of viability was independent of the paternal genotype established that *casp* is a maternal effect gene and its activity is required for viability (**Fig. 1B**). To rule out the possibility that the lethality was induced by non-specific background mutations, we procured multiple deficiency (Df) lines spanning the 52D locus and confirmed that *casp*^{lof}*/Df* mothers also displayed significant embryonic lethality (40%) (**Fig. 1C**). Lastly, as in the case of *casp*^{lof}females, *casp*^{lof} males are also semi-fertile which was assessed by mating the mutant males with w^{1118} virgin females (**Fig. 1B**).

To better understand the function of Casp, we first decided to analyze how *casp* RNA and Casp protein are expressed during embryogenesis. Consistent with the maternal effect lethality, modENCODE (Muers 2011; Chen *et al*. 2014) data suggests that *casp* mRNA is deposited maternally, being highly expressed in the 0-2 hours embryo, with a drop in expression 2-4 hours post fertilization (**Fig. S1B**)(Brown *et al*. 2014). Publicly available *In-Situ* databases such as FlyAtlas, Fly-FISH and BDGP (Weiszmann *et al*. 2009) (Tomancak *et al*. 2002; Lecuyer *et al*. 2007) also confirmed maternal deposition of *casp* mRNA, with ubiquitous expression seen in stage 1-3 embryos. Fly-FISH data (Lecuyer *et al*. 2007; Wilk *et al*. 2016) further suggests that expression in the pole cells persists while maternally deposited somatic transcripts are selectively degraded from stage 4 onwards. We immunostained 0-3 hours old embryos derived from w^{1118}mothers with the anti-Casp antibody (Tendulkar *et al*. 2022) and found that Casp protein was predominantly cytoplasmic, and as in the case of RNA, exhibited a relatively ubiquitous distribution in the somatic compartment (**Fig. 1D**). Again, consistent with the *in-situ* hybridization pattern, Casp-specific antibody staining appeared to be enriched in the posteriorly localized primordial germ cells (PGCs) compared to the surrounding soma (**Fig. 1D**, arrow). To establish the specificity of the anti-Casp antibodies in the embryonic context, we stained the embryos laid by both the *casp*^{lof}*/ casp*^{lof} and *casp*^{lof}*/Df* mothers. As expected, Casp protein was nearly absent in embryos of both these genotypes suggesting that Casp protein is significantly reduced in the 0-3 hours old *casp*^{lof}mutant embryos. Analysis of the embryonic lysates using Western blotting, suggested that trace amounts of Casp protein (estimated 5-10% of w^{1118}), were present in embryos laid by *casp*^{lof}*/casp*^{lof} and *casp*^{lof}*/Df* mothers, supporting the conclusion that *casp*^{lof} is a strong hypomorphic allele and not a null (**Fig.1E**). Interestingly, mature egg chambers of wild-type animals had Casp expression in somatically derived follicle cells, but Casp protein could not be readily detected in the egg itself (**Fig. S1C**). This observation suggested that, unlike the RNA, only trace amounts of Casp protein may be maternally deposited. Thus, the protein detected in the young embryos is likely generated post-fertilization, by the translation of maternally deposited *casp* mRNA.

Casp function is required for early embryonic development

Larval cuticle patterns are an excellent readout for major patterning defects in *Drosophila* (Nusslein-Volhard and Wieschaus 1980). To better understand the function of Casp protein during embryonic development, we analyzed the cuticles of *casp* mutant embryos. 0-3 hours old embryos laid by *casp*^{lof}*/Df* mothers were collected and aged for 22 hours to prepare cuticles (see Material and Methods). The cuticular patterns were visualized under a dark field microscope.

While ~60% of larvae displayed cuticular patterns comparable to wild type larvae, development in the remaining 40% embryos appeared to have stalled before they could deposit cuticle. These data were consistent with the extent of maternal effect lethality.

Next, to define the stage of developmental arrest, we performed time-lapse live Bright-field imaging (Cavey and Lecuit 2008). These data (Movies, Fig. S2A) confirmed that 40% of embryos did not proceed to gastrulation and showed developmental arrest before germ band extension (around stage 6; **Fig. S2**, panels C4, D4). Such embryos displayed irregular, uncoordinated morphogenetic movements with blebbing of the plasma membrane possibly inhibiting both cephalic furrow formation, and germband elongation (Fig. S2 panels C2-C3, D2-D3).

Compromising casp activity leads to centrosomal abnormalities in early embryos

To trace the defects during mid-embryogenesis including failure of gastrulation, we sought to visualize $casp^{lof}$ embryos at blastoderm stage. We labelled the 0-3 hours old $casp^{lof}$ and control embryos with the nuclear dye Hoechst and the cytoskeletal F-actin marker phalloidin (**Fig. 2A, B**). $casp^{lof}$ embryos displayed significant structural abnormalities that are reflected in an irregular actin network that lacks stereotypical organization, which is partially interrupted in several places (**Fig. 2B** 1, compared to **Fig. 2A** 1). Similarly, unlike the age-matched control embryos, regular nuclear spacing is disrupted, and nuclei are unevenly distributed across the embryo with occasional instances of nuclear fusion and possibly mitotic asynchrony (**Fig. 2B** vs **Fig. 2A**).

The nuclear and cytoskeletal defects observed in *casp* embryos prompted us to analyze the centrosomes in embryos deficient in *casp* function. Centrosomes function as the microtubule organizing centers, and actomyosin-based cytoskeletal defects have been correlated with aberrant centrosome activity ((Wu and Akhmanova 2017; Blake-Hedges and Megraw 2019) and references therein). In wild-type nuclei, centrosome duplication occurs simultaneously with the initiation of the nuclear division cycle. After completion of duplication, centrosomes separate and migrate along the nuclear membrane to reach the opposite sides of the nucleus (reviewed by (Lattao *et al*. 2017; Wu and Akhmanova 2017; Blake-Hedges and Megraw 2019)). In the embryos maternally compromised for *casp*, several characteristic aspects of centrosome behavior during mitotic divisions are altered (Compare **Fig. 2B** 3 to **Fig. 2A** 3). At times, centrosomes appeared to divide even without a nucleus (or DNA), and many 'orphan' centrosomes devoid of nuclear DNA were observed (**Fig. 2B** 3, inset). By contrast, in some instances, centrosomes were duplicated but remained in proximity indicating failed or incomplete migration (see arrows in **Figure 2B** 3, inset). Quantitation of the centrosomal abnormalities in $casp^{lof}$ embryos (**Fig. 2C, D**) further revealed that the behavior of the centrosomes, especially their ability to separate and migrate to the opposite poles correctly deteriorated progressively (Compare **Fig. 2D** to **Fig. 2C**), as nuclear division cycles advanced.

To confirm the specificity of the phenotypic consequences induced by the maternal loss of *casp*, we overexpressed *UAS-*$casp^{wt}$ transgene in $casp^{lof}$*/Df* embryos using *nos Gal4*, a maternal Gal4 driver. Embryos derived from such mothers were stained using nuclear dye Hoechst, phalloidin and anti-Gamma tubulin antibodies that mark the centrosomes. As shown in **Fig. 2E**, maternal overexpression of *casp* substantially rescues centrosomal and cytoskeletal abnormalities seen in $casp^{lof}$ (compare panels E1-E3 to A1-A3). This rescue is quantitated in **Fig. 2 G, H** (compare with **Fig. 2C, D**). The $casp^{lof}$*/Df; nos Gal4/UASp-*$casp^{wt}$ showed a rescue of lethality of 20-25% as compared to 40-45% for $casp^{lof}$*/Df*.

Taken together, these data showed that Casp plays an essential role in early embryonic development in *Drosophila*, and loss of *casp* results in conspicuous nuclear and cytoskeletal defects that correlate with incomplete gastrulation movements and developmental arrest, for

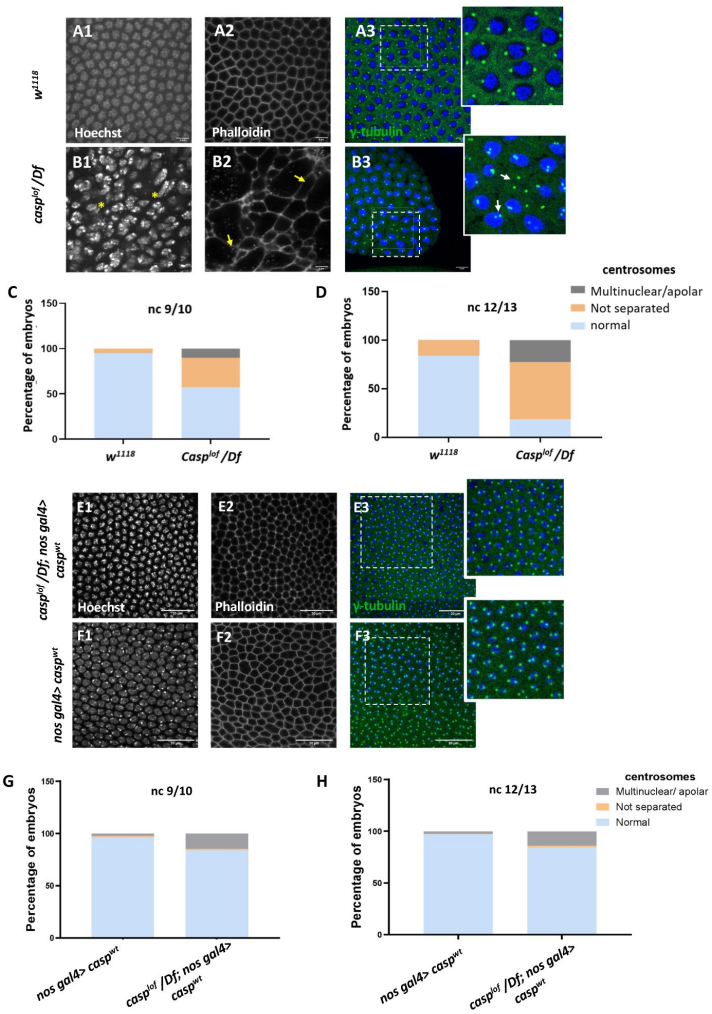

Fig. 2.

A Significant proportion of embryos (~45%) laid by *casp^{lof}/Df* mothers display nuclear division and cytoskeletal defects.

(**A-B**) Confocal images of nuclear cycle 13/14 embryos (single sections) of the indicated genotypes stained with Hoechst, phalloidin, and gamma-tubulin. Both the regular arrangement and uniform density of nuclei are disrupted in the mutants, as indicated by a yellow asterisk. F-actin, marked with phalloidin, shows a regular, hexagonal compartmentalization in *w^{1118}* (panel A2), while disorganized F-actin (white arrows; panel B3) is observed in the mutant. Defective centrosomes marked with gamma-tubulin can also be observed in the *Casp* mutant (inset of B3, compare to inset A3), indicated by yellow arrows. The extent of defects is quantified in nuclear cycle(nc) 9/10 (**C**) and nc 12/13 (**D**). (**E-F**) Confocal images of sections of nc 13/14 embryos of the indicated genotypes stained with Hoechst, phalloidin, and gamma-tubulin. The nuclear, cytoskeletal, and centrosomal defects are rescued (panel E) when wild-type *casp* is expressed in the *casp^{lof}/Df* background (compare panels **G-H** to C-D). Overexpression of *casp* on its own (panel F) does not appear to affect nuclear or cytoskeletal architecture. The bar charts represent the percentage of defective centrosomes in nuclear cycle 9/10 and 12/13 embryos. Number of embryos imaged ~15.

~45% of embryos. Moreover, such defects do not appear to be region-specific or localized within an embryo arguing in support of ubiquitous function across the embryo for Casp protein.

Maternal Casp protein is enriched in pole cells and controls total pole cell count in blastoderm embryos

Primordial germ cells (PGCs) are precursors of the gametes—sperm and eggs. Their specification is essential for the development of the germline, which in turn passes genetic information to the next generation. During early embryonic development, PGCs are specified and segregated from somatic cells. In *Drosophila*, the PGCs are formed in the posterior end of the embryo. As the early embryonic syncytial nuclear division cycles progress, a few nuclei and centrosomes associated with them invade posteriorly localized and anchored pole plasm ahead of the rest of the somatic nuclei (Raff and Glover 1989). The precocious entry of the centrosomes results in the release and microtubule-dependent transport of pole plasm, which is sequestered in newly cellularized PGCs (Raff and Glover 1989; Lerit *et al*. 2017). Thus, the PGCs or the germline stem cell precursors are set aside during early embryogenesis. Moreover, centrosome behavior and dynamics are crucial for proper cellularization and mitotic cell divisions of early germ cells (Lerit and Gavis 2011; Lerit *et al*. 2017). Centrosomes, the microtubule (MT)-organizing centers, ensure the faithful segregation of germ plasm, a reservoir of germ cell determinants, into PGCs. Taken together with the observation that Casp protein is readily detectable in early PGCs, we wondered if it could influence the total pole cell count. To examine this possibility, stage 5 embryos derived from *cs* and $casp^{lof}/Df$ mothers were immunostained for the pole cell marker Vasa and visualized under a confocal fluorescence microscope. Interestingly, while *cs* and *nos-Gal4* embryos had an average of ~30 pole cells (**Fig 3**, panels A1-B3, for quantitation see panel F) $casp^{lof}/Df$ showed a drastic reduction in total PGC count to ~10 (**Fig. 3** panels C1-3, F). By contrast, stage 5 embryos derived from *nosGal4>UAS-casp* mothers, where *casp* was overexpressed (**Fig. 3G**), displayed considerably elevated total number of PGCs (**Fig. 3**, panels E1-E3, F). To confirm that the loss of *casp* is specifically responsible for the reduction in the total number of PGCs, we simultaneously overexpressed *casp* in the $casp^{lof}/Df$ mothers in a germline-specific manner, which resulted in a significant rescue (~26 pole cells; **Fig.3**, panels D1-D3, F). Taken together, these data suggested that the total pole cell number in late syncytial /cellular blastoderm embryos specifically depends on *casp* function. Furthermore, increasing *casp* levels maternally, is sufficient to elevate the total pole cell number substantially.

