A Maternal role for *Drosophila* Caspar/dFAF1

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by

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Certificate

This is to certify the this dissertation entitled "A Maternal role for *Drosophila* Casp/dFAF1" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents study/work carried out by " Neel Vidyadhar Wagh at the IISER Pune under the supervision of Dr. Girish Ratnaparkhi, Associate Professor, IISER Pune during the academic year 2018-2019.

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Committee : Biology

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Declaration

I hereby declare that the matter embodied in the report entitled " A Maternal role for *Drosophila* Casp/dFAF1" are the results of the work carried out by me at the Department of Biology, IISER Pune, under the supervision of Dr. Girish Ratnaparkhi and the same has not been submitted elsewhere for any other degree.

Neel Vidyadhar Wagh

ABSTRACT

Drosophila Caspar (Casp) is an ortholog of mammalian Fas associated factor 1 (FAF1). Analysis of the primary sequence suggests that the protein maintains its function between flies and humans. Casp in flies has been studied in the context of host defence, with Casp negatively regulating the Immune Deficient (IMD) pathway, by modulating activity of Dredd, an endo-protease that cleaves and activates RELISH/NFκB in immune signaling.

In *Drosophila*, *casp* is expressed ubiquitously throughout development, with females also depositing maternal *casp* mRNA in the developing oocyte. In our study , we find that *casp* is a maternal effect gene, with an essential developmental requirement in the 0-3 hour embryo. We prove maternal roles by demonstrating that paternal zygotic expression cannot rescue embryonic lethality due to maternal loss of function (lof) of *casp*. Maternal *casp lof* embryos die in stages 8-15, suggesting that Casp in the early embryo performs critical functions that when absent stalls embryogenesis immediately post gastrulation. We are currently using antibodies and *in-situ* probes to understand cellular roles for Casp.

Caspar/FAF1 contains domains for interactions with p97/VCP and poly-ubiquitin suggesting molecular functions in the ubiquitin-proteasomal degradative pathway. We hypothesize that the developmental stalling is a consequence of aberrant degradation of maternal proteins during the maternal to zygotic transition.

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INTRODUCTION

Development is a complex and a well-orchestrated event. In multicellular animals, the process involves an increase in cell number, morphogenetic movements that place cells in correct locations relative to other cells and restriction in cell fate where the initial totipotent cell divides and becomes a pluripotent or differentiated cell. Interactions, both short-range and long-range between cells are carried out by extracellular molecules that help synchronize development. In *Drosophila*, embryogenesis is initiated post fertilization with rapid cell divisions that form the syncytial embryo, followed by the formation of the blastula which in turn leads to gastrulation and the subsequent system-wide morphogenetic movements of gastrulation (Adapted from Developmental Biology, 6th edition). In order to pattern the developing embryo in a precise manner, the future primary axes, namely the A/P and D/V axis are laid down in the first few hours of development. These two axes are patterned before gastrulation begins, by three independent systems, namely the A/P system, the D/V system, and the terminal system. All three systems are active in the first few hours of development, and they decide all subsequent cell fate. Interestingly, the successful activation of the three systems depends on events in the developing egg, in the mother's body, much before fertilization (Weaver et al. 2018). In the subsequent paragraphs, I describe the events in the egg that set up the A/P, D/V & terminal systems, which in turn specify the fundamental axes post-fertilization and leads to the highly regulated process of embryogenesis.

The assembly line that makes the mature oocyte: *Drosophila* has a pair of ovaries, and each ovary contains approximately 15-16 ovarioles. An ovariole is like an assembly line, wherein the oocyte develops within the egg chamber as a series of fourteen stages which are morphologically distinct (Fig. 1). In each ovariole, 6-7 different egg chamber stages of a developing oocyte can be observed. An egg chamber contains 16 germline cells, one oocyte and 15 nurse cells, which are covered by a layer of somatic follicle cells (Jia et al. 2016). There are two oocyte arrests during oogenesis, first of which is by stage 5 where it is arrested at prophase I. The other 15 nurse cells now undergo endocycling (DNA replication without mitosis) instead and become polyploid (Hong et al. 2003). The oocyte is in arrest till stage 11 after which it undergoes a process called oocyte maturation (stage 12-14)(Fig. 1). Oocyte maturation is synonymous to meiotic maturation where the

oocyte progresses through prophase I until metaphase I (Stetina et al. 2008). The oocyte is arrested for the second time at metaphase I at stage 14. During oocyte maturation, the nurse cells dump huge reserves of mRNA, proteins, lipids and glycogen and eventually undergo cell death.

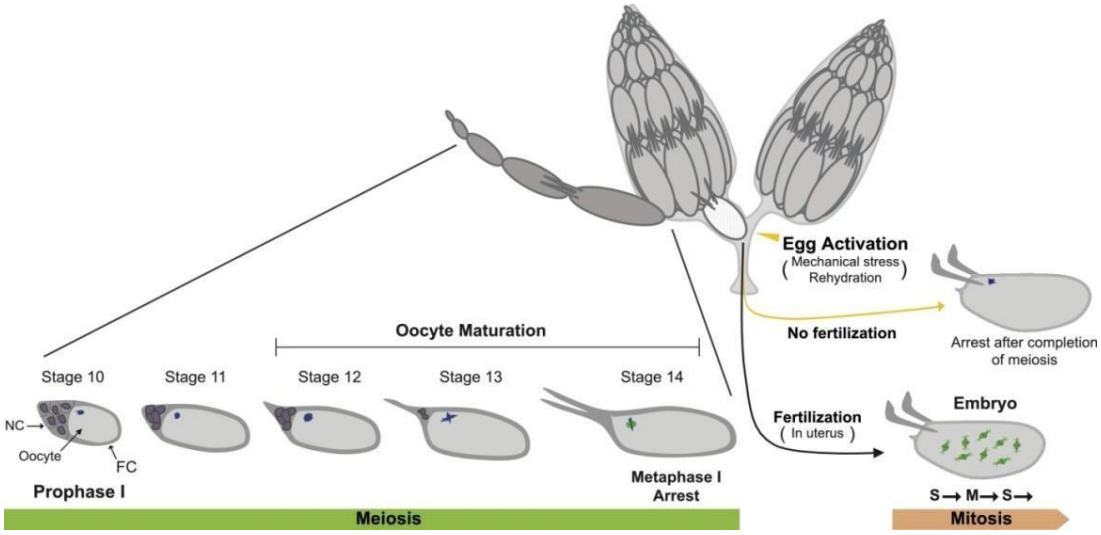

Fig. 1. *Drosophila* oogenesis and early development. Each ovariole has 6-7 morphological distinct stages and is a production line where the oocyte develops. Out of the 16 sister cells, only one is destined to become the oocyte where as the other 15 cell support it by providing nutrient and other maternal factors. The mature is oocyte then descend into the oviduct which leads to egg activation and completion of meiosis. During fertilization the haploid nuclei from the mother and father fuse and set in embryogenesis by undergoing rapid cycles of nuclear divisions, characterized by rapid and accelerated S- and M phases without cytoplasmic divisions. If the egg is unfertilized, the female nuclear material forms a polar body. DNA/nuclei are represented in blue and spindles in green. Figure adapted from Pagan and Orr-Weaver. 2018.

Setting up A/P and D/V polarity in the mature egg: In *Drosophila, the* establishment of cell polarity and anterior - posterior axis is determined during oogenesis (Steinhauer et al. 2006). This is achieved by localization of three main mRNA determinants, *bicoid (bad)* and *oskar (osk)* to anterior and posterior respectively, and *gurken (grk)* to the future dorsal anterior corner (Volhard et al. 1987). Formation of dorsoventral asymmetry in early embryonic development of *Drosophila* is largely controlled by maternal effect genes. Maternal Effect genes are those genes whose gene products, mRNAs and protein are deposited in the developing oocyte by the nurse cells of the mother (Adapted from Pagan and Orr-Weaver. 2018). The D/V axis is determined by activation of the Toll pathway which leads to translocation of transcription factor Dorsal to the nucleus forming a Dorsal

gradient along dorsoventral axis (Moussian et al. 2005). The axis formation is governed by many maternally derived factors like *pipe (pip), slalom (sll), windbeutel (wbl)*, *nudel (ndl),* morhpogen Spatzle (Spz), *gastrulation defective (GD), snake (snk)* to name a few (Schupbach et al. 1987, Stein et al. 1991, Krem et al. 2002). The A/P axis is regulated by gradients of Biciod, Hunchback, Caudal and Nanos. These regulate transcription of gap genes (*giant, kruppel, knrips, huckebein)* and segmentation genes like *even-skipped and engrailed* (Adapted from Developmental Biology, the 6th edition). The terminal structures: the head and the tail, are under the control group of genes called the terminal genes which include *torso, D-raf, tailless* (Lu et a. 1993).

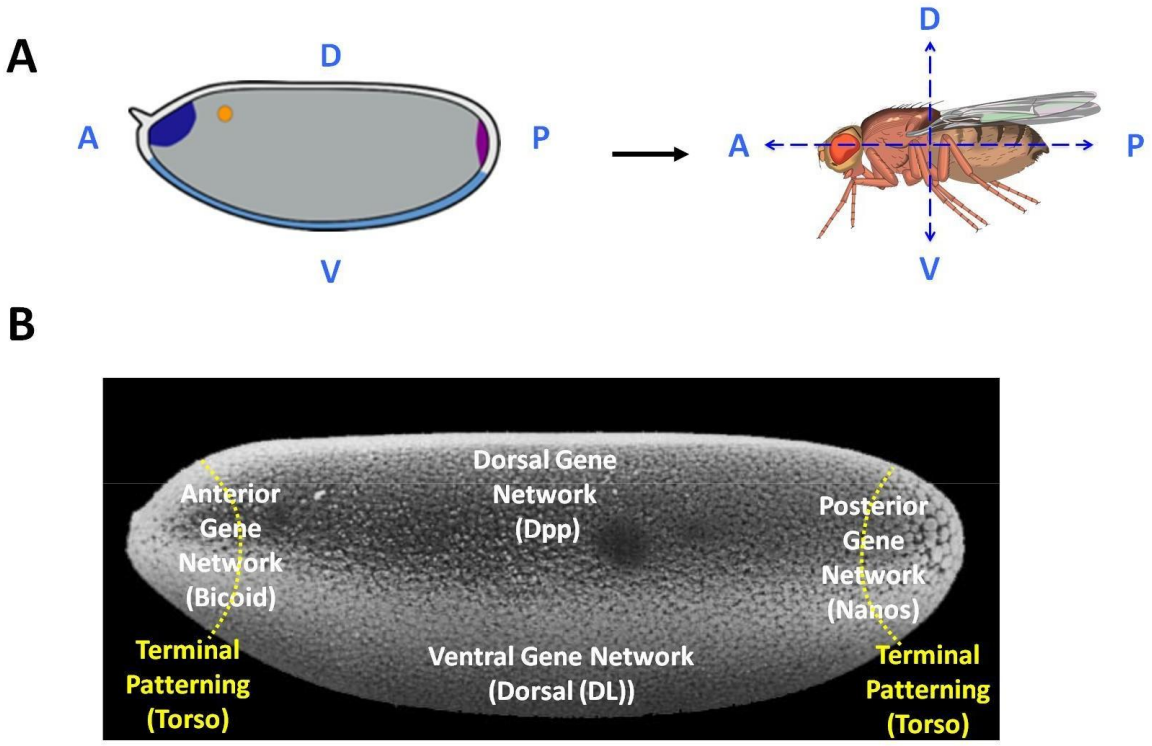

Fig. 2. Establishment of A/P and D/V axes. (A) Anterior (A), Posterior (P), Ventral (V), Dorsal (D) axis are during oogenesis and correspond to A/P and D/V axes of the adult fly. **(B)** A Scanning Electron Micrograph of early *Drosophila* embryo showing players involved in A/P and D/V patterning. Terminal patterning is an independent gene regulatory network.