Casp interacts with TER94 in the early embryo

Data presented in the previous section demonstrated that both the 'loss' and 'gain' of *casp* activity exert reciprocal influence on total PGC numbers in early embryos. As very few germ plasm components have been shown to display this trait (Jongens *et al*. 1994), we decided to explore the phenomenon further. We have previously reported that Casp protein physically associates with both transitional endoplasmic reticulum ATPase (TER94; also called valosin-containing protein, VCP or p97) and vesicle-associated membrane protein-associated protein B (VAPB/VAP33A) (Tendulkar *et al*. 2022). Furthermore, based on interaction studies and biochemical analysis, we proposed that Casp may act as an adapter that either directly or indirectly mediates the physical association between VAPB and TER94 (Tendulkar *et al*. 2022). To arrive at this conclusion, we had primarily relied on S2 cell lysates as well as adult and fly head total protein extracts. To specifically identify the major protein partners of Casp in the early embryos, we performed similar immunoprecipitations using anti-Casp antiserum in early (0-3 hour) embryonic lysates and interactors were identified via mass spectrometry (**Fig. S3A**). Peptides from 122 proteins were recovered in the Casp IP but not the IgG IP and thus, were considered significant interactors. The top 23 interactors are presented in **Fig. S3A**. Interestingly, as in the case of brain and S2 cell lysates, TER94 and VAPB were the top hits, with germ cell determinants also identified as

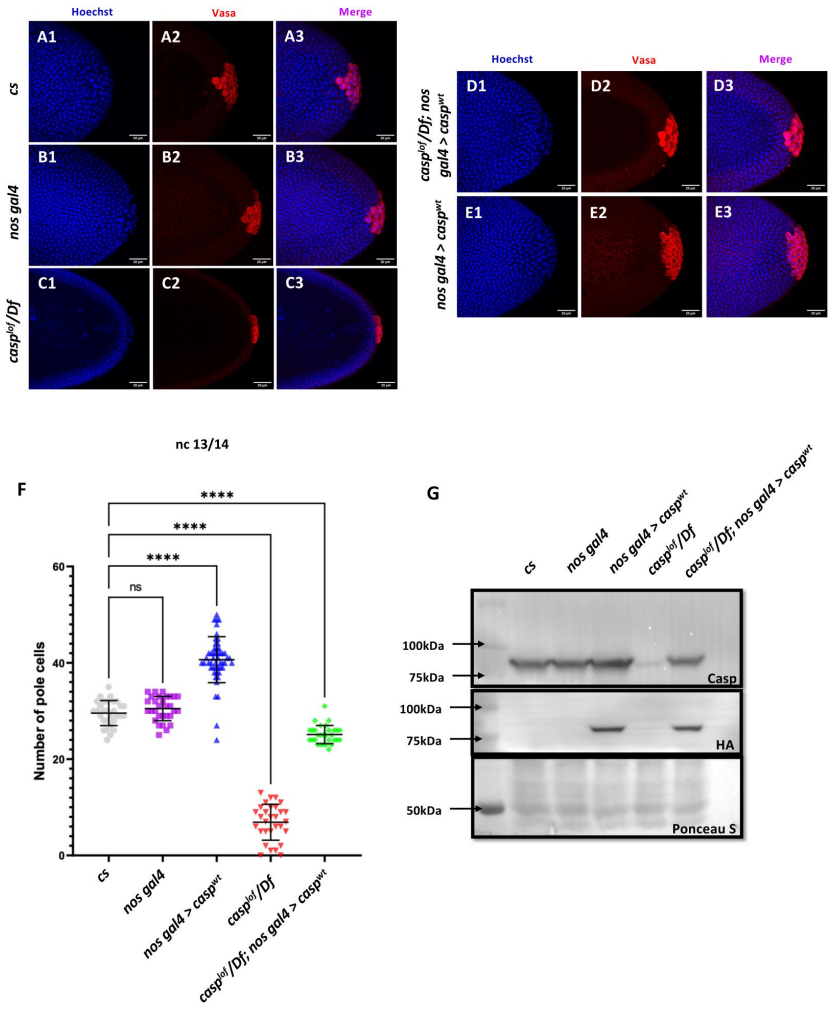

Fig. 3.

Casp influences total PGC count.

(**A-E**): Shown are the Confocal images of the posterior terminii of nuclear cycle 13/14 embryos of the following genotypes: *cs* (A1-A3), *nos gal4* (B1-B3), *casp*^{lof}*/Df* (C1-C3), casp^{lof}*/Df*; *nos gal4>casp*^{wt} (D1-D3) and *nos gal4> casp*^{wt} (E1-E3). Embryos were immunostained with Vasa (1:50) antibody. Hoechst marks the nuclei. (**F**) The total number of germ cells marked with Vasa were quantified and plotted as bar graphs. N (embryos)= 30. Ordinary one-way ANOVA, (****) P <0.0001. (**G**) Casp protein levels were assessed in 0-3 embryos via western blotting. Rabbit anti-Casp (1:10,000) and rabbit anti-HA (1:2000) antibodies were used to probe the blot. Ponceau is used as a loading control. N = 3.

interactors (**Fig. S3C**; Discussed in a subsequent section). These observations suggested that a functional protein complex between Casp, TER94 and VAPB likely exists in many different tissue/cellular contexts, including early embryos.

Maternal requirement of TER94

Mammalian VCP/p97 is an essential chaperone for proteostasis that modulates several ubiquitin-associated processes (Peters *et al*. 1990; Dai *et al*. 1998; Meyer *et al*. 2000; Meyer 2005; Ye 2006; Jentsch and Rumpf 2007; Meyer *et al*. 2012). Its *Drosophila* counterpart TER94 has been studied mostly in the context of neurodegeneration (Griciuc *et al*. 2010; Chang *et al*. 2011; Azuma *et al*. 2014; Kushimura *et al*. 2018; Tendulkar *et al*. 2022; Thulasidharan *et al*. 2024). Loss of TER94 was shown to ameliorate polyQ-induced eye degeneration. Moreover, the overexpression of TER94 promoted the apoptosis of neuronal cells (Higashiyama *et al*. 2002). TER94 has been uncovered in a genetic screen for maternal proteins that are phosphor-regulated (Zhang *et al*. 2018) and have roles in oogenesis and early development.

TER94 is one of the significant interactors of Casp protein in the embryonic context as suggested by the Casp interactome (**Fig. S3A**) and has been implicated in ER-associated degradation (Chang *et al*. 2011; Tendulkar *et al*. 2022). Thus, we wondered if TER94 also has a maternal function and whether embryos maternally compromised for *TER94* display similar phenotypes as *casp*. To achieve this, we employed VALIUM 20/22 maternal RNAi lines (*UAS-TER94i*). First, we used a *mat-α4tubulin:VP16-Gal4* (*Mat-αtubGal4*) driver to deplete *TER94* activity in the late stages of oogenesis (**Fig. 4**).

We sought to observe the consequence of *TER94* knockdown both in the adult females and early embryos derived from such females. In the females compromised for *TER94*, egg-laying behavior remained largely unaffected. Interestingly, however, the viability of such embryos was severely impaired, with >95% of embryos failing to hatch (**Fig 4A**), in agreement with earlier studies (Zhang *et al*. 2018).

A western blot of lysates derived from *Mat-GAL4>UAS-TER94i* mothers probed with anti-TER94 antiserum indicated a robust knockdown of TER94 as compared to control (**Fig. 4B**). Phenotypic analysis of embryos derived from *Mat-GAL4> TER94i* females revealed that >70% of *Mat-GAL4/ TER94i* embryos failed to progress to the syncytial blastoderm stage, with even fewer reaching Stage 5. The late-stage syncytial/ cellular blastoderm embryos were further assessed for cell cycle defects. As in the case of $casp^{lof}/Df$, *Mat-GAL4>TER94i* embryos also displayed defects ranging from irregular nuclear distribution to perturbed F-actin localization, as assessed by HOECHST and phalloidin staining, respectively (Compare **Fig. 4D1-D3** with **Fig. 4C1-C3**).

We also examined if maternal loss of *TER94* activity results in centrosome aberrations like those observed in $casp^{lof}$embryos. Indeed, embryos deficient in *TER94* function showed both the characteristic phenotypes observed in the *casp* embryos including a) inefficient separation of duplicated centrosomes and b) multiple instances of 'orphan' centrosomes devoid of nuclear DNA (**Fig. 4E, F**). An earlier study (Zhang *et al*. 2018) had found that TER94 RNAi leads to multi-polar spindles with supernumerary centrosomes.

Taken together, these data demonstrated that embryos maternally compromised for either Casp or TER94 functions share several phenotypes, suggesting that both the proteins likely perform essential and likely related functions during syncytial nuclear cycles in *Drosophila* embryos. Furthermore, the phenotypic consequences observed upon maternally compromising *casp* and *TER94* overlap but are not identical, with TER94 embryos showing higher penetrance in terms of phenotypes and embryonic lethality. Future experiments will be necessary to resolve the functional distinction between the two.

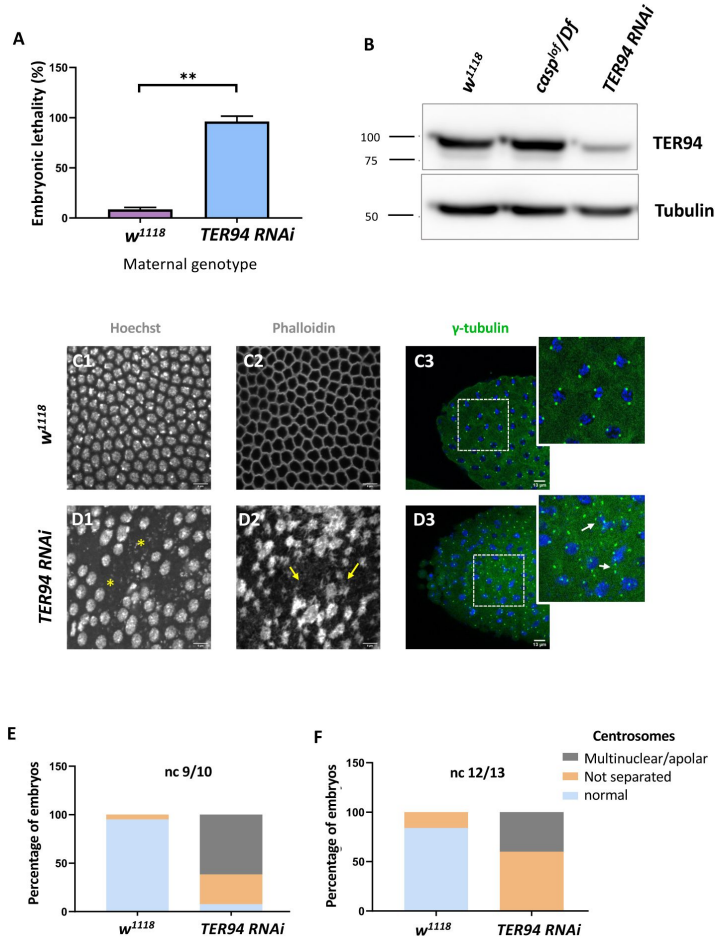

Fig. 4.

Maternal requirement of *TER94* during early embryogenesis.

(**A**) Viability of embryos derived from w^{1118} and *Mat-αtubGAL4* > *TER94i* (referred to as *TER94 RNAi* henceforth) mothers is represented as a bar graph. N = 3, ordinary one-way ANOVA, (***) *P* <0.001. (**B**) Western blot analysis indicates the knockdown efficiency of TER94 in the 0-3 h embryo, estimated to be ~90%., levels remain unaffected in $casp^{lof}/Df$ embryos. Tubulin was used as a loading control. N = 3. (**C, D**) Confocal images of sections of nuclear cycle 13/14 embryos of the indicated genotypes stained with Hoechst, phalloidin, and gamma-tubulin. Nuclei and F-actin are visualized with Hoechst and phalloidin respectively. Nuclear disruption (D, asterisks) and F-actin aggregates (D2, arrows) are observed in the mutant. Defective centrosomes (D3, arrows, inset) can be observed in the *TER94 RNAi* embryos. (**E, F**) The percentage of defective centrosomes in nuclear cycles 9/10 and 12/13 are represented as bar charts. n = 10.

TER94, a known component of pole plasm, is detectible in PGCs

Maternal loss of TER94 function mimicked both the cytoskeletal and corresponding centrosome aberrations observed upon similar loss of *casp* activity. Both centrosomes and germ plasm, are essential for the proper specification and formation of PGCs in *Drosophila* embryos (Lerit and Gavis 2011; Lerit *et al*. 2017). This prompted us to examine the Casp interactome for the possible enrichment of germ plasm components and proteins that may regulate centrosome dynamics and/or behavior. **Fig. S3B** lists Casp interactors that are important constituents of the germplasm, including eIF4A, me31B, and TER94, known constituents of the polar granules (Thomson *et al*. 2008).

Among the different components of the germ plasm, Oskar serves as the principal determinant of the PGC fate (Kim-Ha *et al*. 1991; Snee and Macdonald 2004; Vanzo *et al*. 2007; Lehmann 2016). Supporting the conclusion, the loss and gain of function of *oskar* leads to reciprocal phenotypes. Compromising *oskar* activity maternally, leads to reduction in total number of PGCs whereas anterior ectopic expression of *oskar* using the *bicoid* mRNA localization signal induces pole cell formation at the anterior (Ephrussi and Lehmann 1992). The germ plasm is supplemented with mitochondria and polar granules, which consist of ribonucleoprotein complexes. Downstream of *oskar*, *vasa* and *tudor* are two genes that are essential for the assembly of pole plasm (Breitwieser *et al*. 1996; Arkov *et al*. 2006; Jones and Macdonald 2007). Biochemical proteomic analysis of Vasa (VAS) and Tudor (TUD) containing polar granule complexes identified eIF4A, me31B, and TER94. This indicated that the germ plasm consists of the components of translational machinery and the endoplasmic reticulum assembly (Thomson *et al*. 2008).

TER94 is a component of pole plasm and is also physically associated with Casp. Furthermore, proteomic analysis indicated that Casp could be part of a complex comprising several germ plasm proteins including Tudor and Me31B. To confirm that, as in the case of Casp, TER94 is also detected in PGCs. We used antibodies generated against Casp (**Fig. S3D**) and TER94 (**Fig. S3E**) to label wild-type embryos. Embryos were also co-immunostained for germ cell specific marker, Vasa. As can be seen, both Casp and TER94 proteins are found in PGCs, colocalized with Vasa.

Casp and TER94 regulate embryonic germ cell formation

As we were specifically interested in investigating possible similarities and distinctions between the respective functions of TER94 and Casp during early embryonic germ cell development, we decided to examine their possible functions during PGC formation (also referred to as pole cell budding). This seemed especially pertinent as centrosome behavior and dynamics are crucial for proper formation and cellularization of PGCs. Evidently, a germ plasm component, Germ-cell-less (Cinalli and Lehmann 2013) was shown to be necessary for proper separation of daughter centrosomes in the dividing pole buds (Lerit *et al*. 2017). Similarly, loss of centrosome components such as Centrosomin results in partial loss of early embryonic PGCs. As maternal loss of both *casp* and *TER94* results in aberrant centrosomes in the surrounding somatic nuclei in early blastoderm embryos, we sought to investigate if posteriorly positioned centrosomes in the vicinity of pole plasm also display similar problems. Indeed, maternally compromising *casp* led to significant loss of pole buds and corresponding defective centrosome behavior (**Fig.5**).