The first two hours of development: Following fertilization and completion of 13 cycles of nuclear division, the embryo forms a syncytial blastoderm (Fig. 3A). Further, the plasma membranes folds inwards partitioning each nucleus into a single cell. This process leads to the formation of the cellular blastoderm, in which cells are

arranged around the periphery of the embryo as a single layer around the yolk core of the embryo (Turner et al. 1977). As the embryo forms a cellular blastoderm and the rate of cell division becomes progressively slower over the next four asynchronous divisions (Foe et al. 1989). Soon gastrulation is initiated, which will lead to the formation of a three-layered embryo containing mesoderm, endoderm, and ectoderm.

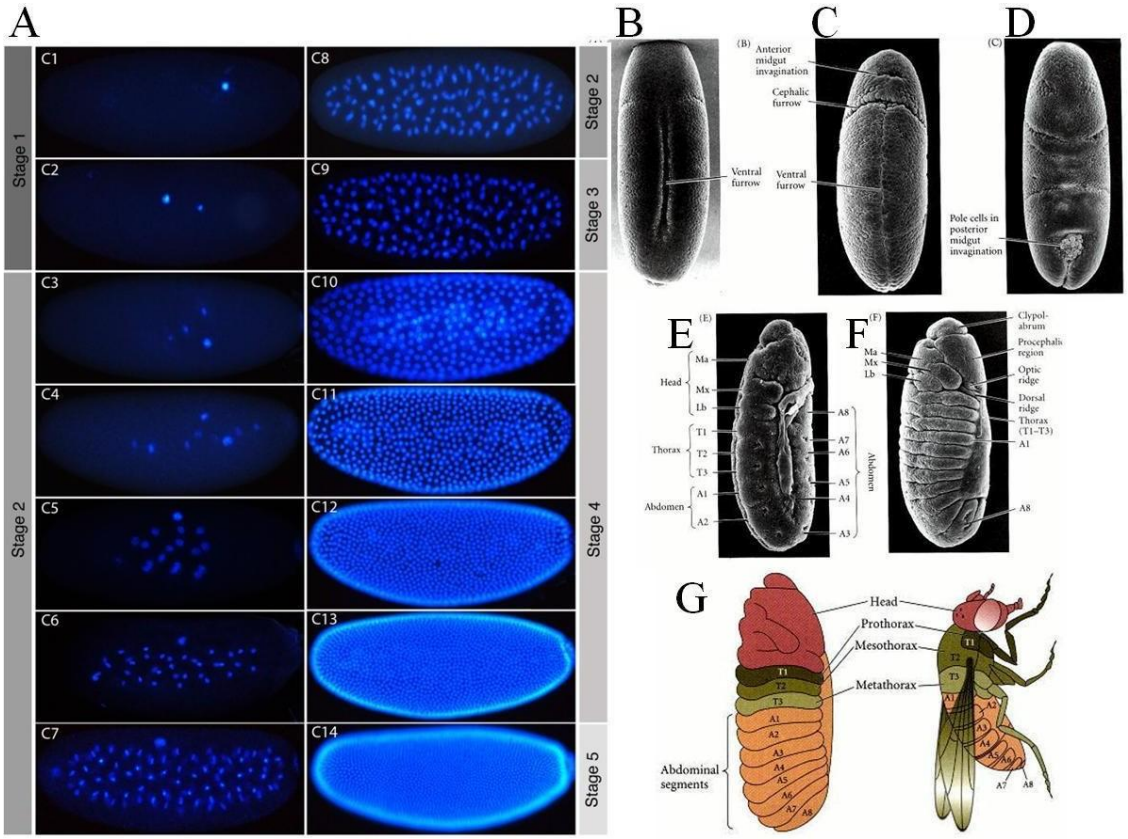

Fig. 3. Embryogenesis. (A) DAPI staining showing cleavage cycles (C1-C14) with formation of blastoderm at C14 (Wotton et al. 2014). **(B)** Cells adjacent to ventral midline invaginate to form Ventral furrow. **(C)** Ventral furrow closed with mesodermal cells within the embryo and ectoderm on the surface. **(D)** Embryo showing pole cells and sinking of posterior endoderm. **(E)** Completion of germband extension and start of segmentation. **(F)** Segments and head structure visible. **(G)** Comparison between larval and adult segmentation. Adapted from Developmental Biology, 6thedition.

The maternal to zygotic transition: The maternally inherited factors govern the early developmental programme of virtually all animals. The maternal mRNAs and

proteins which are deposited into the oocyte during oogenesis regulate the biosynthetic pathways and early nuclear divisions (Tadros et al. 2009), As the developmental programme proceeds, two events are triggered to give the maternal to zygotic transition (MZT): at first the maternal mRNAs are degraded, and this is achieved by maternally encoded proteins (Tadros et al. 2009), second the transcription of the zygotic genome is activated. This transcriptional activation also leads to productions of proteins and micro RNAs (miRNAs) that enhance the maternal mRNA degradation efficiency. Activation of the zygotic genome also leads to the synthesis of transcriptional factors which further enhance the zygotic transcription. As a result, the developmental programme is now under the control of the zygotic genome.

Embryogenesis is a complex and well-orchestrated process where the initial events are solely governed by maternal factors such as *bicoid, hunchback, nanos, caudal, oskar, dorsal* to establish D/V and A/P axis (Russel et al. 2010). Maternal effect gene mutants do not show defects because they receive the necessary wild-type gene products from their mothers and hence develop normally. The developmental defects are observed in their F1 generation because the mutant mother could not provide the maternally required gene product to the embryos. The defects are not rescued even if a wild-type gene copy is provided paternally due the fact that the paternal genome is not transcriptional active at initial hours of embryonic development (0-2.5 hours) and hence the embryo depends completely on the maternally deposited gene products required for its proper development. Many maternal effect genes such as *bicoid, dorsal* were found during the large scale mutagenesis screen performed at EMBL, Heidelberg during 1979-1980 (Volhard et el. 1979). New and novel maternal effect genes are being identified constantly. My gene of interest, *casp* is been shown to deposit high levels of maternal mRNA in the embryos (Fisher et al. 2012). During my study, I found that *casp/dFAF1* is a maternal effect gene.

Casp: Casp (Casp) (encoded by *casp*), a protein found in *Drosophila melanogaster* is a homolog of mammalian Fas-associated factor 1 (FAF1) (Kim et al. 2006). FAF1 is a multi-domain protein and is known to regulate apoptosis, ubiquitination, proteasomal degradation and also NFκB activity (Menges et al. 2009). FAF1, via its UBA domain, interacts with polyubiquitinated proteins and with its C-terminal UBX domain (Song et al. 2005) interacts with VCP (valosin-containing protein). Casp is 695 amino acids long having molecular weight 78,441 Da (UniProt). Casp has been mainly studied in context of immunity in *Drosophila*, and is a suppressor of antibacterial immunity (Kim et al. 2006). Casp negatively regulates the immune deficiency (IMD)- mediated immune responses by blocking nuclear translocation of REL, an NF-kB transcription factor (Fig. 4B). Dredd-dependent cleavage of REL, a prerequisite event for the nuclear entry of REL, is the target of the Casp mediated suppression of the IMD pathway (Kim et al. 2006). Upon infection, with gram-negative bacteria, the peptidoglycan on the bacterial surface which consists of N-acetylglucosamine and N-acetylmuramic acid, activate the peptidoglyca recognition receptors on the cell surface of immune cells in *Drosophila* (Myllymäki et al. 2014), which in turn upregulate IMD signaling.

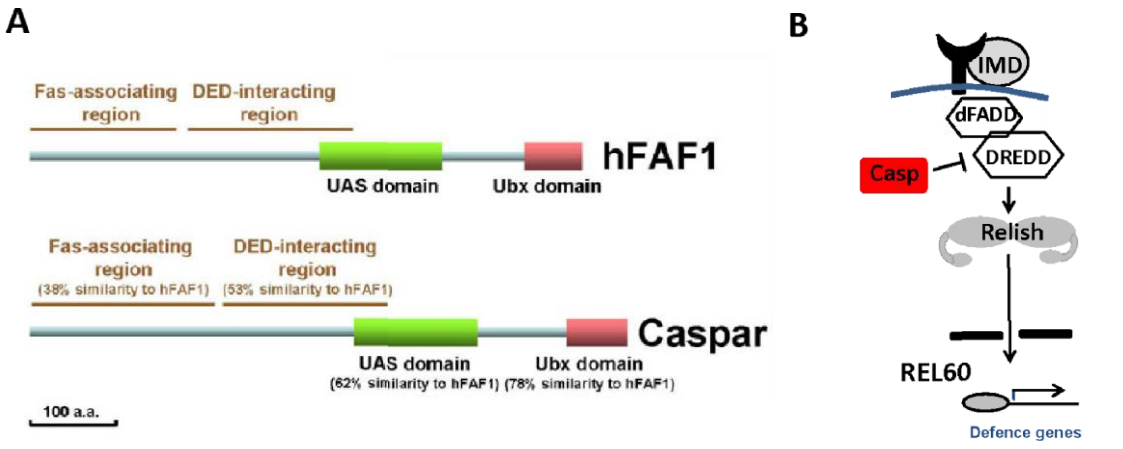

Fig. 4 A. Casp is a homolog of FAF1 A. Structural comparison between human FAF1 (hFAF1) and Casp. The locations of the UAS (ubiquitin-associated) domain and the Ubx (ubiquitin-like) are marked be unstructured. The Fas-associating region and the DED (death effector domain)-interacting regions are also marked. BLAST searches using the sequences of each domain/region identified the corresponding domains/regions in Casp. Casp: Fas-associating region (amino acids 1-214); DED-interacting region (amino acids 231-421); UAS domain (amino acids 376-524); and Ubx domain (amino acids 620-695). **B.** The Immune-deficient (IMD) signaling pathway, along with Toll signaling, is one of the primary pathways

of recognition of pathogens and subsequent activation of a cellular/organismal defense response. Casp negatively regulates the response, presumably by regulating Dredd activity. Loss of function alleles of Casp has higher baseline activities of defense genes. Figures adapted from Kim et al. (2006) and from Handu et al. (2015).

Objectives: The aim of my master's project is to study roles for the gene *casp* in *Drosophila melanogaster* development. I started my research by characterizing a *casp* loss of function (lof) allele, $casp^{c04227}$ (BDSC #11373). Next, based on the developmental lethality of *casp*, I find that *casp* is a maternal-effect gene and I have worked towards a genetic proof for the same. Finally, I address the mechanisms underlying Casp function in the 0-3 hour embryo.

Casp is important for proper embryonic development.

In order to understand roles for Casp in *Drosophila* development, we procured a loss-of-function (lof) allele from the Bloomington Drosophila Stock Centre (BDSC). The available allele, BDSC #11373 had the genotype (w[1118]; PBac{w[+mC]=PB}casp[c04227]) and will be referred to as $casp^{c04227}$ or $casp^{lof}$. *casp* is located on the second chromosome, and the $casp^{lof}$ line was balanced by a floating Cyo balancer. The *lof* was caused by the insertion of a PiggyBac transposon construct in the 5' UTR of *casp* locus (Fig. 5A; Red arrowhead).