Especially these pole buds display defective centrosome separation and/or orphan centrosomes (**Fig. 5B1-B2,D,E**), as observed in the somatic nuclei. Intriguingly, while $casp^{lof}$ embryos show discernible loss of pole buds, *TER94i* embryos displayed an increased number of round-shaped, 'bud-like' cells at the posterior of the stage 3 embryos (**Fig 5 C1-C2**). Typically, such ectopically localized pole buds also showed inadequate separation of centrosomes and could be identified as pole buds due to the enrichment of Vasa. However, Vasa distribution and accumulation in *TER94i* buds was variable and non-uniform. Since TER94 is required for *Osk* mRNA localization in the

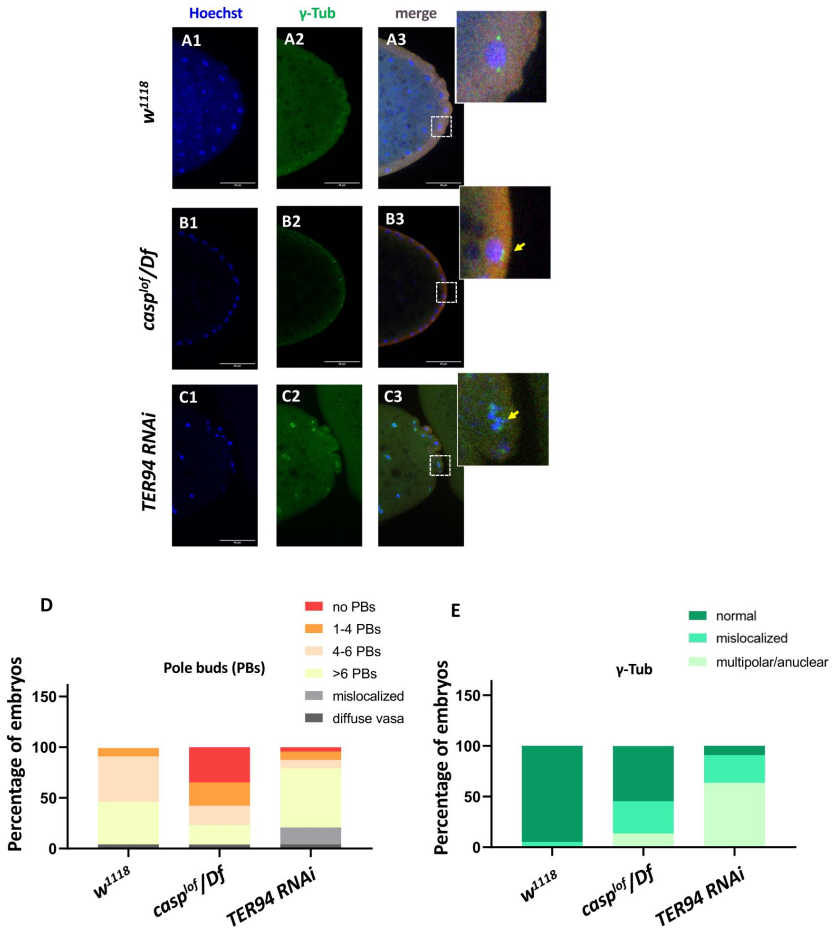

Fig. 5.

***casp and TER94* regulate pole bud formation.**

(A-C) Confocal sections of the posterior termini of nuclear cycle 9/10 embryos derived from w^{1118} (A1-A3), $casp^{lof}/Df$ (B1-B3), and *TER94 RNAi* (C1-C3) mothers immunostained with gamma-tubulin. ψ-tubulin labels centrosomes of pole buds, identified based on Vasa staining. Nuclei are stained with Hoechst. The inset(s) for B3 and C3 highlight the centrosomal defects (arrow) when compared with A3. Defects were quantified and plotted as bar graphs in (**D**) and (**E**) respectively. n = 20.

oocyte, the defects in pole cell budding including their ectopic positioning could be a consequence of inappropriate segregation of *oskar*. Moreover, the early nuclear division cycles and nuclear migration defects seen in are defective in *TER94i* embryos could also contribute to PGC formation/cellularization. Notably, although the pole bud count is elevated in *TER94i* embryos, presumably, these pole buds don't survive through subsequent nuclear cycles, and consequently, late blastoderm *TER94i* embryos almost completely lack PGCs.

Casp activity is needed for the accumulation of Oskar protein at the embryonic posterior pole

Taken together, our data demonstrate that change in Casp levels leads to corresponding alteration in the total PGC count. Moreover, PGC formation is affected by maternal loss of Casp, although the precise nature of its involvement in this process remains to be determined. As Oskar is the master determinant of germ cell fate, we sought to determine if Oskar levels (Kim-Ha *et al*. 1991; Ephrussi and Lehmann 1992) are correspondingly altered upon change in Casp. To assess this possibility, we stained wild-type control embryos and embryos maternally compromised for *casp* using anti-Oskar antibodies (Compare **Fig 6B** to **6A**). Simultaneously, we stained embryos derived from *nosGal4>UASp-casp* mothers (**Fig 6C1-C3**) that display significantly elevated number of PGCs, presumably due to excess levels of Casp protein in the germline.

Satisfyingly, the change in Oskar protein levels is consistent with the alteration in the Casp levels in the maternal germline (**Fig 6D**). Embryos derived from *casp* mutant mothers show considerably diminished levels of Oskar as compared to the control whereas overexpression of Casp in the maternal germline results in elevation in the Oskar protein amount (**Fig 6D**). Furthermore, in all instances, Oskar protein appears to be anchored to the posterior pole as the wild-type control embryos and the only discernible change is observed in its levels.

Casp levels influence total number of phosphor-histone3 (pH3) positive pole buds and PGCs

Changes in Casp protein levels influence the accumulation of Oskar protein. Oskar is necessary and sufficient to assemble pole plasm at the posterior pole which controls the total number of pole buds in an embryo. To directly evaluate the influence of Casp on pole bud formation, total number of pole buds and pole cells were quantitated upon maternal loss and gain of Casp against the control embryos at the same stage (**Fig. 6E**). Consistent with the data presented in the previous sections, total number of pole buds (nc 9 and 10) were reduced in $casp^{lof}$ embryos whereas the number was elevated in the presence of excess Casp (**Fig 6E**).

Similar quantitation was performed in older embryos in two stages (nc11-12 and NC12-13), and a progressive increase was observed in the total number of PGCs upon the gain of Casp function (**Fig. 6E**). By contrast, the total PGC count for $casp^{lof}/Df$ embryos did not increase appreciably at nc11-12 and nc12-13. Primordial germ cell number is determined by limited mitotic cell divisions that each pole bud undergoes in a stochastic and asynchronous manner. As these are mitotic divisions, the dividing PGCs can be identified using antibodies against the miotic marker phosphoHistone3 (pH3). As the total number of pole buds and PGCs change per Casp levels, we wanted to examine if this is also reflected in the total number of pH3-positive germ cells simultaneously labeled with the anti-Vasa antibodies. We decided to focus on syncytial blastoderm embryos between NC12-14. As anticipated, maternal overexpression of Casp led to a corresponding increase. In control (*nos gal4*) embryos, roughly 20% PGCs display either 1 or >1 pH3 positive PGCs whereas, 70% of *nos-gal4>*$Casp^{WT}$embryos show 1 or >1 pH3 positive PGCs (**Fig. 6F**). Conversely, less than 10% of $casp^{lof}$ embryos showed either 1 or >1 pH3 positive PGCs and pole cells with 3 or >3 pH3 positive cells were completely absent in this background as opposed to

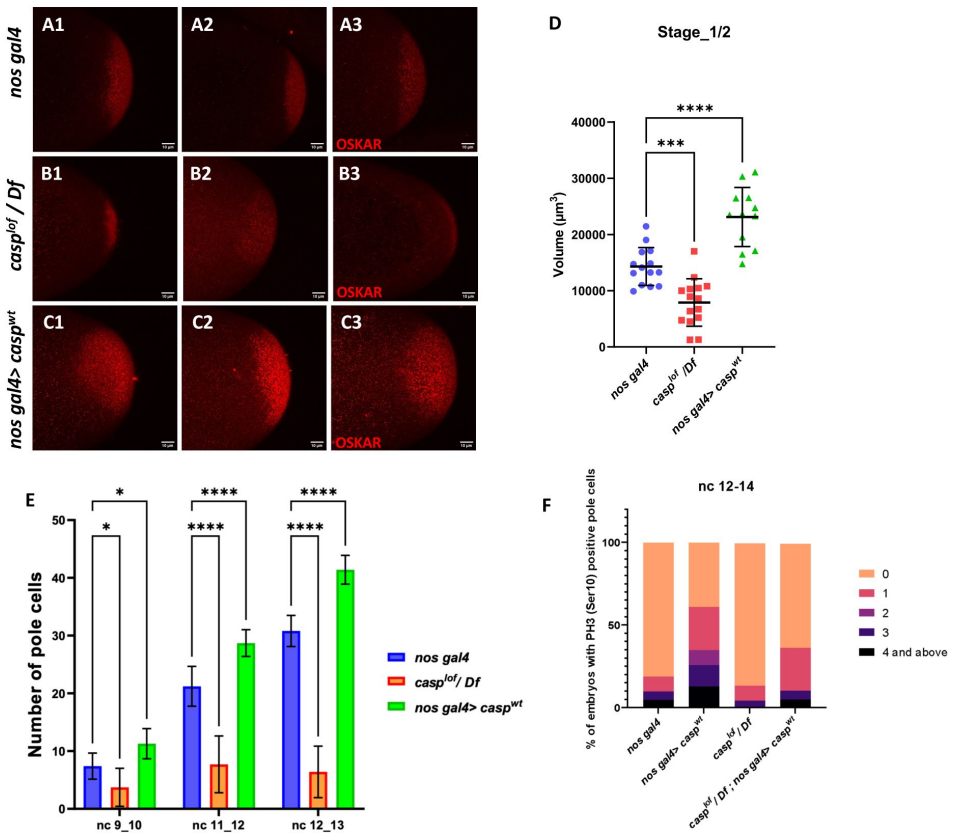

Fig. 6.

Casp regulates Oskar levels and modulates cell division

(A-C) Confocal microscopy images of stage 1/2 embryo laid by w^{1118}, $casp^{lof}/Df$, and *nos gal4*> $casp^{wt}$ females and immunostained with Oskar antibodies. Replicate (1-3) are shown to highlight variable reduction in Oskar levels. In addition, as compared to controls (w^{1118}) embryos (A1-A3), spread of Oskar appears to be restricted in $casp^{lof}/Df$ (B1-B3) whereas it is expanded in *nos gal4*> $casp^{wt}$ (C1-C3). (**D**) Volume occupied by Oskar, per embryo is measured and plotted for all three genotypes (w^{1118}, $casp^{lof}/Df$, and *nos gal4*> $casp^{wt}$), n = 8. Ordinary one-way ANOVA, (**) P <0.01, (*) P <0.05. (**E**)Number of pole buds (nc 9/10) and pole cells (nc 11/12, nc 12/13) in 0-3h old *nos gal4,* $casp^{lof}/Df$ *and nos gal4*> $casp^{wt}$ embryos at different nuclear cycles. Two-way ANOVA, (*) P <0.05, (****) P <0.0001. n = 10 (**F**) Graph showing the distribution of actively dividing pole cells as indicated by the presence of phospho-histone 3 (Ser10) antibody. Quantitative analysis was performed using nuclear cycle 12-14 in 0-3h old, *nos gal4*, $casp^{lof}/Df$, *nos gal4*> $casp^{WT}$ and $casp^{lof}/Df$;*nos gal4*> $casp^{WT}$ embryos.

25% present in the control embryos (**Fig. 6F**). Importantly, maternal expression of the rescue construct in the *casp*^{lof} embryos ameliorated the loss of pH3 positive PGCs seen in just the mutant (*casp*^{lof}) PGCs.

Does Casp function affect canonical MBT regulators?

Our analysis thus far has revealed two related yet distinct phenotypes associated with maternal loss of *casp*. First, it can influence proper PGC formation and specification during early embryonic development via its effect on the accumulation of germ cell determinant, Oskar. Subsequently, it helps orchestrate cellular movements leading up to gastrulation during mid-embryogenesis. While the first activity is likely germ cell autonomous, the second relates to its role in the somatic cells/nuclei. Moreover, both the somatic and germline compartments of *casp*^{lof} embryos share centrosomal as well as cytoskeletal aberrations. We thus wondered whether the two activities are mechanistically connected, and if the possible connection relates to the maternal-to-zygotic transition (MZT). One aspect of the MZT that has been highlighted in recent years is the active degradation of maternal proteins (Cao *et al*. 2020). These proteins comprise 2% of the maternal proteome and are degraded abruptly, at the end of the MZT. In *Drosophila*, ubiquitin-proteasome-based degradation of three repressor proteins, namely Smaug (Smaug), Trailer hitch (Tral), Maternal expression at 31B (Me31B), marks the MZT (Cao *et al*. 2020). We thus decided to probe, using antibodies generated against these proteins (**Fig. 7**) if the degradation of any of these proteins is affected due to maternal loss of Casp/*casp* during the initial hours of embryonic development (0-1, 1-2 & 2-3 hours). TER94 protein is unchanged (Fig. D1, D2) as is the α-tubulin loading control (**Fig. 7E1**, **E2**). Furthermore, pattern of degradation of Me31B and TRAL proteins in the *casp*^{lof} embryos is relatively unaffected, when compared to the *w*^{1118} control samples.

By contrast, Smaug (Fig, C1-C2) is an interesting exception. In control, 0–1hr old embryos, low levels of Smaug protein were observed, which increased in the 1-2hr old embryo extract but decreased the 2-3 hr time-window (**Fig. 7C**), data that agrees with previously published work (Cao *et al*. 2020). However, in the *casp*^{lof}/*Df* embryos levels of Smaug protein are modestly elevated from the start (0-1 and 1-2 hr old extract samples respectively; **Fig. 7C**2). Critically, unlike control embryos, Smaug protein persists in 2-3hr old *casp*^{lof}/*Df* embryonic extracts (**Fig 7C**2, *). Altogether, these data argue that Smaug degradation is specifically adversely influenced in the *casp*^{lof}/*Df* embryos at the end of the MZT.

Germ cell specifc Smaug levels are influenced by casp activity

Since Smaug levels appear to be elevated in embryonic lysates in the MZT, we decided to test if the Smaug levels are elevated specifically in *casp*^{lof}/*Df* pole cells. To this end, pre-syncytial as well as early syncytial blastoderm embryos of both the genotypes (control and *casp*^{lof}/*Df*) were stained with anti-Smaug and anti-Vasa antibodies. Aligned with the Western blot data presented earlier, levels of Smaug protein are significantly elevated in pre-syncytial embryos (**Fig. 8**, compare panel B3 with A3). Also, in *casp*^{lof}/*Df* embryos, Smaug protein seems to accumulate in discernible large puncta which are barely visible at this stage in control embryos (**Fig. 8**, B3 vs A3). At the syncytial blastoderm stage (Stage 4), however, the Smaug positive puncta appear larger and more numerous in the few surviving PGCs from the *casp*^{lof}/*Df* embryos (**Fig. 8**, D3) when compared to controls (C3).

Taken together, our data suggest that Smaug protein levels are appreciably elevated in pole cells from *casp*^{lof}/*Df* embryos. The specific increase in Smaug levels may, in part, be due to inappropriate accumulation and/or stabilization of Smaug. Smaug protein is necessary for translational control of the posterior determinant *nanos*. Early reports indicated that unlocalized *nos* RNA is translationally repressed by Smaug which binds to Smaug Response Elements (SREs) within 3'UTR of *nos* RNA. In addition to *nos*, it also regulates *hsp83* translation. Smaug participates

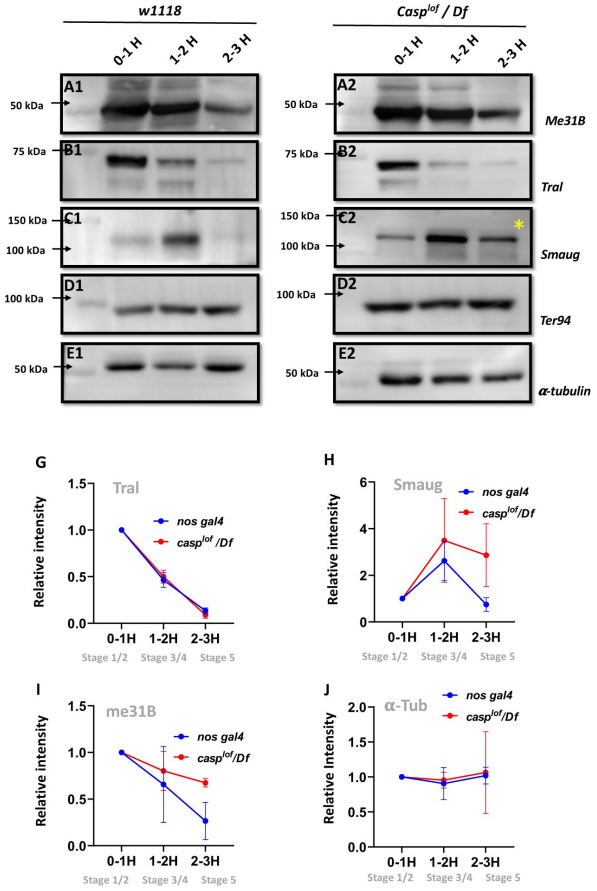

Fig. 7.