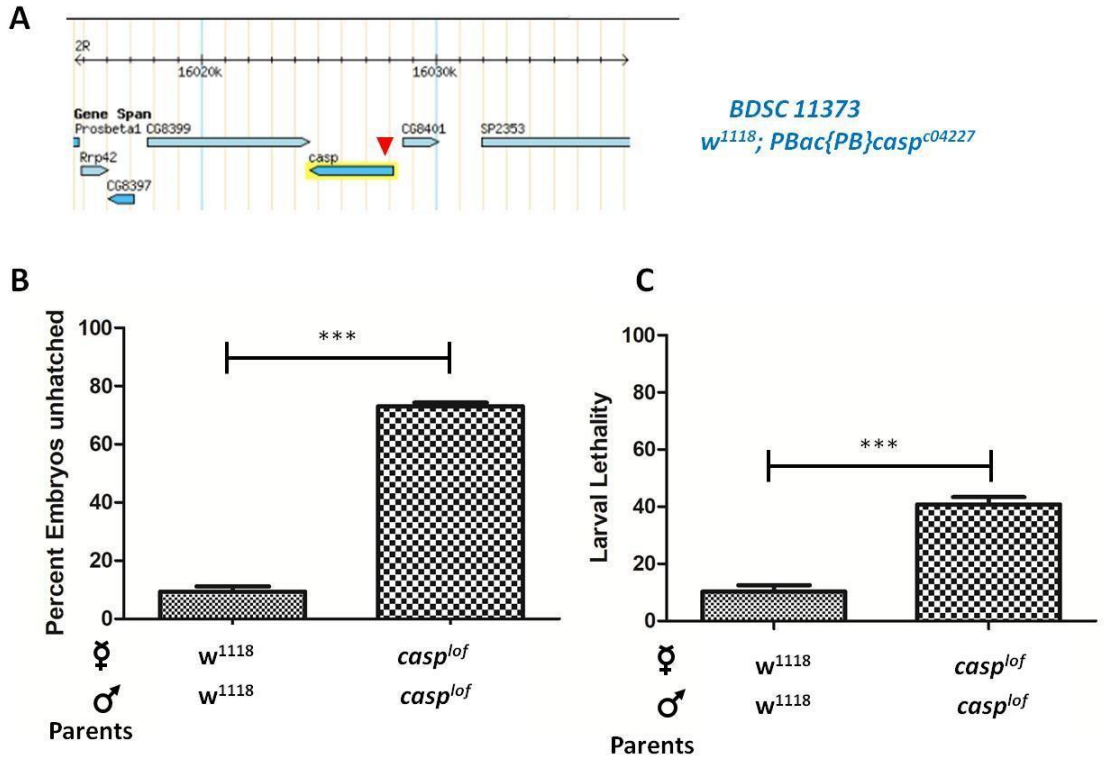

Fig. 5. Casp is necessary for embryonic development. **(A)** $casp^{c04227}$ *Piggyback* insertion in 5'-UTR of *casp*, which causes disruption in the locus and thus may produce Casp protein. **(B)** A graph for percent embryo lethality is shown where wild type (w^{1118}) embryo shows ~ 9 % lethality and $casp^{lof}$ embryo shows ~75 % embryonic lethality. For each replicate (N=3) 30 females were used for egg collection, and 150 embryos were screened. **(C)** A graph for larval lethality, where $casp^{lof}$ larvae show ~ 40% larval lethality as compared to wild type (w^{1118}) larvae which show only ~ 10% lethality. Student's t-Test was used for analysis . '***' indicates a p-value < 0.0001.

Preliminary analysis suggested that $casp^{lof}$ flies are lethal, though the stocks contained homozygous 'escapers'. The stage of lethality of homozygous flies has not

been stated in FlyBase or documented in the literature. *casp* mRNA is present throughout embryonic stages 1-16 (Fisher et al. 2012) but has never been implicated for specific roles in embryo development. The few *casp*^{lof} homozygous escapers flies obtained were self-crossed (male to female) to generate a stable homozygous line. The *casp*^{lof}/ *casp*^{lof} stable line was used for embryo lethality assays. It was observed that 75% of homozygous *casp*^{lof} embryos fail to hatch after 24 hours of egg-laying suggesting an important role for Casp in embryonic development (Fig. 5B). Further,~40% of homozygous *casp*^{lof} died as larvae suggesting a requirement of Casp in larval development.

casp^{c04227} is a near-null allele.

To characterize the *casp*^{lof} allele, an antibody generated against full-length Casp was utilized (Kaduskar et al., Unpublished) to measure levels of Casp protein expression.

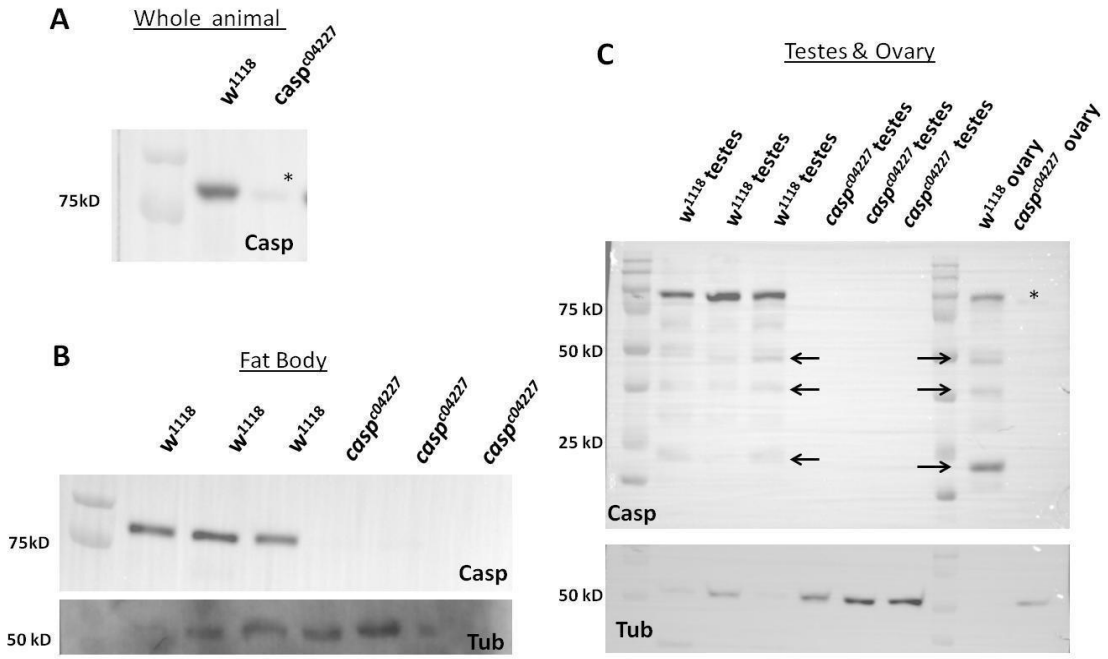

Fig. 6. _casp^{c04227}_ is protein null or near-null. (A) Whole body western (100 flies in 500 µl RIPA) probed with anti-Casp antibody detects Casp (mol. wt. 79 kD) and shows the absence of Casp protein in the *casp*^{c04227}flies. A faint band is observed in lane with *casp*^{c04227} that could either be non-specific or may indicate residual protein being expressed. **(B)** Fat-body western (5 larval fat-body in 150 µl RIPA) probed with anti-Casp antibody detects Casp and shows complete absence of protein in *casp*^{c04227} fat-bodies. **(C)** Testes and Ovary western (7 pairs of testes in 40 µl RIPA and 4 pairs of ovary in 50 µl RIPA) probed with anti-Casp anti-

body detects Casp and also shows lower mol. wt. bands which could be Casp isoforms. Casp is absent from *casp*^{c04227} testes and ovaries.

MODENCODE (Celniker et al. 2009) data suggests moderate levels of transcript expression in the whole body, with moderate to high expression in the fat body, testes and ovary. A whole body western of *casp*^{c04227} shows near-absence of Casp protein when probed with an anti-Casp antibody (Fig. 6A). Based on the absence or very minimal amounts of Casp protein we consider *casp*^{c04227}as a protein null or near null allele, with <10% of Casp expression at the protein level.

Casp **is Maternal Effect Gene**

casp is expressed throughout embryogenesis, with expression seen as early as Stage 1, which is the 0-1/2 hour old embryo. At this stage, all transcripts are a consequence of maternal deposition. *casp* mRNA appears to be deposited maternally (Fig 7A). *Casp* mRNA is thus maternally deposited, and Casp *lof* may have a maternal effect. Maternal roles for any gene in flies can be tested by a set of simple genetic crosses. For a maternal-effect gene, a *lof* allele will usually show an embryonic phenotype which cannot be rescued by a zygotic copy of the gene.

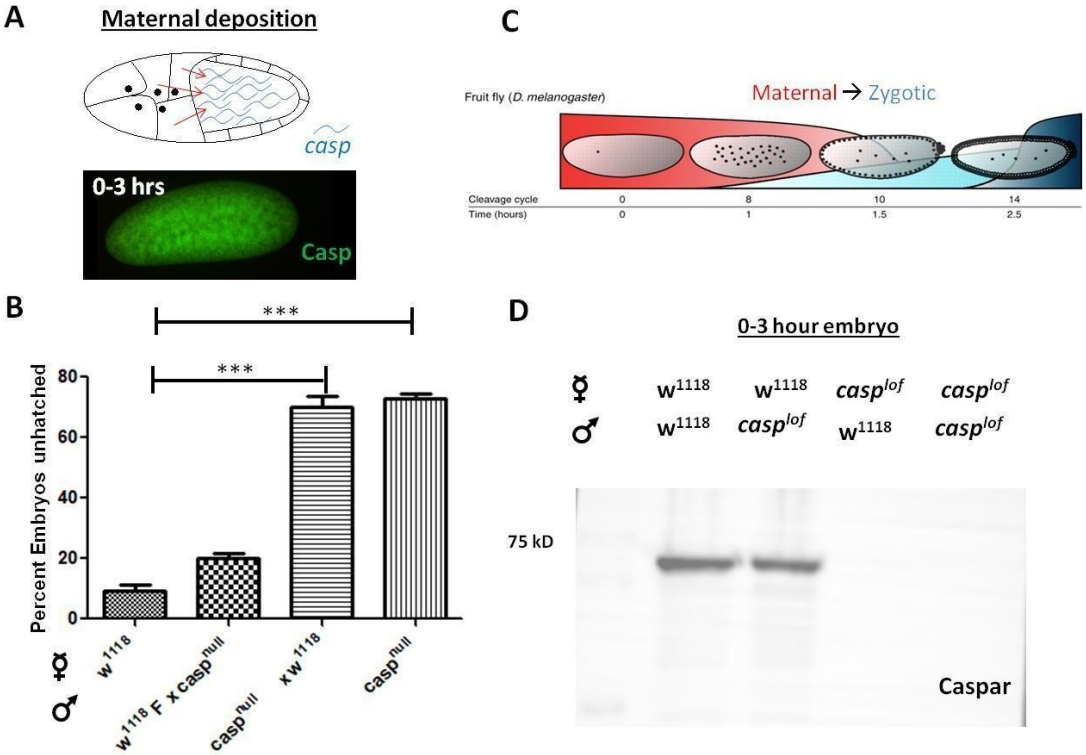

Fig. 7. Maternal deposition of Casp needs for proper embryonic development. (A) *casp* mRNA is deposited maternally into the embryo by the nurse cells during oogenesis. An *in-situ* showing *casp* in the early embryo (Fisher et al. 2012) **(B)** Providing Casp zygotically in the embryo by crossing $casp^{lof}$ female to a w^{1118} male does not rescue the embryonic lethality and shows a lethality of ~ 75 %. A reciprocal cross of w^{1118} female with $casp^{lof}$ male shows normal development of the embryos with lethality ~ 20%, while w^{1118} shows ~ 9% lethality. For each replicate (N=3) 30 females were used for egg collection, and 150 embryos were screened. Student's t-Test was used for analysis . '***' indicates p-value < 0.0001. **(C)** Maternally gene products govern the initial stages of embryonic development, and the zygotic transcription takes over after 2.5 hours, a phenomenon called maternal to zygotic transition (Tadros et al. 2009). **(D)** Western for 0-3 hours embryos.(45 embryos in 30µl RIPA) show the presence of Casp protein when the mother is wild type, but show a complete absence of Casp protein when the mother is $casp^{lof}$ even though $casp^{wt}$ is present paternally.

To test this we crossed a homozygous $casp^{lof}$ virgin females with both a w^{1118} male and a $casp^{lof}$ male. In both cases we found ~75% embryonic lethality, suggesting that a wild-type copy of *casp* from the male (w^{1118}) does not rescue the maternal lethality of the $casp^{lof}$ allele (Fig. 7B). Interestingly, there is small, but significant increase in lethality when a w^{1118} female is crossed to a $casp^{lof}$ male, suggesting a role for zygotic *casp* in either fertilization or sperm development. Our experiment confirms that *casp* is maternally required for the embryonic development. In order to confirm that *casp* mRNA leads to the production of Casp protein in the early embryo, I collected 0-3 hour embryos from $casp^{lof}$ females that have been crossed either with $casp^{lof}$ or w^{1118} males. Western blot showed antibody staining was only seen when the female was wild-type, but no Casp protein was seen when a $casp^{lof}$ female laid eggs (Fig. 7D). Casp, thus has a role in early development and is deposited in the embryos maternally as mRNA, which is translated to functional protein in the early embryo. Thus Casp has a critical, early, maternal role in early embryonic development.