Reduction in Casp activity specifically affects Smaug degradation during the MZT.

At the MZT, a few maternal proteins are actively degraded (C_{AO} *et al*. 2020). Embryos from mothers with genotype w^{1118} **(A1-E1)** and $casp^{lof}/Df$ **(A2-E2)** were collected using three time intervals (0-1, 1-2, 2-3 Hrs), and embryonic lysates were separated on SDS-PAGE gels. The Western blots were probed with antibodies against ME31B (A1-A2), Tral (B1-B2) and Smaug (C1-C2) to assess the extent of protein degradation, as part of the MZT. α-tubulin (E1-E2) was used as a loading control, while TER94 (D1-D2) was used as a negative control as it is a maternal protein that does not undergo degradation at MZT. (**G-J**) Quantitation of the intensity of change of protein band (normalized to 1), for Tral (G), Smaug (H), ME31B (I) and tubulin (J). Each data point is an average of over 5 western blots. The intensities at each time point for *nos gal4* vs $casp^{lof}/Df$ are statistically not significant.

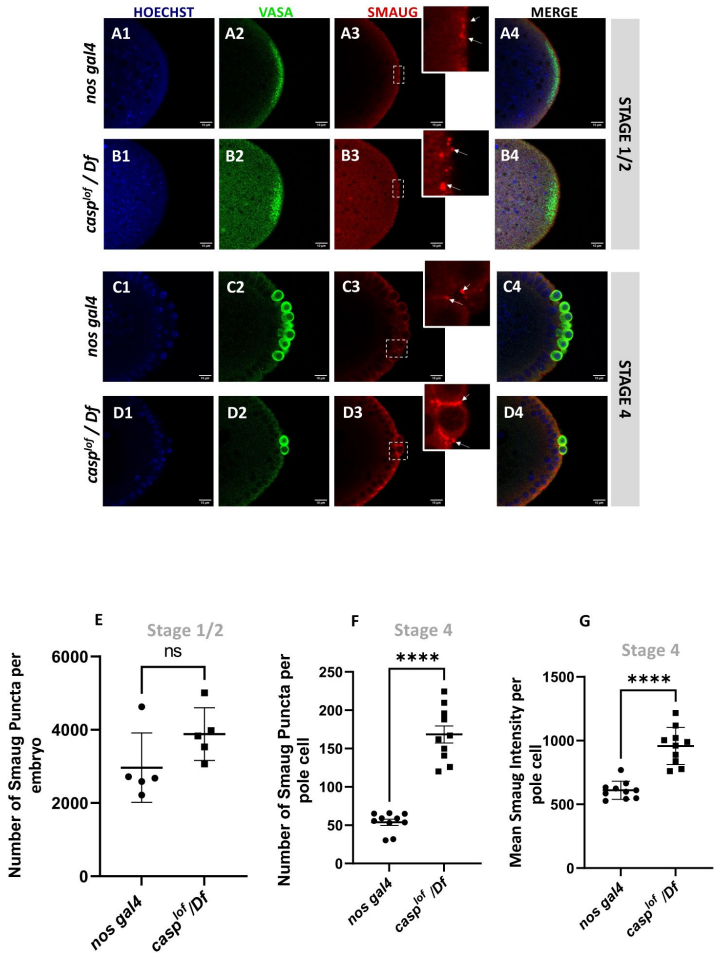

Fig. 8.

Decrease in Casp correlates with an increase in Smaug levels in the pole buds and PGCs.

Confocal microscopy images of the posterior termini of stage 1-2, and 4 embryos derived from *nos gal4* and *casp^{lof}/Df* females. Embryos were immunostained with Vasa (green) and Smaug (red) antibodies. Hoechst marks the nuclei (A-D). Smaug expression was quantified across the two genotypes and plotted as bar graphs (E-G). N=5 embryos (Stage 2) and 10 embryos (Stage 4).

in multiple, overlapping mechanisms including interaction with the components of the translation machinery as well as deadenylation to regulate translation/localization of the target RNAs (Dahanukar *et al*. 1999; Zaessinger *et al*. 2006).

Intriguingly, recent data from Lipshitz lab has, in fact, shown that Smaug protein accumulates in germ granules (Siddiqui *et al*. 2023). Moreover, in addition to its canonical role in regulating *nos* translation, it can also repress *oskar* at the translational level (see Discussion).

As the embryonic PGC count depends upon Oskar levels, *Smaug* mutant embryos show an increase in the total number of PGCs. These data fit nicely with our observations and are entirely consistent with a model incorporating the downregulation of Smaug mediated by Casp.

Functional analysis of different protein domains within Casp

To decipher the specific functions of the individual protein domains of Casp during pole cell formation and division we followed an experimental strategy involving rescue of the loss of function phenotype. We compared the extent of rescue observed in the presence of individual deletion constructs to the full length wild-type control. To this end, fly lines expressing domain deletions of Casp under the *pUASp* promoter were generated (**Fig. 9A**). The *pUASp-casp*$^{\Delta UBA}$, *pUASp-casp*$^{\Delta UBX}$ and *pUASp-casp*$^{\Delta UAS}$ construct represent deletions of the UBA, UBX, and UAS domains, respectively. Additionally, we also generated fly lines that express the *pUASp-casp*$^{\Delta UAS\Delta UBX}$ which deletes both the N and C-terminal domains simultaneously. A full-length coding sequence of Casp was also cloned, and this *pUASp-casp*^{WT} line was used as a positive control carrying the 'rescue' construct. A western blot confirmed the expression of these constructs in adult animals with different domain deletions (Δ), *UAS-casp*$^{\Delta}$ driven by *nos-GAL4* driver (**Fig. 9B**). To assess the domain-specific functions of Casp in isolation without the confounding effects of endogenous Casp, the fly lines were balanced with a *casp*^{lof} allele on the second chromosome. These balanced lines were crossed to a fly line with the maternal driver *nos-GAL4* on the third chromosome and an allele with a deficiency for *casp (Df)*. Embryos laid by mothers of the genotype *casp*^{lof}*/Df; nos-GAL4/pUASp-casp*$^{\Delta}$ were used to determine maternal effects of the domain deletions (**Fig. 9C**).

Embryos were collected from females of different genotypes and stained using anti-Vasa antibodies. As can be seen by the comparison shown in the bar graph (**Fig. 9C**), in terms of the rescue of PGC numbers, deletion of the UBA domain does not affect the rescuing activity of the Casp protein, the UBX domain deletion can rescue, but is weaker than the UBA domain deletion, whereas deletion of the UAS domain is the least effective with regards to the rescue.

To extend these observations, we overexpressed individual Casp deletion mutants in an otherwise wild-type background and counted the total number of PGCs. Neither the maternal overexpression of *pUASp-casp*^{wt} nor any of the *pUASp-casp*$^{\Delta}$increased lethality in terms of decrease in hatching of embryos, in all cases the hatching was in the range 90-95%. Again, as seen before, overexpression of *casp*^{wt} under these conditions led to a significant increase in the total number of PGCs in blastoderm embryos, with average PGC's at 40. The ability to enhance the PGC division is retained in the absence of the UBA domain as, in this particular background, the mean count of the total number of germ cells was similar to embryos that expressed full-length protein. By contrast, deletion of the UAS domain is unable to support the (increased) germ cell division, and the total PGC count dropped to the same level as *nos Gal4* embryos.

Interestingly, deletion of UBX domain, yet again, displayed intermediate activity, with the *ΔUBX-ΔUBA* domain deletion also being unable to drive the increase in PGCs. Proteomic analysis of the embryonic extracts suggested that in addition to TER94 protein, Casp is also associated with VAP (**Fig. S4A**; (Tendulkar *et al*. 2022)). We thus sought to test if physical interaction between Casp and VAP is relevant for pole cell formation.

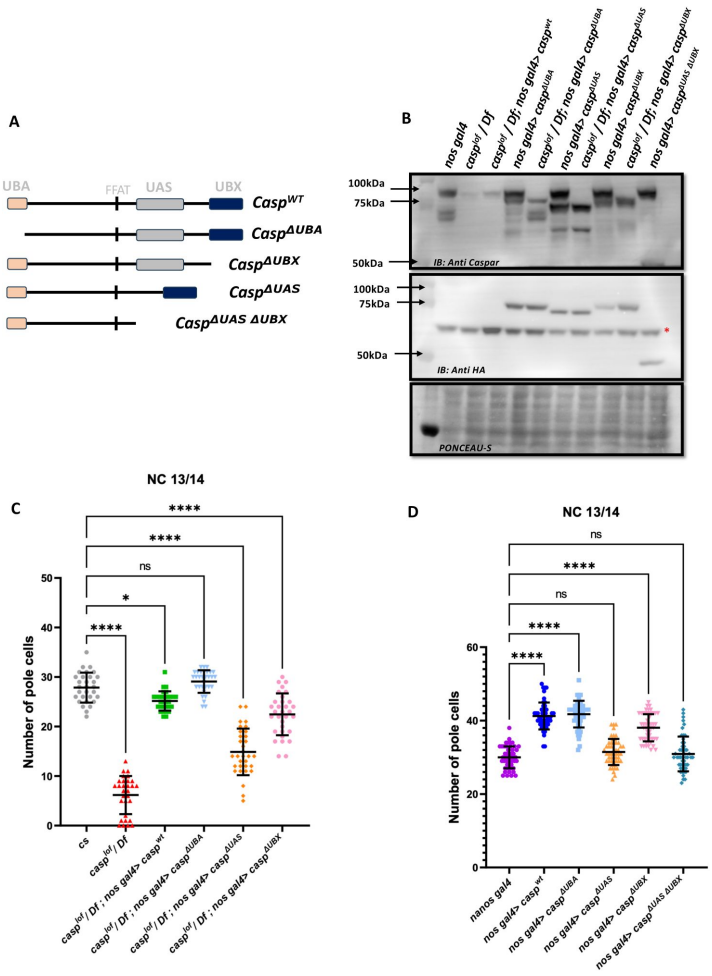

Fig. 9.

Structure-function analysis of Casp protein domains in regulating pole cell number.

(A) Schematic representation of different domain deletion variants of Casp. As shown in the schematic, the WT Casp protein consists of different functional domains including UBA, UAS, UBX and the FFAT-like motif. **(B)** Western blot of the truncated Casp proteins, deficient in the domains indicated, probed with rabbit anti-casp (1:10,000) and rabbit anti-HA (1:2000) antibody. *nos gal4* served as a control. Ponceau staining was used as a loading control. Asterisk (*) denotes non-specific antibody binding. **(C)** The total number of germ cells from nc 13/14 embryos derived from mothers expressing different *casp* domain deletion constructs in the *casp^{lof}/Df* background were marked with Vasa antibodies, quantified and plotted as a bar graph. n = 30, Ordinary one-way ANOVA, (****) *P* <0.0001, (*) *P<0.05*. **(D)** The total number of germ cells from nc 13/14, derived from mothers expressing casp domain deletion, in an otherwise *wild-type* background, were stained with anti-Vasa antibodies. Vasa positive cells (i.e. PGCs) were quantified and plotted as a bar graph. n = 30, Ordinary one-way ANOVA, (****) *P* <0.0001.

To assess this possibility, we used a deletion variant of Casp, Casp$^{\Delta FFAT}$, which cannot interact with VAPB (Tendulkar *et al*. 2022). Expression of *casp*$^{\Delta FFAT}$ under the control of maternal driver, in a *casp*^{lof}/Df mother showed hatching (~60%) frequencies at par with wild type (**Fig. S4B**), with expression levels equal to other *casp* constructs (**Fig. S4C**). Importantly, *nos-Gal4*- dependent maternal overexpression *of UAS-casp*$^{\Delta FFAT}$ led to an increase in the total number of PGCs in syncytial blastoderm embryos, comparable to full-length *UAS-casp*^{wt}(average PGC ~23). Lastly, expression of *casp*$^{\Delta FFAT}$ could rescue the PGC loss upon maternal loss of *casp* (compare **Fig. S4** F1-3 to **Fig. 9C**). These data suggested that interaction between Casp and VAP is either not essential or partially redundant for Casp function in the context of determining PGC. Altogether, the functional analysis of different protein domains within Casp underscores the importance of UAS and UBX domains, especially in embryonic contexts, including PGC development.

Discussion

Proper development of biological systems in an organismal context involves complex interactions between various individual pathways. The qualitative nature of the interaction(s) between pathway components ultimately determines how different pathways intersect in a context-specific manner. Curiously however, there are only a defined number of signaling pathways/circuits that have been elucidated thus far. Consistently, the entire molecular cassettes that constitute a given pathway or a select number of pathway components, are reiteratively used in a variety of biological contexts even within a lifecycle of the same organism. Moreover, while such repurposing is relatively frequent, the corresponding biological outcomes are diverse in nature and insightful in ways more than one. Our data detailing the functional involvement of Casp and TER94 in the PGCs is a case in point. Casp function was initially characterized in the context of *Drosophila* immune response (Kim *et al*. 2006). However, the original study did not investigate the developmental roles of Casp and its interactors. Here, we report novel activities of *Drosophila melanogaster* immune components Casp and TER94 during early embryonic development with a specific focus on germline.

Early embryonic patterning is a dynamic process in metazoans which is primarily regulated by the deposition of maternal gene products including RNAs and proteins. Such maternal determinants are localized in a spatially restricted manner (Schier 2007). On many instances, the unique pattern of localization of specific factors underlies their activities during embryo patterning. To execute their functions properly, patterning determinants heavily rely upon the members of the housekeeping machinery that carefully calibrate the synthesis, stability, transport, and degradation of diverse regulatory components. Here, we have explored novel embryonic functions for the FAF1 ortholog, Casp in *Drosophila*. Initially, we uncovered that maternal loss of *casp* activity results in partially penetrant embryonic lethality. The *casp* allele was determined to be a strong loss of function, but not a null. The partial penetrance could also be due to redundancy. Consistent with either possibility, quantitation of the individual phenotypes yielded significant but variable penetrance.

Our data demonstrate Casp's involvement during cellular movements that lead to gastrulation, a likely cause underlying the lethality. Surprisingly, Casp protein is enriched in the PGCs, which are specified at the posterior pole under the control of the master germ cell determinant, Oskar. PGC formation and specification in a young *Drosophila* embryo depends on the posteriorly anchored specialized cytoplasm (or pole plasm) enriched in RNA and protein components essential to determine germ cell identity and behavior. Two important traits distinguishing early pole cells from the surrounding somatic nuclei include precocious budding and limited mitotic self-renewal. Consistent with its functional involvement in both these processes, maternal loss of either Casp or its protein partner TER94 resulted in a reduced number of buds and subsequent loss of PGCs. While qualitatively similar, the severity of phenotypic consequence due to loss of Casp and TER94

regarding PGC loss, is not identical. Interestingly, however, both proteins appear to influence centrosome behavior and dynamics that are of paramount significance in forming pole cells in *Drosophila* embryos.