Casp does not express in the late stages of the developing germline.

I stained the *Drosophila* Ovary with the anti-Casp antibody. Casp, based on the analysis of its primary sequence is predicted to be a cytoplasmic protein, and expectedly, the protein was found to be localized in the cytoplasm of ovarian cells. Interestingly, Casp was not present in the germline, but showed expression in the ovarian soma (Fig. 9A). The developing oocyte, marked by Orb (Jagut et al. 2013) showed no Casp staining, nor did DAPI stained nurse cells. Casp, in the ovary is present predominantly in the somatic follicle cells (Fig. 8A).

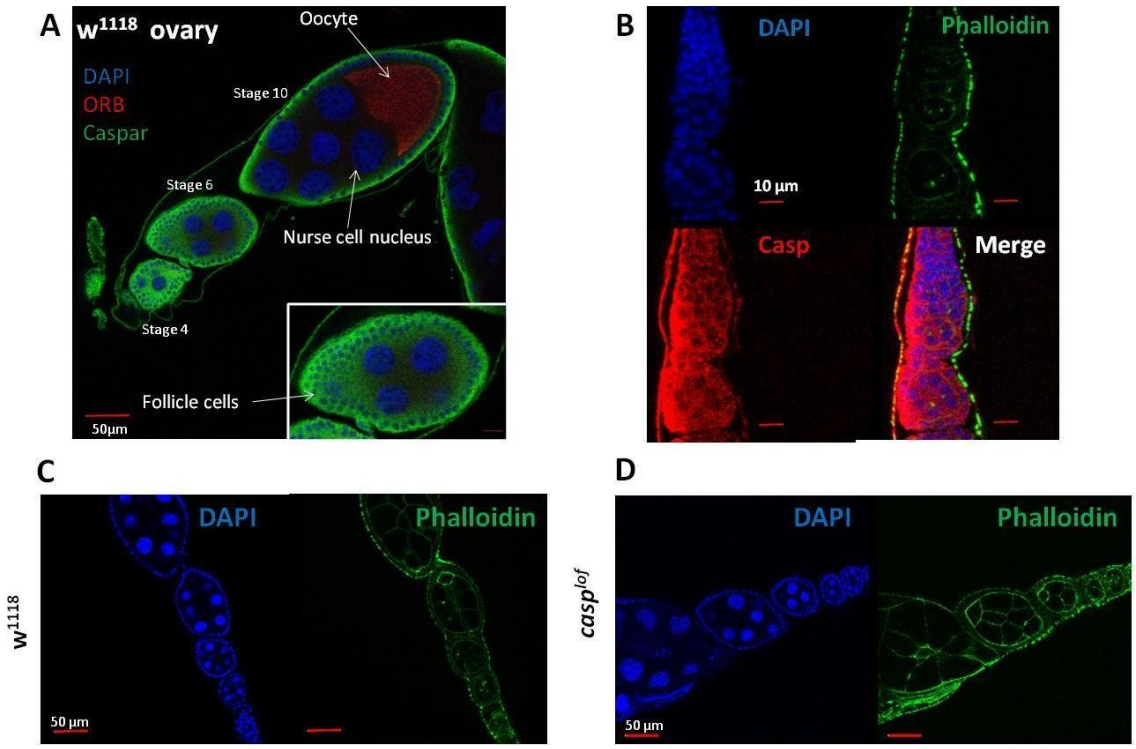

Fig. 8 Casp expresses in the somatic cells of the ovary. (A) wild type ovariole showing Casp (green) is present in early germline cells and is absent for the developing oocyte (marked by Orb, red). Casp is predominately expressed in follicle cells and is cytoplasmic. **(B)** Casp is expressed in the germarium. **(C), (D)** Morphology of the ovariole of wild type and *casp*^{lof}. *casp*^{lof} ovaries show no structural defects either in germline cells or follicle cells.

Cells in the germarium show Casp expression (Fig. 8B). It remains to be tested if any of this early expression includes germline cells. In ovaries of *Casp*^{lof} homozygotes, the Casp staining is absent, notably from the follicle cells.

Casp protein is thus not expressed in the late stages either in the future oocyte or the nurse cells. Nurse cells, however, produce *casp* mRNA which is deposited maternally into the developing egg. The oocyte, post-fertilization shows Casp protein, indicating that all early Casp protein is translated from maternally deposited mRNA.

casp^{lof} ovaries lack Casp from follicle cells as well as germline cells

Anti-Casp staining in *casp*^{lof} ovaries do not detect any Casp protein either in the germline cells or the follicle cells, thus lack Casp protein. *casp*^{lof} ovaries shows developing oocyte and all stages of oogenesis.

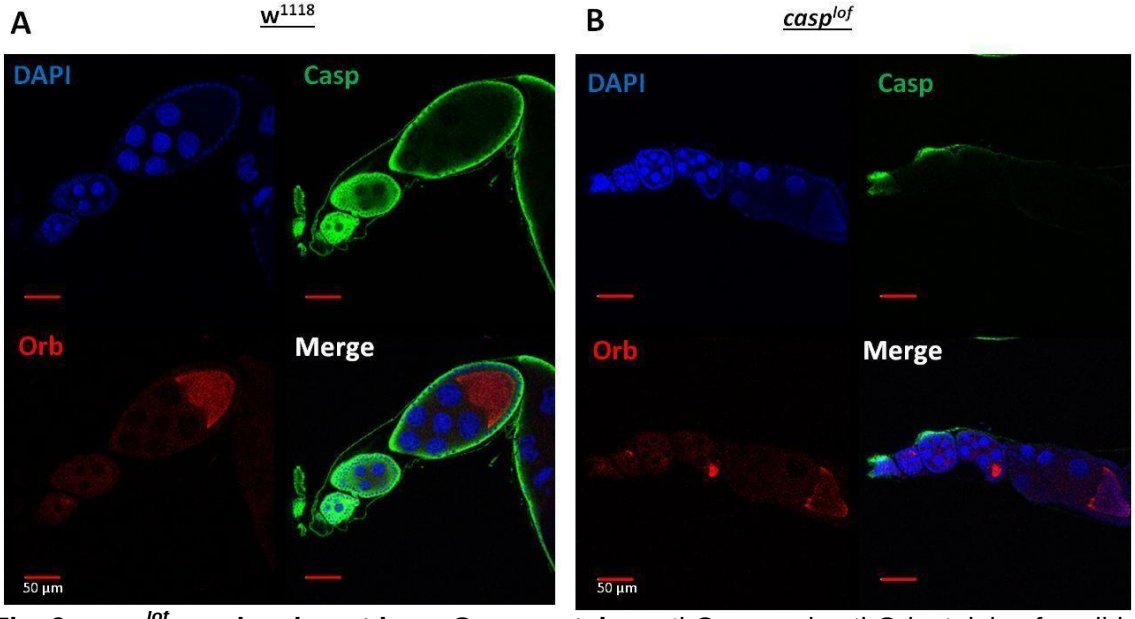

Fig. 9. *casp*^{lof} ovaries do not have Casp protein. anti-Casp and anti-Orb staining for wild type and *casp*^{lof} , Casp is absent from *casp*^{lof} ovaries.

casp RNAi less penetrant than the *casp*^{lof} allele

Knockdown of Casp in female germline was achieved through mating *casp* TRiP (y[1]sc[*]; P{y[+t7.7]v[+t1.8]=TRiP.HMS02742}attP2) (BDSC 44027) to MTD-Gal4 (BDSC 31777, maternal triple driver), which drives the expression of dsRNA in germ cells throughout oogenesis (Petrella et al. 2007), or α-tubulin-Gal4:VP16 (BDSC 7062, mat-Gal4) which drives expression in cystoblast and later stages of oogenesis but not in the female germline stem cell (Radford et al. 2012). The crosses gave progenies having the *casp* dsRNA (*casp* RNAi) driven through the respective Gal4. A lethality assay using these progenies as parents was carried out. Driving the *casp* RNAi expression through MTD Gal4 gave ~25% embryonic lethality whereas driving through Mat Gal4 lethality similar to wild type (Fig. 10A). We believe that the Casp RNAi construct is not effective in a knockdown, either because of excess mRNA or because of the limitations of the Maternal knockdown technology. As Casp is predominantly expressed in follicle cells of the ovary to check whether *casp* in follicle cells have any role in embryonic development we also tried knocking down *casp* in the follicle cell of the ovary using C587-Gal4 (BDSC 67747), which drives expression in the somatic cells of the ovaries. Knocking down *casp* in follicle cells of the mother did not have any effect on the embryonic development of the progeny (Fig. 10A).

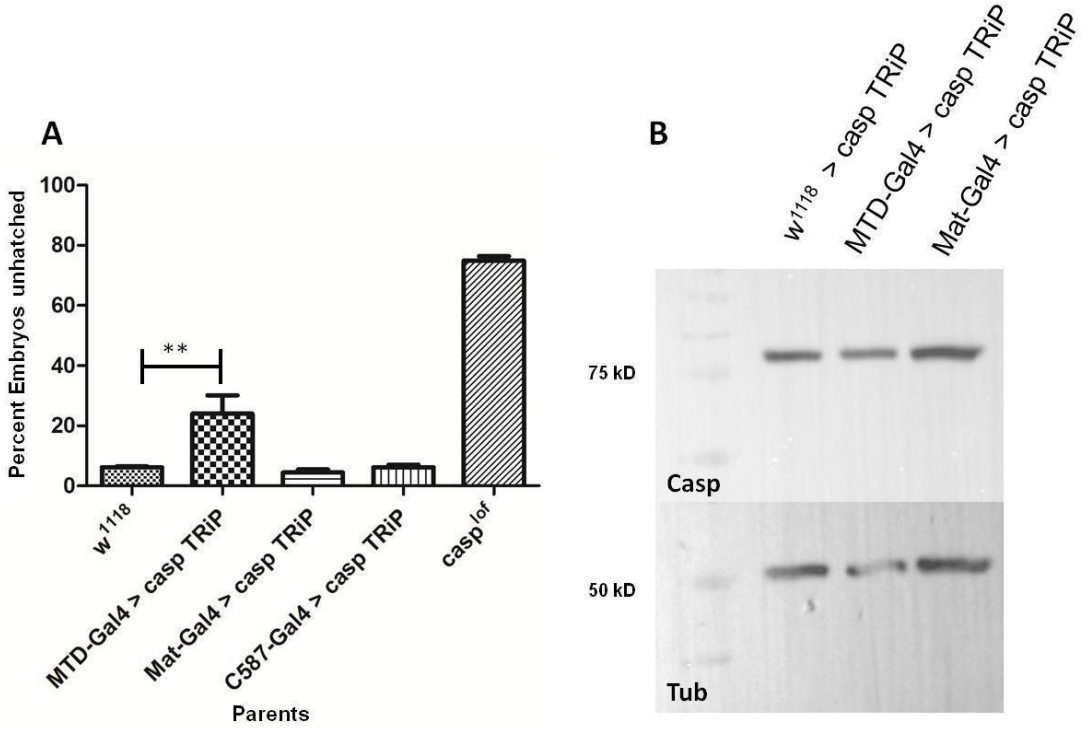

Fig. 10. *casp* knockdown using germline and follicle cells specific drivers (A) A graph showing embryonic lethality when *casp* is knocked down using MTD Gal4, Mat Gal4 and C587 Gal4. Knocking down *casp* in the parents using MTD Gal4 gave ~25% embryonic lethality as compared to wild type which gives ~10% lethality. Knocking down *casp* in the parents using Mat Gal4 or C587 Gal4 give a lethality similar to wild type. . For each replicate (N=2) 200 embryos were screened. Student's t-Test was used for analysis . p-value < 0.0001. **(B)** Western for 0-3 hours embryos from parents having MTD Gal4 > *casp* RNAi and Mat Gal4 > *casp* RNAi.

casp^{lof} females show lowered fecundity

casp^{lof} females show lower fecundity (~20 eggs/ per day/ per female), of about 30% of that of *w^{1118}* (~60 eggs/ per day/ per female) (Fig. 11C). Considering that the progression of egg chambers is normal, the reasons for a lowered egg laying rate are not clear (Fig. 11B). My results till now support a role for Casp in the 0-3 embryo. The 75% embryonic lethality does not appear to have any contributions from oogenesis.