Thus far, Germ cell-less (Gcl) is the only protein shown to control PGC formation in a similar manner (Jongens *et al*. 1992; Cinalli and Lehmann 2013). Importantly Gcl activity depends on its ability to influence proper separation of centrosomes and elaboration of astral microtubules in dividing pole buds (Lerit *et al*. 2017). Aberrant centrosome behavior in *gcl* mutant embryos adversely affects PGC budding and equitable distribution of germ plasm among daughter cells. Intriguingly, these phenotypic traits can be recapitulated by simply engineering defective centrosome separation (Lerit *et al*. 2017). It is thus noteworthy that maternal loss of Casp and TER94 leads to similar defects and future experiments will reveal mechanistic details underlying roles of these two proteins in regulating centrosome dynamics in PGCs and their possible interaction with Gcl and its protein partners. Of note, a germplasm interactome (Thomson *et al*. 2008) included TER94 along with other important germ plasm proteins including Vasa, Tudor, and others.

TER94, an ER protein, is a major Casp interactor. Our data also indicate that in addition to PGC formation, TER94 and Casp regulate early nuclear division cycles in syncytial blastoderm embryos and subsequent cell divisions in the somatic compartment cell cycle processes in the gastrulating embryos. (Casp and TER94 are distinct compared to Gcl in this regard which has a strictly germ cell specific function). Taken together, these observations suggest that Casp and TER94 contribute to critical early developmental functions leading to mid-blastula transition which precedes gastrulation and germband extension. In the mammalian context, the FAF1-VCP interaction is mediated by the UBX domain. Thus, it is unsurprising that deletion of the protein domain crucial for association between Casp and TER94 resulted in a somewhat diminished function as compared to a full-length version. Curiously however, deleting UAS fragment, a protein domain of unknown function, implicated in self-association, resulted in substantially compromised activity as compared to native protein. Future experiments will focus on the specific molecular interactions this (and other) individual domain(s) are involved in. It will be also important to determine how protein degradation especially engineered via ubiquitin modification contributes to Casp stability and function.

Intriguingly our data also argue that regulation mediated by *casp* is likely critical for maintenance of PGC fate via maintenance of Oskar levels. Importantly, several independent observations suggest that this influence is likely zygotic. First, barely detectable amount of Casp protein is deposited in the egg and bulk of the Casp protein is generated by translation post-fertilization. Second, Casp protein controls overall levels of Smaug at the post-transcriptional level including in the early embryonic PGCs. It was recently reported that Smaug protein accumulates in the germ granules where it controls Oskar (and Bruno) translation negatively by binding to Smaug response elements present in the untranslated regions within the respective RNAs (Siddiqui *et al*. 2023). Consistently loss of function mutations in *Smaug* result in a modestly elevated PGC count in stage 4-5 embryos whereas a reciprocal phenotype is observed in $casp^{lof}$ embryos. Taken together with the increase in Smaug levels in $casp^{lof}$ embryos, it would be reasonable to propose that PGC-specific phenotypes observed upon loss of *casp* are, in part, mediated by excess accumulation of Smaug. Moreover, these authors also suggest that Smaug dependent regulation of *oskar* RNA likely has a significant zygotic i.e. embryonic component which aligns well with our data. Future experiments will be necessary to elucidate the mechanism underlying regulation of Smaug levels by Casp. It will also be interesting to examine if Smaug and Casp regulate centrosome behavior reciprocally to control the final PGC count.

Degradation of maternally deposited RNAs and proteins constitutes an important transition during embryonic development. Zygotic Genome Activation (ZGA) and turning over of maternal determinants (Both RNAs and proteins) happen almost simultaneously. Together these two events

constitute MZT which is delayed in the germ cell compartment as opposed to soma. Nonetheless, recent data have suggested that the two events possibly occur in a coordinated manner possibly via shared components (Colonnetta *et al*. 2023). Many of the embryonic phenotypes that *casp*^{lof} embryos display (aberrant nuclear migration, cellularization defects, defective gastrulation etc.), are shared by mutations in gene products either directly or indirectly involved in one of these processes. It will be important to elucidate new interactors of Casp as its function likely impacts protein degradation and/or stability of the target proteins. Recent reports have indicated potential involvement of ZGA regulators such as Zelda and CLAMP in germline/soma distinction. Critically this function of the components of ZGA depends on proper anchoring, release and transmission of posteriorly anchored pole plasm that involves centrosome function. Maternally compromising ZGA components resulted in inappropriate release and transmission of pole plasm RNAs, a phenotype that is partially recapitulated in *casp*^{lof} and *TER94i* embryos.

While our data have clearly established the involvement of Casp and TER94 during PGC formation and specification, further studies will be necessary to elucidate their precise molecular function(s) underlying this activity. Several observations are noteworthy in this regard and will guide the course of future investigation. The first set of results points to the possible participation of both Casp and TER94 during ubiquitin-dependent protein degradation.

A regulatory network incorporating Bru1, Cup, Oskar and Smaug are key to PGC specification. The Skp Cullin and F-box (SCF) containing complex marks proteins such as Smaug for degradation by functioning as an E3 ubiquitin ligase ((Cao *et al*. 2020; Cao *et al*. 2022); **Fig. 10A**). The ubiquitinated Smaug is then degraded by the proteasome. In the *mammalian* context, the Casp/TER94 orthologs FAF1/VCP (in *green* font; **Fig. 10A**) have been found to interact with the SCF complex. Our data supports a similar scenario in *Drosophila*.

It would be reasonable to propose that Casp recruits TER94 via Casp's UBX domain (**Fig. 10B**). Independent proteomic studies ((Thomson *et al*. 2008; Dehaan *et al*. 2017; Tendulkar *et al*. 2022); **Fig 10 B**) suggest multiple overlaps between Casp/TER94 and germ-cell specific protein complexes (**Fig. 10B**), again suggesting functional relationships between Casp/TER94 and germ cell determinants. In addition, the SCF complex contributes to cell cycle progression and integrity of centrosomes ((Wojcik *et al*. 2000; Murphy 2003; Phuong Thao *et al*. 2006); **Fig 10C**). Total PGC count in early embryos depends on centrosome function and efficient mitotic divisions. So SCF complex based degradation may have multiple targets that participate during germ cell formation and specification (**Fig. 10C**).

Casp likely functions as an adapter protein that works in conjunction with the SCF complex ((Cao *et al*. 2020; Cao *et al*. 2022); **Fig. 10D**) and TER94 (**Fig. 10D**). The binary complex between TER94 and Casp subsequently targets ubiquitinated proteins such as Smaug which are present in a complexed form with other proteins. Homeostatic maintenance of Smaug levels via Casp-mediated proteolytic degradation could explain many of our observations. Consistently, increased Smaug levels, due to diminished Casp function leads to loss of PGCs whereas, *smaug* mutant embryos show the opposite phenotype (Siddiqui *et al*. 2023). Intriguingly, even though Smaug and Oskar are members of a complex, the reduction in Casp activity results in the stabilization of Smaug. By contrast, Oskar levels are diminished resulting in loss of PGCs. The mechanism underlying the substrate-dependent divergent activities is not known at this point.

The similarity between the phenotypic consequences due to loss of ZGA components and Casp/TER94 also needs a special mention. The only study implicating TER94 during early embryogenesis (Zeng *et al*. 2014) suggested that TER94 can potentiate BMP signaling. Interestingly, *decapentaplegic* (*dpp*), a BMP ligand which is one of the important targets of ZGA regulators including *Zelda*, can also influence the specification of embryonic PGCs (Colonnetta *et al*. 2023). PGCs need BMP signals (Dpp) to maintain their identity.

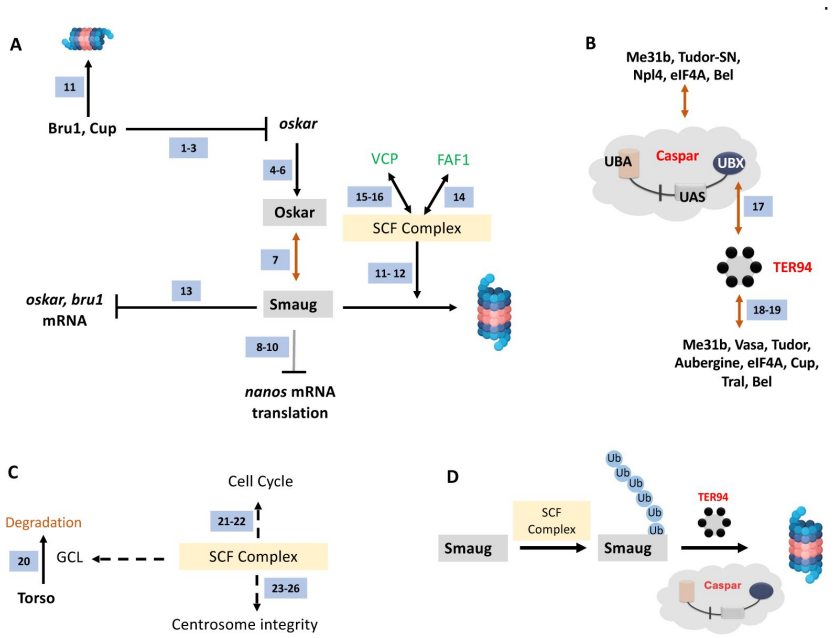

Figure 10.

A model for Casp function in PGCs.

(**A**) Smaug is marked for degradation by the SCF complex. Specific pole plasm components including Cup, Oskar and Smaug are actively degraded. *oskar* is regulated by Bru1 and Cup (*ref 1-3*; ($N_{AKAMURA}$ *et al*. 2004; K_{IM} *et al*. 2015; B_{AYER} *et al*. 2023)). Oskar (*ref 4-6*; ($M_{AHOWALD}$ 2001; $H_{UYNH\ AND}$ S_T $J_{OHNSTON}$ 2004; L_{EHMANN} 2016)) is the master determinant of PGC fate, and hence the stability of the Oskar:Smaug complex (*ref 7*;($K_{UBIKOVA}$ et al. 2023)) is a key to PGC determination and proper specification. Oskar also regulates posterior cell fate by regulating *nos* translation which is modulated by Smaug (*ref 8-10;* ($D_{AHANUKAR}$ et al. 1999; $Z_{AESSINGER}$ et al. 2006; J_{ESKE} et al. 2011)). The SCF complex is a multi-protein E3 ubiquitin ligase complex, and Smaug is one of its prominent targets (*ref 11-12;* (C_{AO} et al. 2020; C_{AO} et al. 2022)). Smaug appears to repress the translation of *oskar* and *Bruno 1* mRNA (*ref 13*; ($S_{IDDIQUI}$ *et al*. 2023)). In mammals (marked with green font), FAF1 modulates the SCF complex (*ref 14*; (M_{ORAIS}-$_{DE}$-S_A *et al*. 2013)) with VCP/p97 assisting in the degradation of ubiquitinated Smaug (*ref 15-16;* (L_I et al. 2014; R_{EIM} et al. 2014)), suggesting potential conservation of the activities in the fly orthologs, Casp and TER94.

(**B**) Casp/TER94 interact with an overlapping set of proteins. TER94 has earlier been demonstrated to be associated with Oskar (R_{UDEN} *et al*. 2000). Our data points to an association between *Drosophila* Casp and TER94 (this study and *ref 17;* ($T_{ENDULKAR}$ et al. 2022)) that participates in protein degradation. In a proteomic analysis of germ cell components, TER94 was identified (this study and *ref 18*; (T_{HOMSON} *et al*. 2008)), along with other bona-fide germ cell constituents including Cup, Tral, Bel, eIF4A, Tud and Vasa (*ref 18* and *19* (T_{HOMSON} *et al*. 2008; D_EH_{AAN} *et al*. 2017)). Both, Casp and TER94 are thus enriched in the pole cells and interact with proteins that specify PGC fate.

(**C**). The SCF Complex, Gcl and Centrosome integrity. The SCF complex is localized to the centrosome. Gcl interacts with Cullin 3 to degrade Torso receptor to promote PGC fate (*ref 20*; (P_{AE} *et al*. 2017)). The SCF complex regulates the cell cycle (*ref 21-22*; ($M_{ARGOTTIN}$-G_{OGUET} *et al*. 2003; R_{OGERS} *et al*. 2009)) and is a known regulator of centrosomal integrity (*ref 23-26*;(W_{OJCIK} *et al*. 2000; M_{URPHY} 2003; P_{HUONG} T_{HAO} *et al*. 2006; C_{UNHA}-$F_{ERREIRA}$ *et al*. 2009)), thereby influencing PGC specification (L_{ERIT} *et al*. 2017).

(**D**) Casp and TER94 assist in the degradation of Smaug. The SCF complex works as a ubiquitin E3 ligase to mark Smaug for Degradation and PolyUb-Smaug is degraded by the action of Casp and TER94 during the late-MZT.

Furthermore, the exposure to BMP signals needs careful calibration as excess BMP signaling in the surrounding soma leads to PGC loss. Importantly, components of protein degradation machinery including those involved in ubiquitination (Smurf, a Ubiquitin ligase) and sumoylation (Ubc9) appear to be involved in fine-tuning the signaling (Deshpande *et al*. 2014). Thus, it will be of interest to determine if the aberrant germ cell specification observed due to loss of *casp* and the components of BMP signaling pathway is mechanistically connected. This seems especially relevant in the light of their respective dependence on the components of the protein degradation machinery. It will be of considerable interest to investigate whether and how, zygotic activity of Smaug, one of important canonical MBT regulators, fits into this picture. In sum, while the specific details await detailed examination, it is apparent that possible recapitulation of maternal regulation in the zygotic context may be a recurrent theme rather than an isolated anomaly.

Materials & Methods

Fly husbandry and stocks

Flies were raised on standard cornmeal agar at 25 °C unless stated otherwise. *Casp^{lof}* (11373), *Casp Df* (23691), *nos Gal4* (4937), *Mat α4-tubulinGal4:VP16* (7063) and *TER94 RNAi* (32869) lines were procured from the Bloomington *Drosophila* Stock Centre, with numbers in brackets indicated Stock numbers.

Cloning of *casp* deletion constructs and generation of transgenic

UASp-casp$^{\Delta}$ constructs (*pUASp casp$^{\Delta UBA}$*, *pUASp casp$^{\Delta UAS}$*, *pUASp casp$^{\Delta UBX}$*, *pUASp casp$^{\Delta UAS\Delta UBX}$*) were generated, starting from a *pUASp-attB-Casp* construct (Tendulkar *et al*. 2022), with the design including an N-terminal HA tag. The constructs were injected into a *w^{1118}; Attp2* embryo in the NCBS-CAMP transgenic injection facility (*https://www.ncbs.res.in/research-facilities/drosophila-services*), and stable lines were generated by balancing against a *w^{1118}; TM3Sb/TM6Tb* animal.

For cloning and amplification, a functional N-terminal HA tag was introduced with the 5'-forward primers and a 3'-homology arm was introduced using the 3'-reverse primers. The exception was the *pUASp Casp$^{\Delta UAS}$* construct where two fragments, one upstream and one downstream of the UAS domain were amplified separately with 22 nucleotide homologous overhang between them to mediate homologous recombination. The upstream fragment contained the HA sequence while the downstream fragment contained the 3'-homology arm.