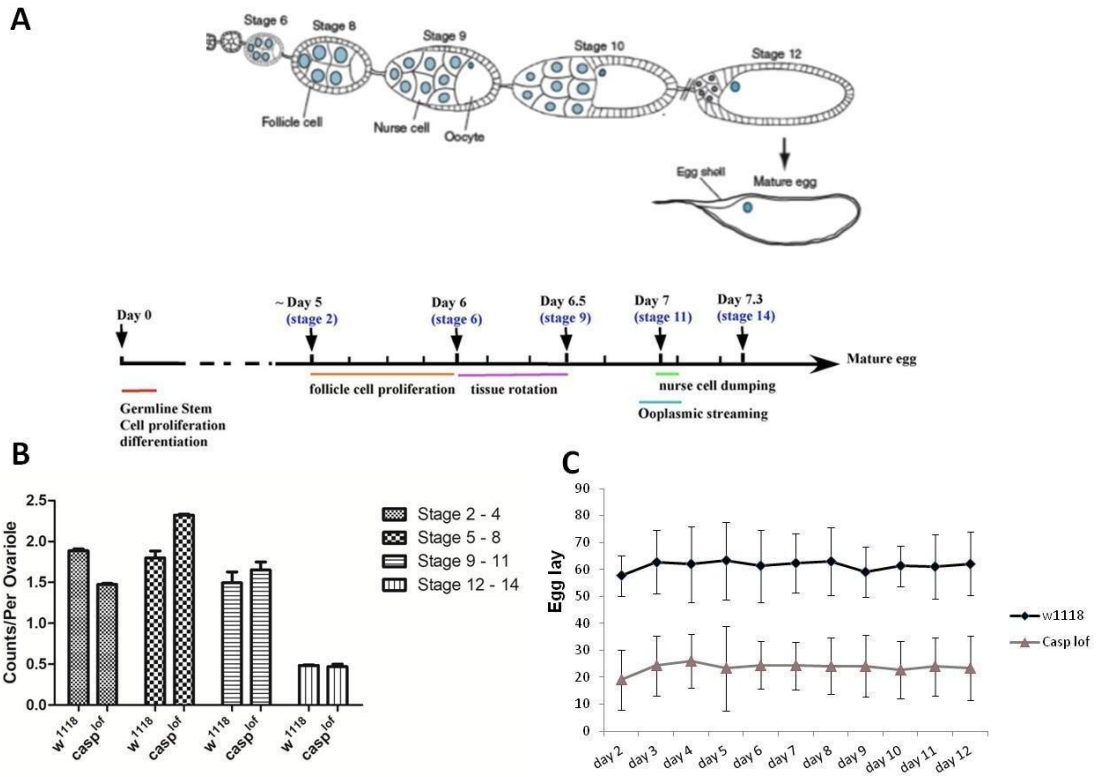

Fig. 11. Ovary staging and female fecundity assay (A) Schematic of ovariole and oogenesis (Adapted from Upadhyai et al. 2013). **(B)** Ovary staging for wild type and *casp*^{lof} shows that all oogenesis stages are present in *casp*^{lof} females and do not show any ovary defects or stalling of oocyte at any stage. N=2, n= number of ovarioles = 100. Stages are grouped as 2-4, 5-8, 9-11, 12-14 and counts of a stage per ovariole is plotted. **(C)** Fecundity assay showing egg lay per day across 12 days shows that *casp*^{lof} female lay lesser (~30% of wild type) number of eggs per day but the egg count does not decline with age. N = 3 , n = number of single-parent crosses = 6.

casp^{lof} embryos fail to deposit cuticle

The cuticle is deposited during stage 16 of the embryonic development of *Drosophila* (Ostrowski et al. 2002). Patterning defects can be observed using cuticle preparation by inspecting differences in the number of denticle bands, morphology and absence/presence of head and tail structures (Adapted from Wieschaus and Volhard. 2016). Cuticle preps of 24 hours old maternally *casp*^{lof} embryo (embryonic development completed in 22-24 hours) showed a complete absence of cuticle deposition (Fig. 12A). The absence of cuticle suggests that the embryos are dying before stage 16 of embryonic development. These results suggest that Casp protein

has critical a role in early embryonic development and the embryos die before stage 16 of embryonic development.

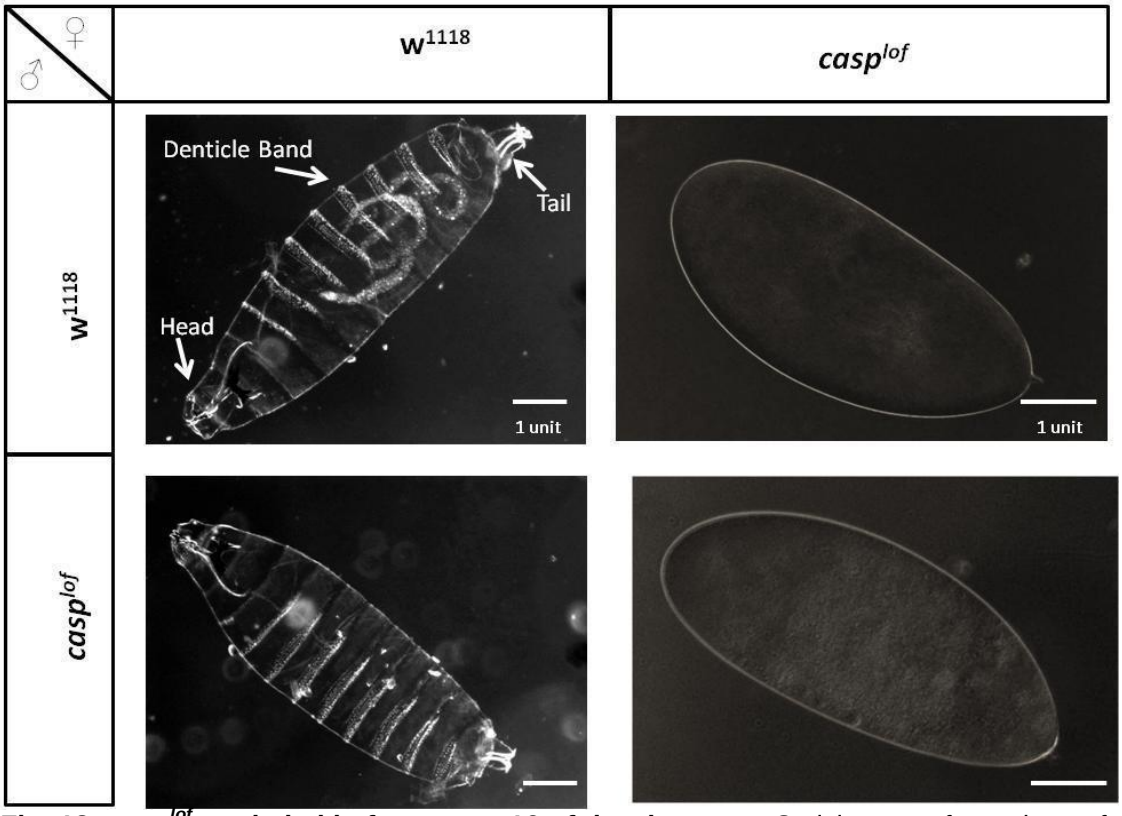

Fig. 12. *casp^{lof}* are lethal before stage 16 of development. Cuticle preps for embryos for crosses described in Fig. 8. B, result 3. Maternally deficient Casp embryos do not show any denticle band fail to deposit the cuticle. N = 3 , n = number of embryos = 100.

casp^{lof} embryos are fertilized and progress to gastrulation

In order of confirm that the lethality in *casp^{lof}* embryos is due to the absence of Casp and not a fertilization defect, we checked for nuclear divisions in 0-3 hours embryos. Maternally *casp^{lof}* show nuclear divisions and are thus fertilized (Fig. 13). DAPI staining for 2-4 hours old embryos show that *casp^{lof}* embryos undergo gastrulation.

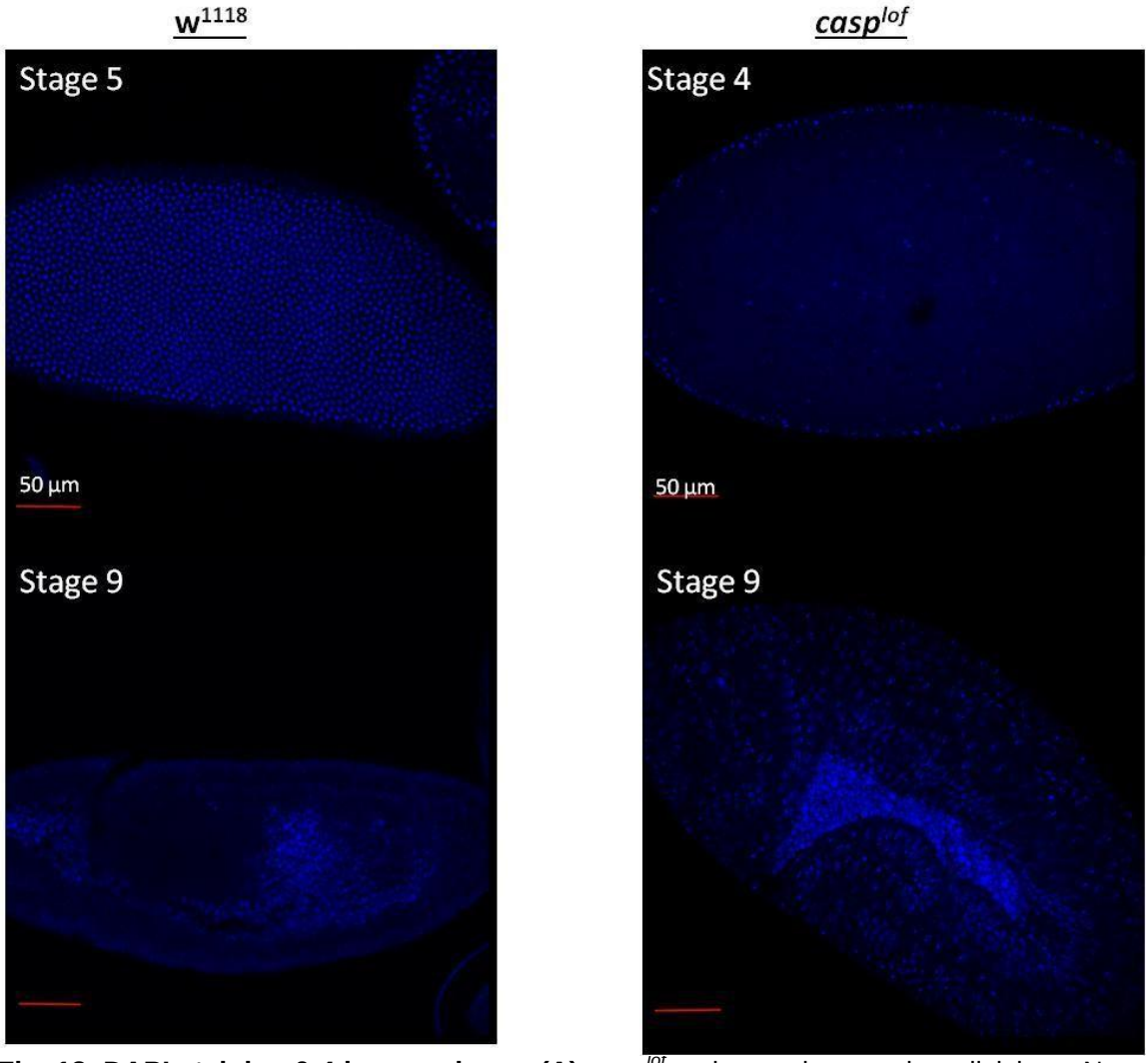

Fig. 13. DAPI staining 0-4 hour embryos (A) *casp*^{lof} embryos show nuclear divisions. N = 2, n = number of embryos = 20. **(B)** *casp*^{lof} undergo gastrulation.

casp^{lof} embryos do not form a nervous system

When do *casp*^{lof} animals die? To answer this question, I used markers for the nervous system, specifically HRP, Repo and Futsch which stain embryos as early as Stage 10 with staining enhanced by Stage 16. The peripheral nervous system starts forming around stage 11 (age ~7 hours) and the ventral nerve cord around stage 13 (age ~ 7 - 10 hours). As each *casp*^{lof} is post 12 hours (age 12 - 22 hours) the nervous system should under normal circumstances have been formed. Using the two different CNS marker, Hrp and Futsch and the glial cell marker Repo, I find that I see very few *casp*^{lof} embryos (<10%) marked with antibodies against these proteins

indicating absence of nervous system in most *casp*^{lof} embryos (stage 14-15) (Fig. 14. A, B). The few (<10%) I see have disorganized or malformed nervous system. Also elevated cleaved Caspase-3 levels suggest there is active apoptosis in these *casp*^{lof} embryos (Fig. 14. B). This suggests that *casp*^{lof} embryos are no longer in the normal track of development by Stage 10 and the developmental is aborted the embryo is undergoing apoptosis. *casp*^{lof} embryos show *the* presence of furrows, and thus it seems that these embryos are undergoing gastrulation (Fig. 14. A, B, arrows pointing furrows) but stalling at some later stage of gastrulation and undergoing apoptosis. Checking for gastrulation defects is one of the future studies to dissects the mechanism of Casp and the stage at which the embryo is lethal.