The sequences of the primers used are as follows. *TGTTCCAGATTACGCTGGCGGC* was used as the 5'-forward primer for the *pUASp casp^{wt}*, *pUASp casp$^{\Delta UBX}$*, *pUASp casp$^{\Delta UAS\Delta UBX}$* and the upstream fragment of the *pUASp casp$^{\Delta UAS}$* constructs.

TGTTCCAGATTACGCTGGCGGCCCCCATCCTATCCTGGTGCC was used as the 5'-forward primer for the *pUASp casp$^{\Delta UBA}$* construct.

CGGATGATGAGATAAGTGGCTCCACGGAAACATGCGAAATGTTTGAGGAGCAG was used as forward primer for amplifying downstream fragment of the *pUASp casp$^{\Delta UAS}$* construct.

ACCATGGGTTTAGGTATAATGTTATCAAGCTCC was the reverse primer for *pUASp casp^{wt}*, *pUASp casp$^{\Delta UBA}$*, and downstream fragment of *pUASp casp$^{\Delta UAS}$*.
TTAGGTATAATGTTATCAAGCTCCTCATTCGGACGGCTCCTGAGGTAG was the reverse primer for *pUASp casp$^{\Delta UBX}$*.

TTAGGTATAATGTTATCAAGCTCCTCACGTGGAGCCACTTATCTCATCATCCG was used as the reverse primer for *pUASp casp$^{\Delta UAS\Delta UBX}$*.

CGTGGAGCCACTTATCTCATCATCCG was the reverse primer for upstream fragment of *pUASp casp$^{\Delta UAS}$*. PCR products were further amplified with a common 5'-primer *ATAGGCCACTAGTGGATCTGATGTACCCATACGATGTTCCAGATTACGCTGGCGGC* and their respective reverse primers to introduce the 5'-homology arm. Inserts were recombined into *pUASp AttB* vector linearised at the BamH1 site, using a variation of the SLiCE cloning method (Zhang *et al*. 2012; Zhang *et al*. 2014). In short, E.coli DH10B expressing the optimised λ-prophage red recombinase system (PPY cells) was cultured in the presence of arabinose to induce recombinase expression and subsequently used for preparing competent cells. DNA fragments containing homologous overhangs were co-transformed into the competent bacteria and resultant colonies were screened through PCR to identify proper recombinants and subsequently confirmed through sequencing.The pUASp *casp$^{\Delta FFAT}$*was PCR amplified from *pRM-HA:casp $^{\Delta FFAT}$*(Tendulkar *et al*. 2022), cloned into pUASp-AttB, sequenced for validation and injected into a *w^{1118}; Attp2* animal.

Embryonic lethality

0-3 hr embryos were collected, transferred to a fresh sugar-agar plate, and unhatched larvae were scored after 48 hours to determine viability.

Immunoprecipitatio

0–3-hour embryos were lysed in Co-IP Lysis Buffer (20mM Tris pH 8.0, 137mM NaCl, 1% IGEPAL, 2mM EDTA, 1X PIC) using a Dounce homogenizer, and centrifuged at 21,000 g for 30 minutes. 3 mg of total lysate was incubated with 5 µg of primary antibody (Rb anti-Casp) and 5 µg of Normal Rabbit IgG overnight at 4 °C. Antigen-antibody complexes were captured using 50 µL of BioRad SureBeads Protein A (1614013) at 4 °C for 4 hours. Beads were washed six times with Co-IP Lysis Buffer and protein complexes eluted by boiling in 1X Laemmli Sample Buffer. Eluted proteins were resolved on a 10% polyacrylamide gel followed by western blotting or in-gel trypsin digestion, described in the following sections.

Western blot analysis

Embryos collected at varied time points (0-3, 0-1, 1-2, 2-3 hours) were lysed in RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 0.1% SDS, 0.01% Sodium azide, 0.5% sodium deoxycholate, 1 mM EDTA, 1% Triton X-100, 1X PIC) with a pellet pestle (Kontes). Lysates were cleared by centrifugation at 21,000 g at 4 °C for 30 minutes. Protein concentration was estimated using a BCA assay (Pierce) and 30-40 µg of total protein was loaded onto the gel after boiling in 1X Laemmli Sample Buffer. Proteins separated by 10% SDS-PAGE were transferred onto a PVDF membrane (Immobilon-E, Merck) and blocked in 5% milk in Tris-Buffer Saline (TBS) with 0.1% Tween 20 (TBS-T) for an hour. Blots were then incubated overnight with primary antibody diluted in 5% milk in TBS-T, at 4 °C. Following three washes with TBS-T, blots were incubated with secondary antibodies diluted in 5% milk in TBS-T, for 1 hour at room temperature. Blots were washed thrice with TBS-T and visualized on a LAS4000 Fuji imaging system after incubating with Immobilon Western Chemiluminescent HRP substrate (Merck). The following antibodies were used: Rabbit anti-VAP, 1:10000 (Tendulkar *et al*. 2022), Mouse anti-α-Tubulin, 1:10000 (T6074, Sigma), Mouse anti-Ubiquitin, 1:1000 (P4D1, Santa Cruz Biotechnology), Mouse anti-HA, 1:5000 (H3663 HA-7, Sigma-Aldrich), Rabbit anti-Casp, 1:10000 (Tendulkar *et al*. 2022), 1:2000 (04-902 DW-2, Sigma-Aldrich) Goat anti-rabbit HRP and Goat anti-mouse HRP secondary antibodies, each at 1:10000 (Jackson ImmunoResearch). Rabbit anti-me31B, 1:1000, Rabbit anti-Smaug, 1:500, Rat anti-Tral, 1:1000 were a kind gift from Elmar Whale (Cao *et al*. 2020).

In-gel Trypsin Digestion and LC-MS/MS Analysis

Before in-gel trypsin digestion of the Co-IP eluate, the antibody was crosslinked to the SureBeads using DMP (Sigma) according to the NEB crosslinking protocol to avoid elution of the antibody. After crosslinking 10µg Casp antibody, Co-IP was performed as described above. In-gel trypsin digestion was carried out as previously described (SHEVCHENKO et al. 2006). Briefly, Coomassie-

stained bands on the gel were excised and cut into 1 mm cubes. Gel pieces were transferred to a clean microcentrifuge tube and destained with buffer containing 50% acetonitrile in 50 mM Ammonium bicarbonate.

Reduction and alkylation were carried out on the destained gel pieces by incubating with 10mM dithiothreitol (DTT) followed by incubating with 20mM iodoacetamide. Gel pieces were saturated with sequencing grade Trypsin (Promega) at a concentration of 10 ng/µL and incubated overnight at 37 °C. Peptides were extracted by sequential addition of 100 µl of 0.4% Trifluoroacetic acid (TFA) in 10% ACN, 100 µl of 0.4% TFA in 60% ACN and 100 µl of ACN. The pooled extract was dried in a vacuum centrifuge and reconstituted with 50 µl of 0.1% TFA. The peptides in TFA were purified using the StageTip protocol (RAPPSILBER et al. 2007). LC– MS/MS analysis was performed on the Sciex TripleTOF6600 mass spectrometer interfaced with an Eksigent nano-LC 425. Tryptic peptides (1 µg) were loaded onto an Eksigent C18 trap (5 µg capacity) and subsequently eluted with a linear acetonitrile gradient on an Eksigent C18 analytical column (15 cm × 75-µm internal diameter). A typical LC run lasted 2 h post loading onto the trap at a constant flow rate of 300 nL/min with solvent A consisting of water + 0.1% formic acid and solvent B consisting of acetonitrile. The gradient schedule for the LC run was 5% (vol/vol) B for 10 min, a linear gradient of B from 0% to 80% (vol/vol) over 80 min, 80% (vol/vol) B for 15 min and equilibration with 5% (vol/vol) B for 15 min. Data was acquired in an information-dependent acquisition (IDA) mode over a mass range of 300–2,000 m/z. Each full MS survey scan was followed by MS/MS of the 15 most intense peptides. Dynamic exclusion was enabled for all experiments (repeat count 1; exclusion duration 6 s). Peptides were identified and quantified using the SCIEX ProteinPilot software at a false discovery rate (FDR) of 1%. A RefSeq *Drosophila* protein database (release 6) was used for peptide identification. Proteins that were identified in two or more replicates were tabulated.

Fixation, immunostaining, and imaging of embryos

0-3 hr embryos were collected in a sieve and dechorionated in 4% sodium hypochlorite for 90 seconds. After thorough washes with distilled water, embryos were fixed in a 1:1 heptane:4% PFA solution for 20 minutes. The PFA layer was removed, and embryos in heptane were re-constituted with an equal volume of methanol. Embryos were devitellinized by vigorous shaking in the 1:1 heptane:methanol mixture. The heptane layer and the interphase containing non-devitellinized embryos were carefully removed. Devitellinized embryos in the bottom methanol phase were washed twice with methanol and stored at -20 °C till they were ready to be imunostained. For phalloidin and Smaug staining, embryos fixed in heptane: 4% PFA were hand de-vitellinized, after which the standard immunostaining procedure was followed. For immunostaining, embryos were rehydrated by washing thrice with 0.3% PBS-TritonX 100 (PBS-T) for 15 minutes each. Embryos were blocked in 2% BSA in 0.3% PBS-T for 1 hour at room temperature (RT). Embryos were incubated at 4 °C overnight with primary antibodies diluted in 2% BSA in 0.3% PBS-T at the appropriate dilutions. Following three 15-minute washes with 0.3% PBS-T, embryos were incubated in the appropriate secondary antibodies for 1 hour at room temperature. The following antibodies were used: Rabbit anti-Casp, 1:1000 (Tendulkar *et al*. 2022); Rat anti-α-vasa; 1:50 (DSHB); Rabbit anti-VCP, 1:200 (#2648, Cell Signaling Technology); Rabbit anti-Oskar, 1:1000 (generated by Mandy Jeske, Anne Ephrussi lab); Rabbit anti-Smaug, 1:50; Mouse anti-γ-tubulin, 1:1000 (T6557, Sigma), Rabbit P-Histone H3 (S10) 1:300 (#9701, Cell Signalling); Alexa Fluor 568 Phalloidin, 1:1000 (Invitrogen) The following secondary antibodies were used, goat anti-mouse Alexa488/ goat anti-rabbit Alexa568/ goat anti-rat Alexa647/ goat anti-rabbit Alexa647/ goat anti-rabbit Alexa480, 1:1000 (DSHB). Embryos were washed thrice with 0.3% PBS-T, and DAPI/Hoechst (1:500) was added in the penultimate wash. Embryos were mounted in 70% glycerol and observed under a Leica sp8 confocal microscope with a 20X objective.

Live imaging of embryos

Embryos at the appropriate stage were washed and dechorionated as described previously. A 2-well Nunc™ Lab-Tek™ II Chamber Slide™ System (ThermoFisher Scientific) was affixed with a 3M Scotch double-sided tape, and dechorionated embryos with intact vitelline membranes were mounted on the tape under a dissecting microscope. Halocarbon oil 200 was used to cover the embryos to prevent dehydration during imaging. Time-lapse imaging of embryos was performed on an inverted LSM confocal system (Zeiss multiphoton 710) at 20X for ~8 hours. Embryo images were acquired at 5 min intervals.

Quantification of western blots

Western blots were quantified using ImageJ. Each protein was normalized to the tubulin loading control, and the highest signal for each was set to 1 and all other tubulin band intensities were normalized against it. For MZT experiments, western blots were used to determine protein degradation dynamics. 3-5 biological replicates, representing 3-5 independent embryo collection were assayed. Band intensities were quantified using ImageJ and normalized to α-tubulin as loading control. For each replicate intensities were normalized to the first time point (0-1 hour). Significance thresholds are presented in the figures and figure captions.

Quantification of the Smaug Immunostaining

All quantification of Smaug stained images were done on ImageJ. ROIs were made for a single pole cell through the z-stacks, thereby only analysing one pole cell at a time. For each embryo five such pole cells were measured. To quantify intensity, mean intensity was measured for each ROI per stack and added to get the final intensity. Each data point on the graph represents an average of all five cell intensities per embryo. For Smaug punctae measurement, a combined ROI for each cell was taken and duplicated. Each of the stack was then analysed using 3D objects counter, where thresholding for all images was kept within the range of 80-90 with a minimum size filter kept at zero. The number of objects counted was taken as the total number of Smaug punctae per cell. Five such pole cells were analysed per embryo and the average was plotted as a single data point on the graph. For quantification of poleplasm volume in the embryos, embryos were stained with Oskar. An ROI (kept same for all images) encompassing the posterior part of the embryo was drawn. Gaussian blur 3D (X sigma = 2, Y sigma = 2 and Z sigma = 2) was applied to each image. The volume of pole plasm was analysed by using 3D objects counter after appropriate thresholding.

Competing interests

The authors declare no competing or financial interests.

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Contributions

SH and SD performed most of the experiments, with contributions from NW, JS, and AR. GR and GD conceptualised the project with input from SH and SD. SH, SD, GR, and GD analyzed and interpreted the data. GD, GR, SH, and SH wrote the manuscript. GR and GD supervised the project and acquired funding.

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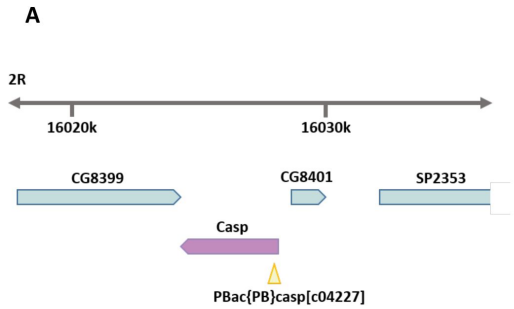
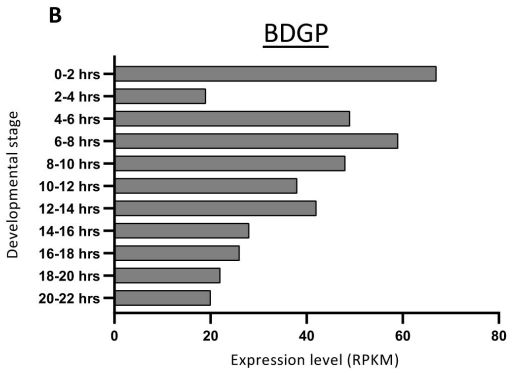


Source: FlyBase (flybase.org)



Source: Brown et. al. (2014), *Nature* 512: 393.

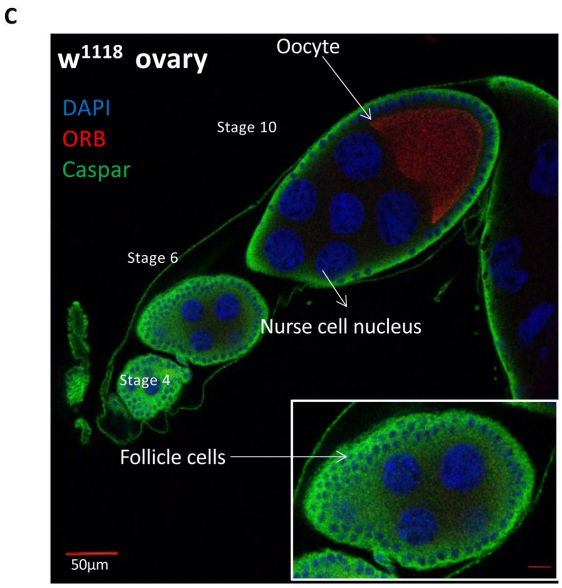


Fig. S1.