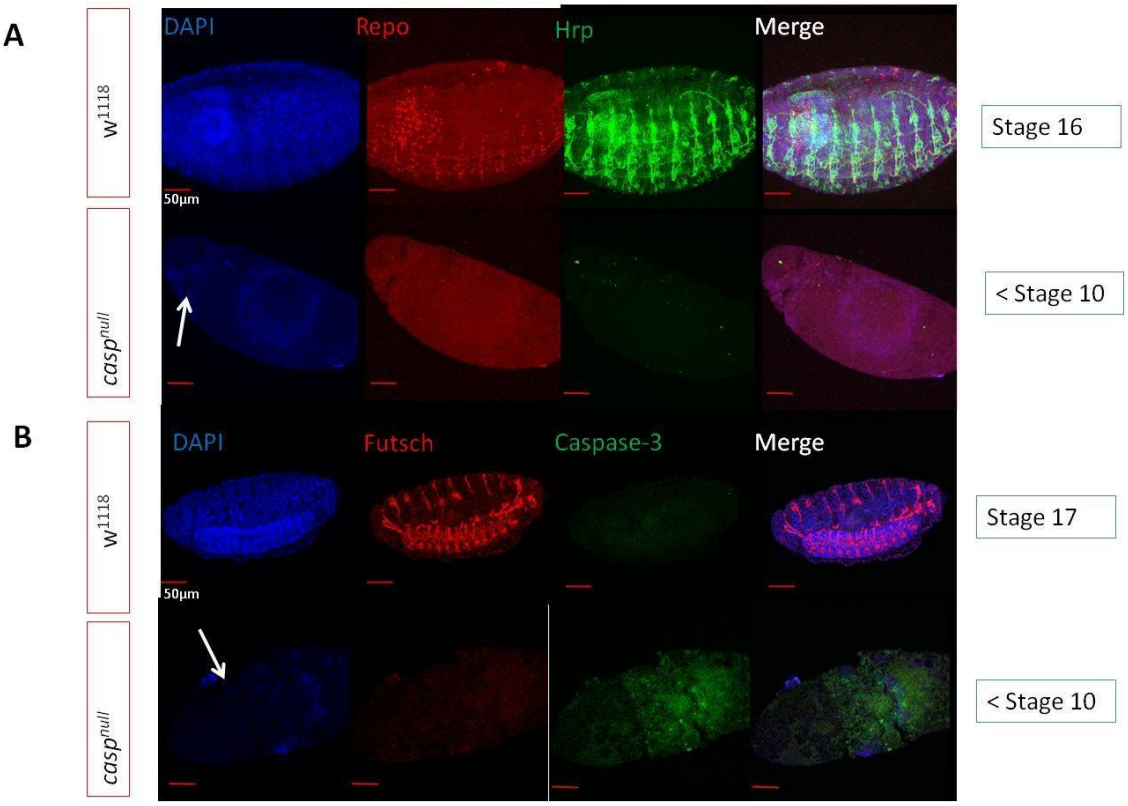

Fig. 14. *casp*^{lof} embryo show absence of nervous system and elevated apoptosis. (A) Immunohistochemistry staining using Repo (a marker for glia) and Hrp (a marker for CNS) shows that *casp*^{lof} do not have a nervous system. Arrow anterior furrow. n = 100 embryos **(B)** Immunohistochemistry staining using Futsch (marker CNS and peripheral nervous system) and Cleaved Caspase-3 (marker apoptosis) shows that *casp*^{lof} embryos do not have a nervous and also elevated apoptosis. Arrow points to cephalic furrow. n = 100 embryos.

DISCUSSION

My study shows that *casp* is a maternal effect gene and loss-of-function of Casp leads to ~ 75 % embryonic lethality. *casp*^{lof} embryos seem to undergo gastrulation but die between 10-15 of embryonic development as indicated by complete absence of nervous system. This result is also supported by the fact the these embryos do not deposit any cuticle (Stage 16). The Casp protein itself is not deposited maternally but the *casp* mRNA which is maternally deposited gets translated early during embryonic development. Even though casp^{lof} females show lowered fecundity, the oogenesis progression and ovary morphology seems to be completely normal and thus the defect in the embryos is independent of oogenesis. DAPI staining of *casp*^{lof} embryos show that they are undergoing nuclear divisions and progressing to gastrulation (stage 7-8) but do not develop past stage 10-15. This suggests that maternal loss of *casp*/Casp is in some ways affecting the developmental programme late during gastrulation.

Domain structure of Casp (Fig. 4A) shows that it has a FAS associating region (amino acids 1-214) for functions similar to FAF1 protein; DED-interacting region (amino acids 231-421) for interaction with proteins having death effector domain (DED); UAS domain (amino acids 376-524), which falls under thioredoxin-like superfamily known to regulate protein activity through changes in the thiol groups redox state (S2 to SH2) (Buchanan et al. 2005); a UBX domain (amino acids 620-695), which is found in ubiquitin-regulatory proteins (Kim et al. 2006). A molecular/physical interactome (Fig. 15A) shows that Casp and FAF1 interact with many proteins of the proteasomal degradation pathway, most notably p97 (Ter94 in *Drosophila*, VCP in humans) and it is well-established in literature that UBX domain proteins are in general cofactors of p97 (Schuberth et al. 2008). Presence of UBX and UBA domains similar to that of FAF1 strongly suggests that Casp might be involved in similar molecular pathways as FAF1. FAF1 has a non-canonical FFAT-like domain with which it interacts with the MSP domain of VAPB and simultaneously interacts with ubiquitinated ER proteins like RPN2 through its UBX domain and recruits p97 (VCP) for proteasomal mediated degradation of the misfolded RPN2 proteins (Baron et al. 2014).

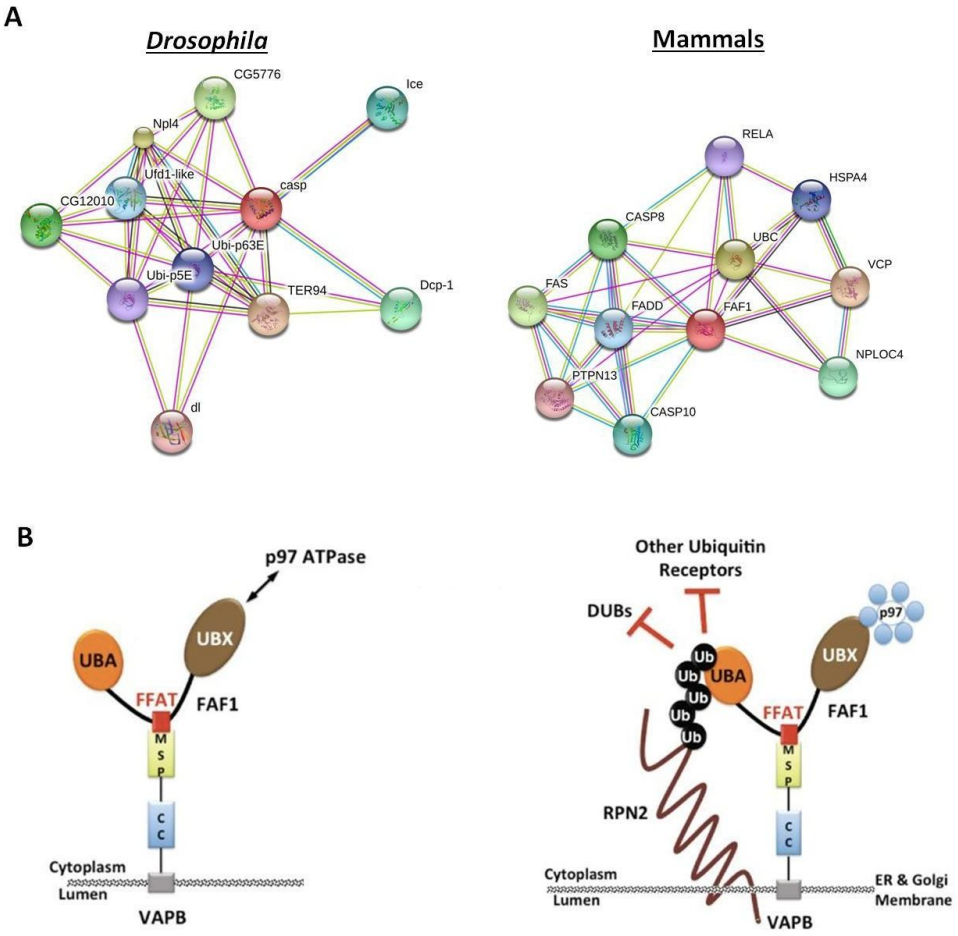

Fig. 15. Casp and FAF1 interactome (A) Inter-actors maps for Casp and FAF1(Adpated from STRING CONSORTIUM) **(B)** FAF1 interacts with VAPB. FAF1 through its FFAT-like motifs interacts with MSP domain of VAPB to recruit p97 (VCP) to ER membrane. FAF1 recruits p97 hexamer for extracting ubiquitinated RPN2 from ER and taking it to proteasome mediated degradation (Adapted from Baron et al. 2014).

Interaction of FAF1 with VAPB helps regulate ER protein homeostasis. ER being a major platform for protein synthesis requires stringent quality control to ensure its homeostasis and proper cell functioning. ER quality control include: unfolded response (UPR), Endoplasmic Reticulum associated Degradation (ERAD), and autophagy (Qi et al. 2017). VCP-Ufd1-Npl4 complex is associated with proteasomal degradation of proteins from ER membrane (ERAD) (Fig. 16A), mitochondrial membrane, ribosome and other multi-protein assemblies (Le et al. 2016). FAF1 plays a key role as an adaptor protein ERAD pathway by modulating the domain-domain interactions. VCP-Npl1-Ufd1 complex through VCP interacts with UBX domain of FAF1 which facilitating the recruitment of polyubiquitinated proteins

to the UBA domain of FAF1 (Fig. 16B), thus promotes endoplasmic reticulum associated degradation(Lee et al. 2012).

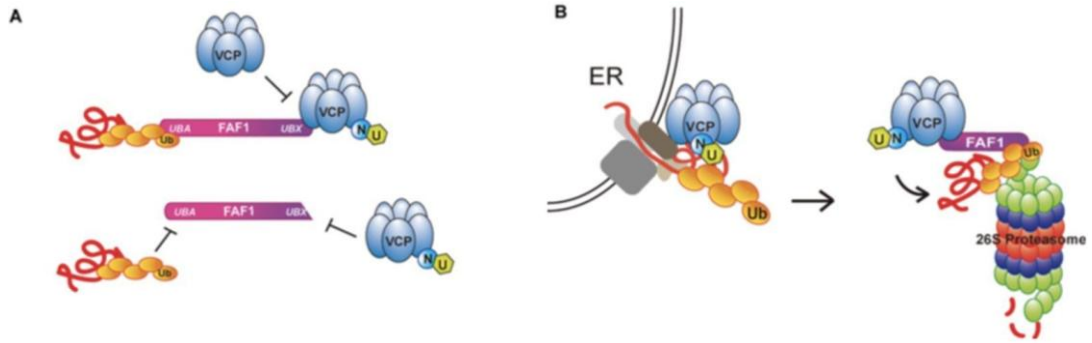

Fig. 16. FAF1 regulates the VCP:Npl4:Ufd1 Complex. (A) FAF1 interaction with the VCP-Npl4-Ufd1 complex through its UBX domain regulates recruitment of the polyubiquitinated proteins to UBA domain. **(B)** FAF1 acts as scaffolding protein to promote ERAD through UBX and UBA domain interaction. N, Npl1; U, Ufd1; Ub, Ubiquitin (Adapted from Lee et al. 2012).