(A) Schematic of the the chromosomal location of the *casp* locus and the associated transposon insertion that generates the $casp^{c04227}$ allele. This allele is referred to as $casp^{lof}$ hereafter. **(B)** RNA expression data from the modENCODE database, plotted as a bar chart **(C)**. DAPI marks the nuclei, orb marks the oocyte. Casp marks the follicle cells, but is not significantly enriched in the germline (egg, nurse cells).

A

Protein	Peptide count
casp	73.5
TER94	18
Vap33	2.5
Tudor-SN	2.25
alphaTub85E	2
eIF4A	2
Npl4	2
SERCA	1.75
bel	1.75
CCT2	1.5
mRpS14	1.5
CCT6	1.5
Hacl	1.25
mAcon1	1.25
ATPsyngamma	1.25
mRpL27	1.25
alphaTub67C	1.25
eIF3b	1
ScsbetaA	1
26-29-p	1
lig	1
me31B	1
nocte	1
Ufd1-like	1

C

Protein	Known function in the germline	Reference
TER94	polar granule component; associated with vasa and Tudor	Thomson et al., 2008
Tudor-SN	regulates spermatogenesis	Ku et al., 2016
eIF4A	polar granule component; associated with vasa and Tudor	Thomson et al., 2008
belle	required for female fertility and maintenance of male germline stem cells	Johnstone et al., 2005
CCT6	expressed in primordial germ cells	Fisher et al., 2012
α-Tubulin at 67C	regulation of male germline stem cells, centrosome positioning and segregation	Tao et al., 2021
Lingerer	interacts with germline component vasa	Durdevic and Ephrussi, 2019
me31B	polar granule component; associated with vasa and Tudor	Thomson et al., 2008

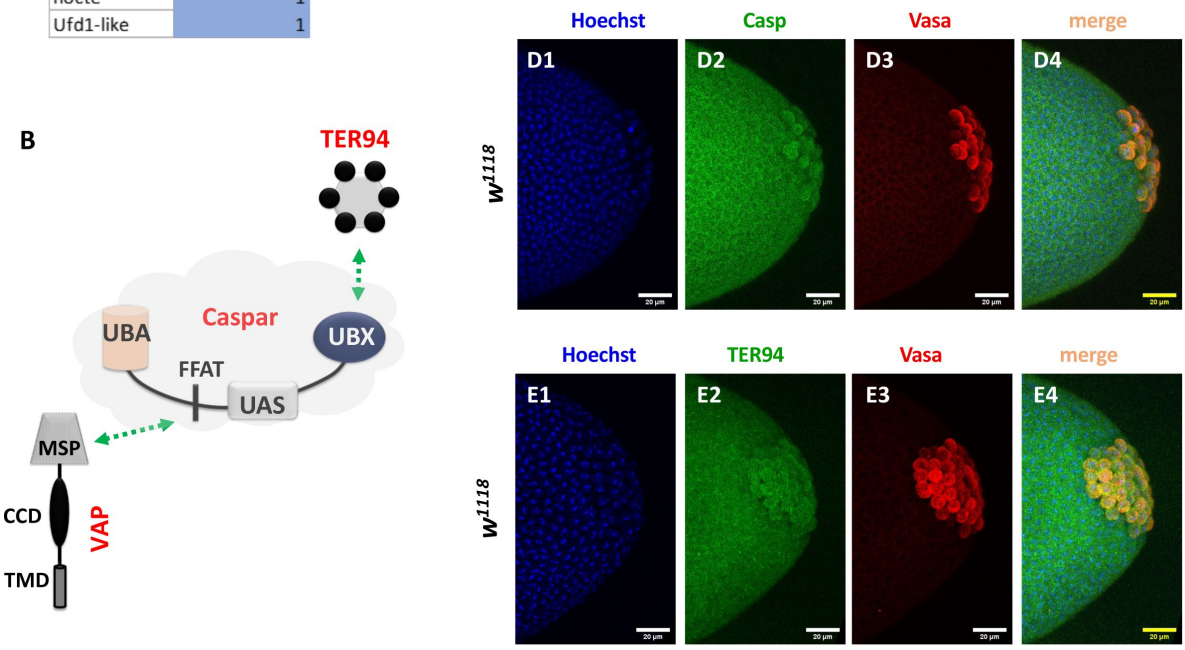


Fig. S3.

TER94 and germline components are major interactors of Casp.

(**A**) shows a list of the 24 proteins enriched after a Casp IP, followed by mass spectrometry, listed in the order of their peptide counts. The peptide counts are averaged from 4 biological replicates. A subset of interactors with known functions or expression in the germline are tabulated in (**B**) A schematic of the interaction of Casp with both TER94 and VAPB (Tendulkar et al., 2022). TER94 interacts with the Casp UBX domain, while VAPB with the FFAT motif (**C**) List of proteins that are associated with Casp and are previously identified as pole-cell components (**D-E**) Immunostaining of wild-type nuclear cycle 13/14 embryos with Casp (D2) and TER94-specific (E2) antibodies indicates expression in the

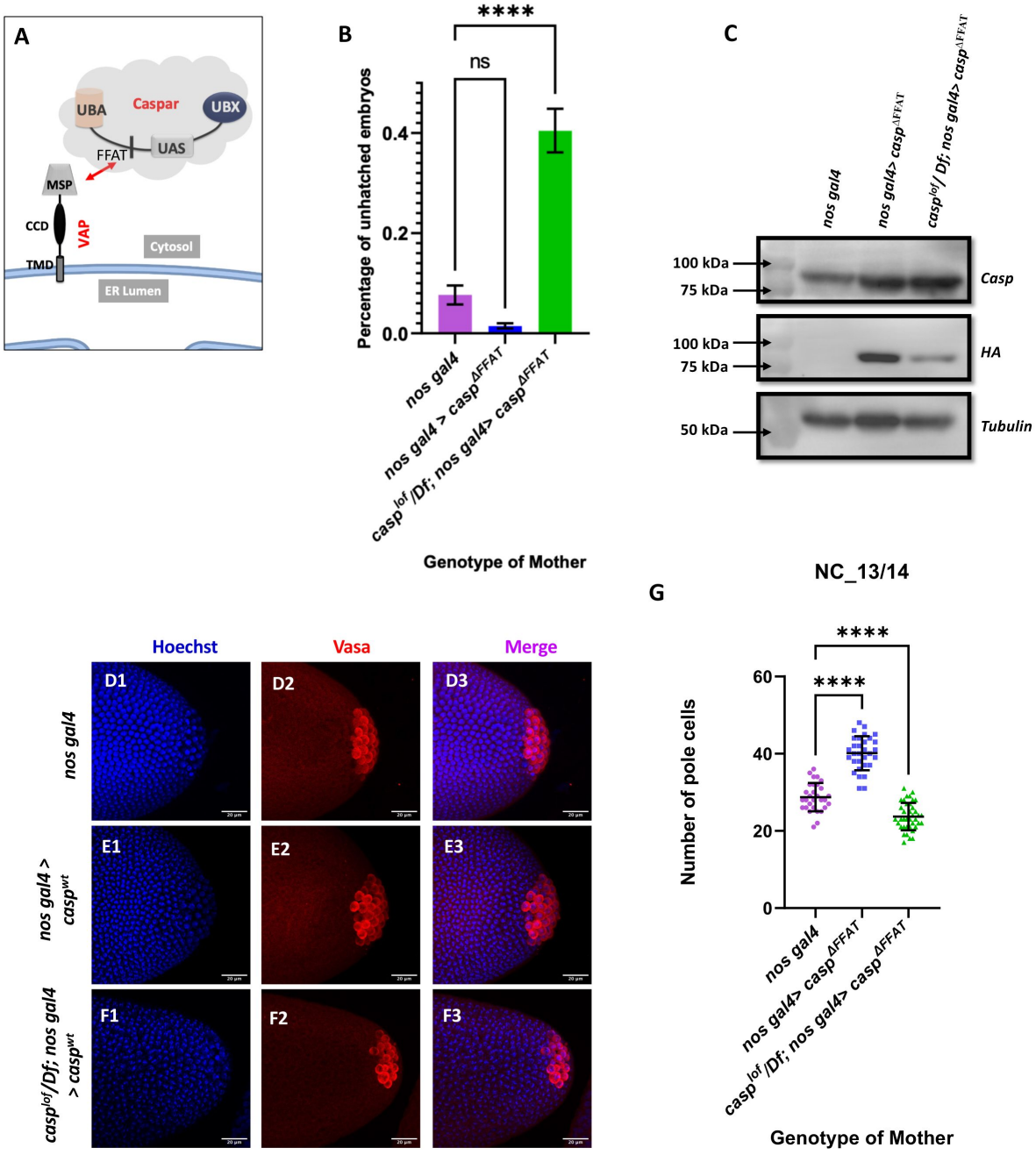


Fig S4.

Casp:VAP interaction is not required for defining pole cell number.

(**A**) Casp interacts with VAPB via its FFAT-like motif (**B**) represents embryonic lethality at 25° C plotted as a bar graph. The genotype of the mother is listed on the X-axis. N = 3. n = 200, ordinary one-way ANOVA, (****) P <0.0001. (**C**) Casp protein levels were assessed in 0-3h embryos via western blotting. Rabbit anti Casp (1:10,000) and rabbit anti HA (1:2000) antibodies were used to probe the blot. Mouse anti Tubulin (1:10,000)is used as a loading control. (**D-F**) Confocal microscopy images of the posterior of nuclear cycle 13/14 embryos from mated *nos gal4* (D1-D3), *nos gal4> casp$^{\Delta FFAT}$* (E1-E3), and *casp^{lof}/Df; nos gal4> casp$^{\Delta FFAT}$* (F1-F3) females immunostained with vasa antibody (1:50). Hoechst marks the nuclei. (**G**) The germ cells at the posterior marked by vasa quantified and plotted as bar graphs. n = 30-40.

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Editors

Reviewing Editor
Michael Buszczak
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Senior Editor
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Reviewer #1 (Public review):

Summary:

The authors were seeking to define the roles of the Drosophila caspar gene in embryonic development and primordial germ cell (PGC) formation. They demonstrate that PGC number, and the distribution of the germ cell determinant Oskar, change as a result of changes in caspar expression; reduction of caspar reduces PGC number and the domain of Oskar protein expression, while overexpression of caspar does the reverse. They also observe defects in syncytial nuclear divisions in embryos produced from caspar mutant mothers. Previous work from the same group demonstrated that Caspar protein interacts with two partners, TER94 and Vap33. In this paper, they show that maternal knockdown of TER94 results in embryonic lethality and some overlap of phenotypes with reduction of caspar, supporting the idea may work together in their developmental roles. The authors propose models for how Caspar might carry out its developmental functions. The most specific of these is that Caspar and its partners might regulate oskar mRNA stability by recruiting ubiquitin to the translational regulator Smaug.

Strengths:

The work identifies a new factor that is involved in PGC specification and points toward an additional pathway that may be involved in establishing and maintaining an appropriate distribution of Oskar at the posterior pole of the embryo. It also ties together earlier observations about the presence of TER94 in the pole plasm that have not heretofore been linked to a function.

Weaknesses:

(1) A PiggyBac insertion allele casp[c04227] is used throughout the paper and referred to as a loss-of-function allele (casp[lof]). While the authors avoid the terms 'null' or 'amorph' and on one occasion refer to the allele as a 'strong hypomorph', nevertheless terming it a 'loss-of-function' allele is misleading. This is because the phenotype of the allele when homozygous is different from the phenotype produced when heterozygous over a deficiency.

(2) The peptide counts in the mass spectrometry experiment aimed at finding protein partners for Casp are extremely low, except for Casp itself and TER94. Peptide counts of 1-2 seem to me to be of questionable significance.

(3) The pole bud phenotypes from TER94 knockdown and casp mutant shown in Fig 5 appear to be quite different. These differences are unexplained and seem inconsistent with the model proposed that the two proteins work in a common pathway. Whole embryos should also be shown, as the TER94 KD phenotype could result from a more general dysmorphism.

(4) Fig 6 is not quantitative, lacking even a second control staining to check for intensity variation artifacts. Therefore it shows that the distribution of Oskar protein changes in the various genotypes, but not convincingly that the level of Oskar changes as the paper claims.

(5) The error bars are huge in the graphs in Fig 7H, I, and J, and in fact these changes are not statistically significant. Therefore the conclusion that 'Reduction in Casp activity specifically affects Smaug degradation during the MZT' is not supported by the data in this figure.

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Reviewer #2 (Public review):

Summary:

This study investigated the role of the Caspar (Casp) gene, a Drosophila homolog of human Fas-associated factor-1. It revealed that maternal loss of Casp led to centrosomal and cytoskeletal abnormalities during nuclear cycles in Drosophila early embryogenesis, resulting in defective gastrulation. Moreover, Casp regulates PGC numbers, likely by regulating the levels of Smaug and then Oskar. They demonstrate that Casp protein levels are linearly correlated to the PGC number. The partner protein TER94, an ER protein, shows similar but slightly distinct phenotypes. Based on the deletion mutant analysis, TER94 seems functionally relevant for the observed Casp phenotype. Additionally, it is likely involved in regulating protein degradation during PGC specification.

Strengths:

This paper uncovers a new function of the Casper (Casp) gene, previously known for its role in immune response regulation and NF-kB signaling inhibition. This new function includes nuclear division and PGC formation in early fly embryos. The findings provide crucial insights into how this pathway contributes to the proper establishment of both somatic cells and the germline, particularly in the context of early embryogenesis. This research is therefore of significant interest to cell and developmental biologists.

Future Research:

While this study has made significant strides in understanding the role of the Casp gene in early embryogenesis, the functional relationships among molecules shown here (Casp, TER94, Osk) and other genes previously known to regulate these processes remain unclear. This underscores the need for future studies to delve deeper into these relationships and their implications.

https://doi.org/10.7554/eLife.98584.2.sa2

Reviewer #3 (Public review):

Summary:

Das et al. discovered a maternal role for Caspar (Casp), the Drosophila orthologue of human Fas-associated factor-1 (FAF1), in embryonic development and germ cell formation. They find that Casp interacts with Transitional endoplasmic reticulum 94 (TER94). Loss of Casp or TER94 leads to partial embryonic lethality, correlated with aberrant centrosome behavior and cytoskeletal abnormalities. This suggests that Casp, along with TER94, promotes embryonic development through a still unidentified mechanism. They also find that Casp regulates germ cell number by controlling a key determinant of germ cell formation, Oskar, through its negative regulator, Smaug.

Strengths:

Overall, the experiments are well-conducted, and the conclusions of this paper are mostly well-supported by data.

Weaknesses:

Some additional controls could be included, and the language could be clarified for accuracy.

https://doi.org/10.7554/eLife.98584.2.sa1

Author response:

The following is the authors' response to the original reviews.

> *This study investigates the role of Caspar (Casp), an orthologue of human Fas- associated factor-1, in regulating the number of primordial germ cells that form during Drosophila embryogenesis. The findings are important in that they reveal an additional pathway involved in germ cell specification and maintenance. The evidence supporting the conclusions is solid, as the authors identify Casp and its binding partner Transitional endoplasmic reticulum 94 (TER94) as factors that influence germ cell numbers. Minor changes to the title, text, and experimental design are recommended.*

We thank the Editors and Reviewers for their overall positive and thoughtful feedback. Based on these comments, we have revised our manuscript. The changes in the manuscript have been highlighted in 'blue' font for easy visualisation.

> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *The authors were seeking to define the roles of the Drosophila caspar gene in embryonic development and primordial germ cell (PGC) formation. They demonstrate that PGC number, and the distribution of the germ cell determinant Oskar, change as a result of changes in caspar expression; reduction of caspar reduces PGC number and the domain of Oskar protein expression, while overexpression of caspar does the reverse. They also observe defects in syncytial nuclear divisions in embryos produced from caspar mutant mothers. Previous work from the same group demonstrated that Caspar protein interacts with two partners, TER94 and Vap33. In this paper, they show that maternal knockdown of TER94 results in embryonic lethality and some overlap of phenotypes with reduction of caspar, supporting the idea may work together in their developmental roles. The authors propose models for how Caspar might carry out its developmental functions. The most specific of these is that Caspar and its partners might regulate oskar mRNA stability by recruiting ubiquitin to the translational regulator Smaug.*
>
> *Strengths:*
>
> *The work identifies a new factor that is involved in PGC specification and points toward an additional pathway that may be involved in establishing and maintaining an appropriate distribution of Oskar at the posterior pole of the embryo. It also ties together earlier observations about the presence of TER94 in the pole plasm that have not heretofore been linked to a function.*
>
> *Weaknesses:*
>
> *(1) A PiggyBac insertion allele casp[c04227] is used throughout the paper and referred to as a loss-of-function allele (casp[lof]). However, this allele does not appear to act strictly as a loss-of-function. Figure 1E shows that some residual Casp protein is present in early embryos produced by casp[lof]/Df females, and this protein is presumably functional as the PiggyBac insertion does not affect the coding region. Also, Figures 1B and 1C show that the phenotypes of casp[lof] homozygotes and casp[lof]/Df are not the same; surprisingly, the homozygous phenotypes are more severe. These observations are unexplained and inconsistent with the insertion being simply a loss-of-function allele. Might there be a second-site mutation in casp[c04227]?*

The term loss-of-function (lof) is used rather than null or amorph. *casplof* is a strong hypomorph, with residual (and functional) protein estimated in the range 5-10% when

compared to the wild type. The *caspc04227* was procured from BDSC, and based on the decrease in lethality of the *casplof/casp(Df)* compared to *casplof*, we assume that second site hits in the *casplof* line are the reason for the enhanced lethality. For this very reason, we have used *casplof/ casp(Df)* for all subsequent experiments. We also conducted rescue experiments wherever possible to confirm the specificity with *caspWT* and various deletion variants of *casp*.

> *(2) TER94 knockdown phenotypes have been previously published (Zhang et al 2018 PMID 30012668), and their effects on embryonic viability and syncytial mitotic divisions were described there. This paper is inappropriately not cited, and the data in Figure 4 should be presented in the context of what has been published before.*

We apologize for the oversight. Indeed, Zhang *et al.* (2018) highlighted TER94 as one of the loci uncovered in their screen and some of the relevant phenotypes are described there. We have referred to their findings at the appropriate junctures as suggested (pg 11, pg13, pg 15).

> *(3) The peptide counts in the mass spectrometry experiment aimed at finding protein partners for Casp are extremely low, except for Casp itself and TER94. Peptide counts of 1-2 seem to me to be of questionable significance.*

Peptide counts are indeed low, but the fact that they are enriched at all, in comparison to controls, considering that we are using whole embryo lysates rather than isolated PGC lysates, suggests interaction with Casp could be biologically/ functionally meaningful. The data is restricted to the supplementary material and is not analyzed in isolation; we have combined data from multiple mass spectrometry experiments by other researchers to link Casp to pole plasm components.

> *(4) The pole bud phenotypes from TER94 knockdown and casp mutant shown in Fig 5 appear to be quite different. These differences are unexplained and seem inconsistent with the model proposed that the two proteins work in a common pathway. Whole embryos should also be shown, as the TER94 KD phenotype could result from a more general dysmorphism.*

We agree that TER94 KD is a stronger phenotype, with TER94 having essential cell division and patterning roles. In fact, the *TER94* RNAi embryos, unlike *casplof*, stall in terms of their developmental program before Stage 4. This has been noted in the earlier study (Zhang et al., 2018). As a result, we focused on pole bud stage embryos that were rare - but present in the collections. We report that PGC from very early *TER94 RNAi* embryos have fewer pole buds.

The rationale behind the presumption that these two proteins may work in a common pathway is clear-cut. We have validated the physical interaction using protein lysates from two developmental time points. Satisfyingly, an affinity purification using antibodies against TER94 or Casp invariably enriches the other protein as the primary interacting partner. Our model integrates data from mammalian and fly systems to support the idea that there must be an overlap between TER94/Casp function, with these two proteins working together to engineer the degradation of ubiquitinated Smaug. Future experiments are necessary to confirm and extend this claim.

> *(5) Figure 6 is not quantitative, lacking even a second control staining to check for intensity variation artifacts. Therefore, it shows that the distribution of Oskar protein changes in the various genotypes, but not convincingly that the level of Oskar changes as the paper claims.*

We appreciate that *oskar* RNA localization is also somewhat altered due to change in *casp* levels. We have acknowledged the variability in the various phenotypes, and as such, it is unsurprising that it has also reflected in the Oskar levels. However, it is evident that a statistically significant number of mutant embryos show a decrease in Oskar levels.

> *(6) The error bars are huge in the graphs in Figure 7H, I, and J, leading me to question whether these changes are statistically significant. Calculations of statistical significance are missing from these graphs and need to be added.*

The data in the Western blots represents the whole embryo, as the lysates used are from embryos 0-1, 1-2, 2-3 hrs. We have averaged and plotted data from 5 Western blots. The changes are not statistically significant. Even without the statistical significance, the data for Fig. 7I led us to examine Smaug in the pole cells, rather than in the whole embryo. The pole cell data (Fig8-D3) is striking and led to the conclusion – that Smaug protein perdures in the pole cells during the stages of syncytial/cellular blastoderm.

> *(7) There are many instances of fuzzy and confusing language when describing casp phenotypes. For example, on lines 211-212, it is stated that 'casp[lof] adults are only partially homozygous viable as ~70% embryos laid by the homozygous mutant females failed to hatch into larvae'. Isn't this more accurately described as 'casp[c04227] is a maternal-effect lethal allele with incomplete penetrance'? Another example is on line 1165, what exactly is a 'semi-vital function'?*

We thank the reviewer for reading the manuscript in detail. We have tried to pay attention to reduce the ambiguity and fixed the text accordingly (pg 7, line 214; pg 33, line 1169, word semi-vital is deleted).

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *This study investigated the role of the Caspar (Casp) gene, a Drosophila homolog of human Fas-associated factor-1. It revealed that maternal loss of Casp led to centrosomal and cytoskeletal abnormalities during nuclear cycles in Drosophila early embryogenesis, resulting in defective gastrulation. Moreover, Casp regulates PGC numbers, likely by regulating the levels of Smaug and then Oskar. They demonstrate that Casp protein levels are linearly correlated to the PGC number. The partner protein TER94, an ER protein, shows similar but slightly distinct phenotypes. Based on the deletion mutant analysis, TER94 seems functionally relevant for the observed Casp phenotype. Additionally, it is likely involved in regulating protein degradation during PGC specification.*
>
> *Strengths:*
>
> *The paper reveals an unexpected function of the maternally produced Casp gene, previously implicated in immune response regulation and NF-kB signaling inhibition, in nuclear division and PGC formation in early fly embryos. Experiments are properly conducted and strongly support the conclusion. The rescue experiment using deletion mutant form is particularly informative as it suggests the requirement of each domain function.*
>
> *Weaknesses:*
>
> *Functional relationships among molecules shown here (and other genes known to regulate these processes) are still unclear.*

We completely agree with this assessment. In our view this is an interesting albeit initial report. We also appreciate that understanding the mechanistic underpinnings of these results will be critical. We have ensured that our present claims are backed up by data, however, are fully sensitive to the fact that newer observations will refine or even alter these claims. We are continuing to work on the problem and will hopefully make further inroads in mechanism in the coming years.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *Das et al. discovered a maternal role for Caspar (Casp), the Drosophila orthologue of human Fas-associated factor-1 (FAF1), in embryonic development and germ cell formation. They find that Casp interacts with Transitional endoplasmic reticulum 94 (TER94). Loss of Casp or TER94 leads to partial embryonic lethality, correlated with aberrant centrosome behavior and cytoskeletal abnormalities. This suggests that Casp, along with TER94, promotes embryonic development through a still unidentified mechanism. They also find that Casp regulates germ cell number by controlling a key determinant of germ cell formation, Oskar, through its negative regulator, Smaug.*
>
> *Strengths:*
>
> *Overall, the experiments are well-conducted, and the conclusions of this paper are mostly well-supported by data.*
>
> *Weaknesses:*
>
> *Some additional controls could be included, and the language could be clarified for accuracy.*
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) The paper is inconsistent in using standard Drosophila nomenclature. Often the name of the mammalian counterpart is used instead. This needs to be cleaned up as it is very confusing to the reader.*

The names of the mammalian counterpart are explicitly used, when we intended, to underscore the parallels between mammalian vs *Drosophila* function, specifically in the context of the major players in this study, TER94 vs VCP; Caspar vs FAF1. Since we do not have direct biochemical data indicating that TER94/Casp degrades Smaug, we use published mammalian literature to draw parallels. At no point have we swapped terminology casually.

> *(2) The Discussion is far too long and in my view extends too far beyond the experimental data in the paper. As a start for editing, its first two paragraphs (lines 1138-1164) include mostly general statements and could be greatly reduced or eliminated.*

Our aim was to emphasize the repurposing of factors between early development and later/adult stages for different functional contexts. Our laboratory (Ratnaparkhi) works on Casp in terms of its roles in NF-kappa B signalling. We serendipitously stumbled on the embryonic lethality while characterizing the *casplof* allele, which, later, led us to examine the function of Casp during embryonic germ cell development.

> *(3) The Introduction is weak in its description of the developmental function of Toll and Dorsal. This could be summarized in a sentence or two.*

As suggested, a few sentences that highlight the developmental function of Toll/Dorsal signalling have been added to the text (pg 3, line 90-92).

> *(4) Even if correctly cited, it is not appropriate to simply reproduce an image from a public database, as was done in Figure S1C. This should be removed.*

Figure S1C has been deleted.

> *(5) The Materials and Methods section should be moved to after the Discussion so it does not interrupt the flow of the Results.*

The Section has been moved as suggested.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *For general readers, more detailed information about the PGC specification will be helpful in the Introduction or Results section.*

PGC specification is introduced in the text as the story transits from global embryonic effects of *casp* knockdown to specific effects on PGCs. A few additional sentences have been added to bolster the text (pg 11, first paragraph).

> *The Methods section talks about live imaging, but I could not find the experiments in the figures. Are the data available for asynchronous nuclear divisions in the live imaging?*

The live imaging relates to DIC movies that are part of Suppl. Fig 2A. The movies are embedded in an MS PowerPoint slide, which has been uploaded as a PowerPoint (and not a PDF).

> *To ensure that the mutant changes the Osk translation rate, showing the Osk RNA level may be helpful.*

oskar RNA localization is quite distinct as compared to Oskar and Vasa protein. It has been shown that *oskar* RNA is localized to the founder granules and is, in fact, excluded from the germ granules that contain Vasa, Oskar and *nos* RNA etc. Gavis lab recently reported (Eichler *et al.*, 2020) that ectopic localization of *osk* RNA in the germ granules is toxic to pole cells. Thus, it will be of interest to analyze whether and how *oskar* RNA is localized in *casp* embryos.

> *More discussion about the difference between Casp and ter94 phenotypes and potential reasons would be informative.*

TER94 appears to be an essential maternal gene. Hypomorphic knockdown of TER94 using RNAi is sufficient to induce early embryonic lethality. In fact, Zhang *et. al.*, 2018 et al., using stronger/earlier maternal drivers highlighted the lethality and somatic cell division defects caused due to the severe loss of TER94. The UBX domain is present in multiple proteins, in addition to Casp. TER94 possible plays a vital role in protein degradation of critical cell cycle proteins, such as cyclins that need to be degraded for efficient genomic duplications in the 10' nuclear division cycles that predominate the first few hours of embryogenesis.

> *N=3 (Fig1 legend) and N =15 (Fig2). What are those numbers?*

N=3 indicated the number of repeats of the western blot. This reference has been deleted. N=15, represents the number of embryos imaged for data in panels G and H.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *Major Suggestion:*
>
> *(1) Oskar (Osk) mRNA Localization: Does Osk mRNA localization change upon overexpression or LOF of Casp? Since TER94 has been implicated in Osk mRNA localization (Ruden et al., 2000), this would be a good control to include.*

As mentioned earlier, in the response to editors, data presented in our manuscript indicates that Caspar is unique in its ability to regulate both Oskar levels and centrosome dynamics. As the reviewer pointed out, we are in the process of analysing the possible localization defects in *oskar* mRNA in the embryos. Since the preliminary data are promising, we are pursuing this carefully to better understand the involvement of Caspar. We are focusing on the ability of Caspar to regulate early nuclear divisions prior to pole cell formation. It is possible that in *casp* mutant embryos the nuclei/centrosomes that enter the pole plasm are already defective and thus can influence release of the pole plasm components. This needs to be examined carefully, and we are conducting these experiments.

> *(2) Western Blot for Osk Protein: It would also be beneficial to perform a western blot for Osk protein to demonstrate that it is indeed increased upon Casp overexpression.*

This is a good suggestion. However, Oskar antibodies are not readily available, and we have a very limited supply which have been used for embryo staining experiments. We considered these more useful as in addition to the absolute levels, staining experiment can reveal localization pattern. It was thus possible to correlate Oskar function with the pole cell counts in respective genetic backgrounds.

> *(3) Title Clarification: The title states, "Caspar determines primordial germ cell identity in Drosophila melanogaster." The current experiments do not show that Casp determines germ cell identity. It would be more accurate to conclude that Casp regulates germ cell numbers.*

Please refer to the introductory paragraphs where we explain our views in this regard. We have modified our title to “Caspar specifies primordial germ cell count and identity in *Drosophila melanogaster*."

> *Minor Suggestions:*
>
> *(1) Line 69: Delete the use of "recent" for papers published in 2001 and 2007. These papers are around 20 years old.*

The word has been deleted.

> *(2) Paragraph from Line 110: Consider splitting this paragraph into two for better readability and clarity.*

Paragraph has been split into two; this has improved readability.

> *(3) Line 266: Check and correct the formatting issues in this line.*

Edited, based on suggestion. A line break was added after the title.

> *(4) Line 328: Adding references to earlier studies here will be useful for providing context and supporting information.*

References that introduce Centrosomes and their roles as organizing centres have been added in line 336.

> *(5) Line 564: It is best to avoid using the word "master." Please consider using other terms such as "key" or "principal."*

Edited, based on suggestion.

> *(6) Citations: The authors should also cite Cinalli et al., 2013 for the Gcl reference to ensure comprehensive citation of relevant literature.*

Thank you for the suggestion. The reference has been added on pages 16 and 29.

> *(7) Overall Length: The paper is quite long. If it can be shortened, it will be easier to read. Consider condensing sections where possible without losing essential information.*

The paper is indeed longer than average, but the choice of *eLife* as the home for this study was, in part, determined by the platform's flexibility regarding length/ word count. It seemed worthwhile to elaborate the text in places to accentuate the novelty of the findings.

> *These additions and adjustments would help to further substantiate the claims and improve the clarity of the paper.*

We hope that the claims made in our manuscript are substantiated by the data that are presented. Wherever possible, we have tried to modify the text suitably to improve clarity.

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