As the embryonic development progresses there is a maternal to zygotic transition wherein all the maternally deposited products such as mRNAs and proteins are degraded (Tadros et al. 2009), (Fig. 17). As FAF1 plays a major role in proteasomal degradation pathways such ERAD, we hypothesize that loss of Casp/dFAF1 in the embryo might be playing a critical role in the degradation of the maternally derived proteins.

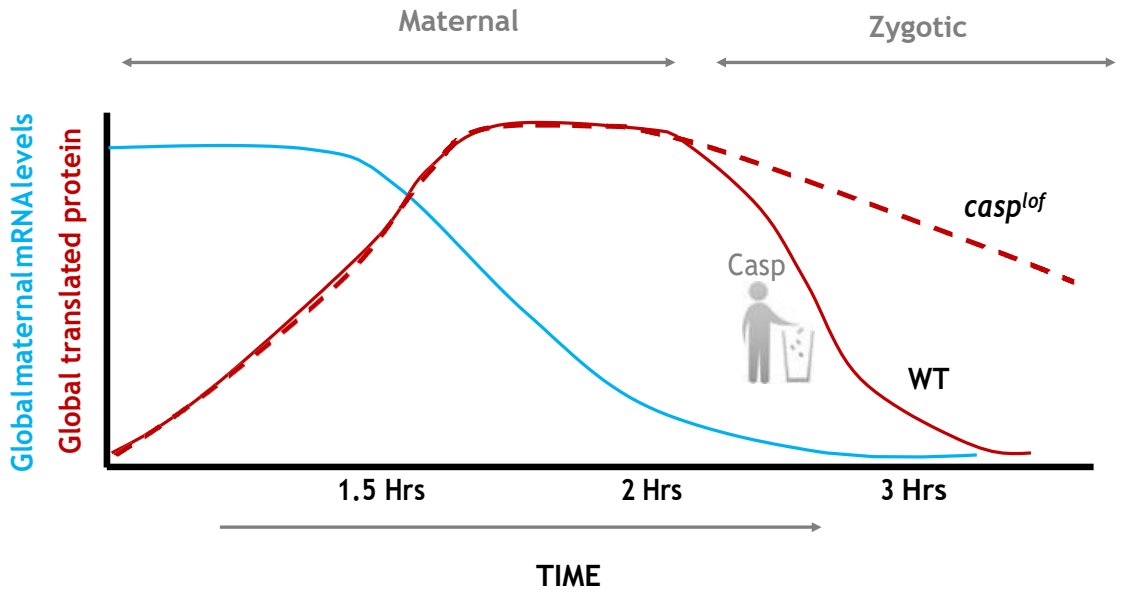

Fig. 17. Casp regulates degradation of Maternal proteins. A hypothetical model for the effect of casp^{lof} in early development. The graph represents total maternal mRNA (blue) and

maternal protein (brick red; i.e., protein made by translating maternal mRNA). Loss of *casp* mRNA leads to loss of the ability of the embryo to degrade maternal proteins through the proteasomal pathway (Garbage graphic). In the absence of Maternal Casp protein (made from maternal mRNA), maternal proteins are not degraded effectively (dotted line) allowing them to persist in the embryo, post gastrulation. These long lived maternal proteins perturb the normal course of development – leading to stalling of the developmental program post-gastrulation.

This may lead to the accumulation of these proteins, generating ER stress, thus resulting in the death of the embryos. Prolonged ER-stress often leads to apoptosis (Szegezdi et al. 2006), and the fact that $casp^{lof}$ embryos show increased levels of cleaved Caspase-3 indicating apoptosis, further strengthens our hypothesis. In-order to test our hypothesis we are planning to delete UBX domain of Casp using CRISPR/Cas9 technique. If the deletion of UBX domain alone gives same results as the $casp^{lof}$ it will confirm the role of Casp as adaptor of proteasomal degradation pathway. Also profiling for amount ubiquitinated proteins in $casp^{lof}$ embryos will be a further focus of our study.

CONCLUSION

In my Master's project I studied the role of Casp in the *Drosophila melanogaster* early embryonic development using the *casp* loss of function allele, $casp^{c04227}$. I find that *casp* is a Maternal Effect gene, which is a novel function for *casp.* Casp protein appears to play a critical role in the early embryonic (0-3 hour) development that affects the development post gastrulation. The Casp protein is itself not maternally deposited, but is actively translated from the maternally deposited *casp* mRNA. $casp^{lof}$ embryos fail to deposit any cuticle and also fail to form a major organ system, the central nervous system. The 75% of the $casp^{lof}$ embryos which are lethal stall between embryonic stage 7-11. $casp^{lof}$ females show lowered fecundity as compared to the wild type, but do not show any ovary or oogenesis defects. The reason to why do 25% of the $casp^{lof}$ escape the embryonic lethality needs to be further evaluated. We predict, based on known functions for the Casp mammalian ortholog FAF1, that Casp works to actively degrade maternal protein products during the maternal to zygotic transition.

MATERIALS & METHODS

***Drosophila* Strains and Fly Genetics**. Flies were maintained on standard yeast-cornmeal media. *casp*^{lof} was generated using a p-element insertion in 5'-UTR of *casp* (w[1118]; PBac{w[+mC]=PB}casp[c04227]) and VALIUM20 *casp* RNAi (y[1]sc[*]; P{y[+t7.7]v[+t1.8]=TRiP.HMS02742}attP2) were obtained from Bloomington Stock Center. Wild-type control used was w^{1118} (Dmel\w^{1118}). Knockdown of Casp in female germline was achieved through mating *casp* TRiP to MTD-Gal4 (BDSC 31777, maternal triple driver), which drives the expression of dsRNA throughout oogenesis (Petrella et al. 2007), or α-tubulin-Gal4:VP16 (BDSC 7062, mat-Gal4) which drives expression in cystoblast and later stages of oogenesis but not in the female germline stem cell (Radford et al. 2012). Also knocking down *casp* in the follicle cell of the ovary was achieved using C587-Gal4 (BDSC 67747), which drives expression in the somatic cells of the ovaries. The crosses will yield progenies having the *casp* dsRNA (*casp* RNAi) driven through the respective Gal4, and these flies were used for further studies.

Embryonic Lethality Assay. Approximately 40-50 pairs of flies of required genotype were placed in an egg collecting chamber. A small sucrose agar plates (3% w/v agar, 2.5% w/v sucrose) with some yeast paste was placed at the chamber's opening. Flies were allowed to acclimatize for 24 hours at 25 ^{o}C. Eggs were collected after 3 hours after placing a fresh sucrose agar plate. A 100 embryos in 10X10 grid were arranged on a new plate and keep them at 25 ^{o}C for 24 hours (larvae emerge after 22-24 hrs of fertilization). After 24 hours the number of embryos hatched was counted.

Larval Lethality Assay. A similar egg collection and arranging the embryos in a 10X10 grid followed by 24-hour incubation at 25 ^{o}C was performed. The number of hatched embryos were counted which are equivalent to a total number of larval present in the sucrose agar plate. The sucrose agar gel from these plates were transferred to individual fly media bottles and checked for number of pupas developed and flies eclosed to give the larval lethality count.

Cuticle Preparation*. Drosophila* embryos of required genotype were obtained using the egg collection chamber and were allowed to age for 24 hours. The embryos/larvae were collected and washed using water and dechorionated using

100% bleach. The embryos/larvae were then transferred to scintillation vials containing ethanol. The embryos/larvae were then mounted on a glass slide using 80% lactic acid (to dissolve internal organs and leave the cuticle intact) and kept on a heating pad at 55 ^{o}C overnight. The cuticles were then visualized under Apotome microscope in dark field at 10X magnification.

Embryo Collection and Immunostaining. Embryos of required age and genotype were collected using egg collection chambers and washed using water. Embryos were dechorionated using 100% bleach and fixed for 20 minutes using equal volumes of heptane : 4% paraformaldehyde in 0.3%PBST (0.3% Triton X-100 in PBS {137 mM NaCl, 10mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4}) at room temperature. Embryos were devitellinized by vigorously shaking the embryos in heptane : methanol (in ration 1:1). The embryos were re-hydrated using PBS and washed 5X using PBS. Blocking was done using 2% BSA (Bovine Serum Albumin) in 0.3% PBST and incubated in primary antibody solution (primary antibodies diluted in blocking solution) overnight: 1:500 Casp (BIOKLONE), 1:150 cleaved caspase 3 (Cell-signalling), 1:500 HRP (Sigma-Aldrich), 1:20 Futsch (DSHB), 1:10 Repo (DSHB). After incubation in primary antibody solution, the embryos were washed using 0.3% PBST and incubated in secondary antibody solution (Alexa Fluor, ThermoFisher Scientific) for 1 hour and then washed with 0.3% PBST thrice, with DAPI in the second wash. The embryos were then mounted using anti-fade mounting media on a glass slide and imaged using Leica-SP8 confocal microscope.

Ovary Immunostaining. Flies were placed in vials containing yeast powder for a day and ovaries were dissected out the following day. Ovaries were immediately fixed using 4% paraformaldehyde in 0.3% PBST for 20 minutes and washed using 0.3% PBST six times. The ovaries were blocked using 2% BSA in 0.3% PBST solution for an hour and incubated with primary antibody solution overnight: 1:500 Casp (BIOKLONE), 1:20 Orb (DSHB), 1:200 488 Phalloidin (Sigma-Aldrich). The ovaries were then washed using 0.3% PBST and incubated in secondary antibody solution for an hour. After washing the ovaries with 0.3% PBST and addition of DAPI the ovaries were mounted by separating out the ovarioles in anti-fade mounting media on the glass slide and imaged using Apotome and Leica-SP8 confocal microscopes.

Western and Immunoblotting. Tissue samples were crushed in RIPA (10mM Tris-Cl [pH 8.0], 1mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1%, 0.1% SDS, 140 mM NaCl) with 1X PIC (protease inhibitoe cocktail). Samples were processed by adding SDS loading dye (62.5 mM Tris-HCl [pH 6.8], 2.5% SDS, 0.002% Bromophenol Blue, 5% β-mercaptoethanol, 10% glycerol) and heating at 95 ^{o}C. Processed samples were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel and electrophoresed according to lab protocol. The proteins were then transferred to an activated polyvinylidene difluoride (PVDF) membrane and blocked using 5% milk in 0.1% TBST (0.1% Tween 20 in TBS {50mM Tris-Cl [pH 7.5], 150 mM NaCl}) The blot was then kept in primary antibody solution overnight: 1:20,00 Casp (BIOKLONE), 1:10,000 α-tubulin (DSHB). After washing the blot with 0.1%TBST thrice, the blot was kept in secondary anti mouse-HRP or anti-rabbit HRP antibody solution for an hour. After 3 washes with 0.1%TBST the blot was developed using HRP substrate and luminol and visualized using chemi-luminescence.

Female Fecundity Assay. To assay for fecundity (Eggs per female per day), single parent crosses (w^{1118} x w^{1118} and $casp^{lof}$ *x* $casp^{lof}$) were set and placed in an egg collection chamber. A day was given for acclimatization and mating. Day 2 onwards fresh sucrose agar plates with yeast paste were placed in the egg collection chamber and after 24 hours were replaced with a fresh sucrose agar plate with yeast paste, repeating the same for next 11 days until day 12. Each day number of eggs laid were counted for each single parent cross to give a data point.

Casp SUMOylation in not required for embryonic development

Casp is known to be SUMOylated (Handu et al. 2015) and *casp*^{lof} being protein null inherently lacks SUMOylation. In order to check whether loss of Casp SUMOylation is involved in the embryonic lethality, we repeated lethality and cuticle preps using SUMO resistant casp^{K551R} lines. SUMOylation of Casp doesn't seem to be necessary for embryonic development as a SUMO resistant casp^{K551R} lines do not show defects as shown by the casp^{null} flies (Fig. 18 A). This also suggests that casp^{K551R} alleles are at par with wild type in early embryonic development.

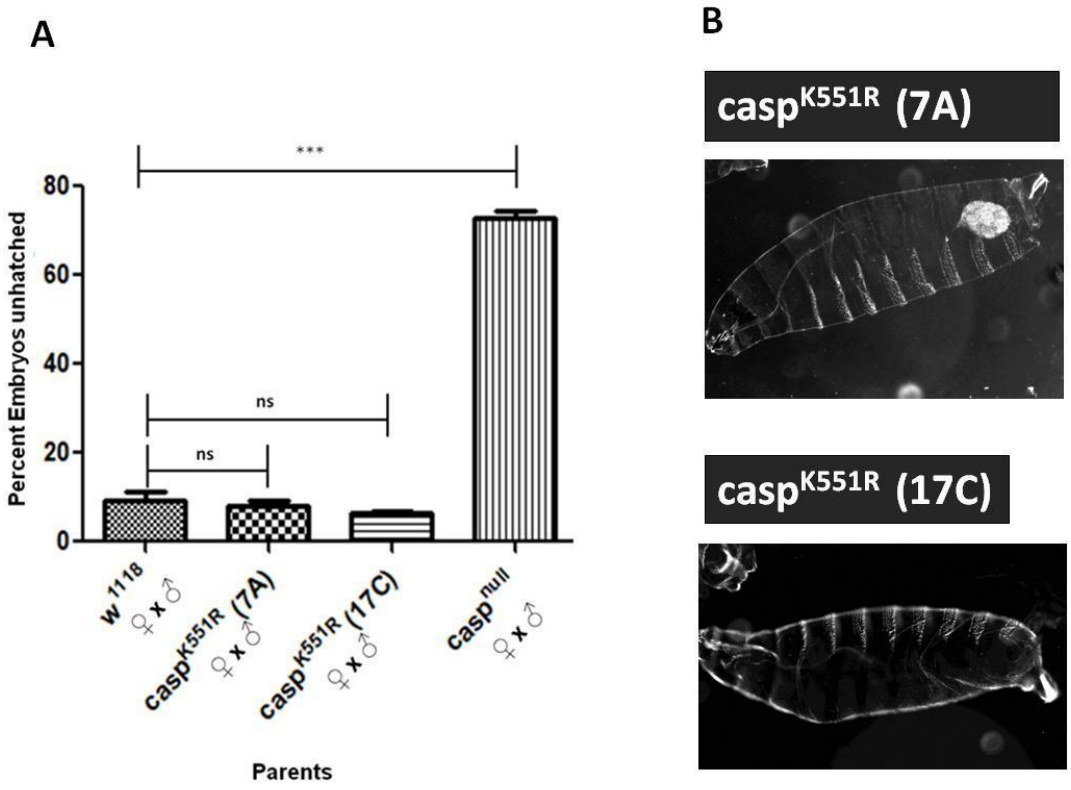

Fig. 18. SUMOylation of Casp is not required for embryonic development.**(A)** A graph showing percent embryonic lethality where w^{1118} has ~ 9% lethality, *casp*^{K551R} 7A line has ~ 8% lethality, *casp*^{K551R} 17C line ~ 6 % lethality and *casp*^{lof} ~ 75% lethality. Loss of SUMOylation of Casp does not seem to affect embryonic development as the lethality for w^{1118}, *casp*^{K551R} 7A line and *casp*^{K551R} 17C line are similar. For each replicate (N=3), n = number of embryos = 300. Students t-Test was used for analysis. p-value > 0.1. **(B)** Cuticle preps for *casp*^{K551R} 7A and *casp*^{K551R} 17C. N = 2 , n = number of embryos = 100

No patterning defects are observed in SUMO resistant $casp^{K551R}$ lines, and all denticle bands and head and tail structures are present (Fig. 18B). The number of eggs laid by the $casp^{K551R}$ lines is similar to w^{1118}. All these results point to the fact that its Casp and not SUMOylation of Casp that is necessary for the embryonic development and fecundity and/or fertility of the flies.

Contributions: Bhagyashree Kaduskar for SUMO resistant $casp^{K551R}$ lines using CRISPR/Cas9 technique.

Appendix II

Casp in the Innate Immune Response

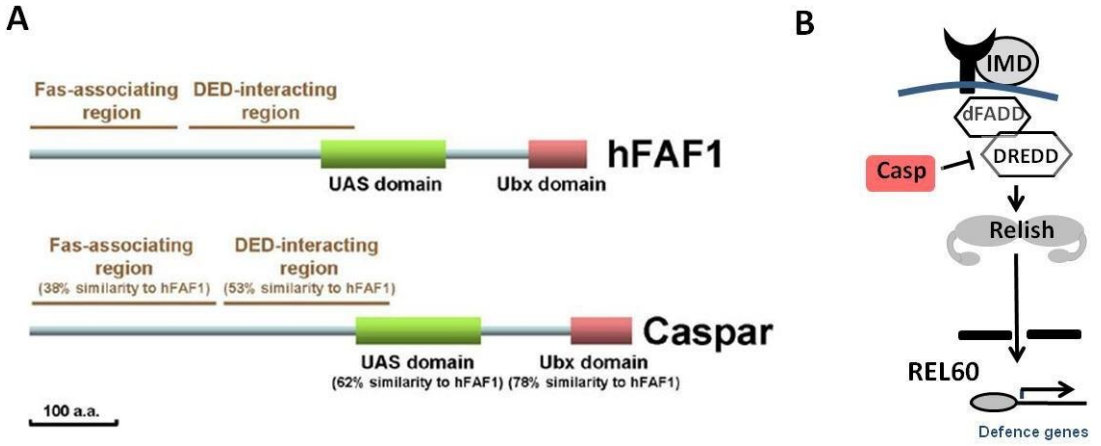

Fig. 19. Casp in *Drosophila* (A) Structural comparison between *Drosophila* Casp and Human FAF1. A 38% similarity of fas-associating region and 53% similarity of DED-interacting region to hFAF1 and highly conserved and similar UAS domian and Ubx domain with similarity to hFAF1 of 62% and 78% respectively.**(B) The IMD signaling pathway.** Casp has been demonstrated to be a negative regulator of IMD/REL signaling. It is believed to act by suppressing Dredd cleavage of REL. Adapted from Kim et al. 2006

REL is important and central to the Imd Pathway, gets cleaved upon activation into two fragments: an N-terminus fragment REL-68 which gets translocated to the nucleus and activates transcription of anti-microbial peptides like Diptericin (Myllymäki et al. 2014) (Fig. 19B). Casp regulates the levels of immune activation and anti-microbial peptide synthesis by inhibiting the cleavage and subsequent activation of REL (Kim et al. 2006). Casp is not involved in the Toll pathway, which is an independent immune pathway that regulates antifungal as well as anti-Gram-positive bacteria immunity (Kim et al. 2006). Casp is known to be SUMOylated at K551 (Handu et al. 2015) (Fig. 20). **S**mall **U**biquitin-like **Mo**difier (or **SUMO**) is a post-translational modification involved in various cellular processes, such as nuclear-cytosolic transport, transcriptional regulation, protein stability, response to stress and progression through the cell cycle (Hay et al. 2005). The addition of SUMO to its target proteins occurs with the assistance of the E1, E2, and E3 enzymes similar to that of the ubiquitin pathway, the enzymatic machinery, however, being distinct from the ubiquitin cycle (Hay et al. 2005).

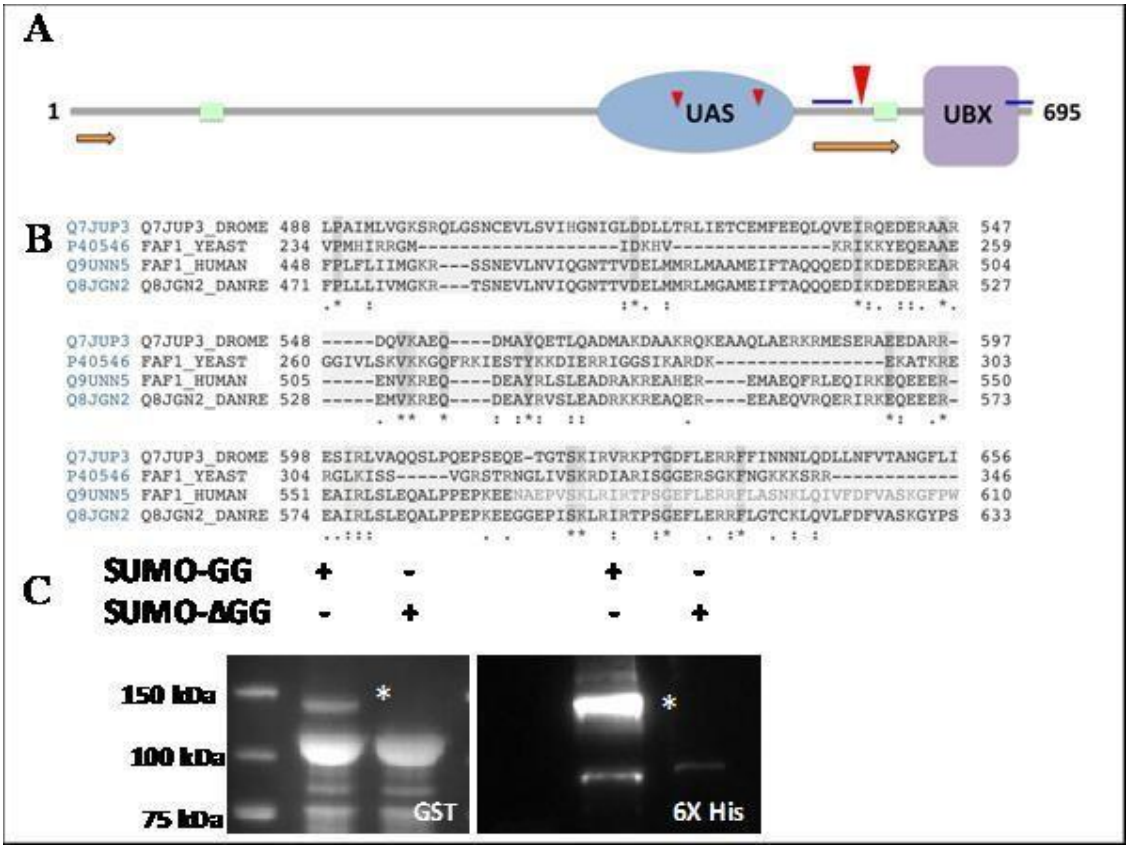

Fig. 20. *Drosophila* Casp SUMOylation. (A) Domain structure of Casp .The UAS and UBX domains are marked with potential SUMOylated sites (red arrowheads). The largest arrowhead (K551) has a consensus SUMO acceptor site sequence. The grey line indicates disordered regions, green boxes are low complexity regions, while the orange bars indicate regions with a predicted helical structure. **(B)Sequence alignment of FAF1 proteins from *Drosophila*, yeast, human and zebrafish.** Figure shows a section of the aligned sequences that contains the predicted SUMO acceptor site (VK551AE) and is conserved from yeast to man. **(C)Casp is SUMOylated.** Casp is SUMOylated when tested using the in-bacto system. An additional band (*) is seen for GST-SUMO in the presence of activated SUMO (SUMO-GG) but not when SUMO-GG, an inactive form of SUMO that cannot conjugate is used in the SUMOylation assay. Data adapted from Handu et al., 2015.

Studies done in S2 cells in our lab shows that both the SUMOylated and non-SUMOylated form of Casp exist in S2/529SU cells with stress causing a transition to the SUMOylated state (Handu et al. 2015). Mutation of lysine K551 to arginine showed loss of SUMO-modified form of the protein. The Casp SUMO-resistant mutant fly, *casp(K551R)* has been generated using CRISPR-Cas technique by Bhagyashree Kaduskar in our lab. How SUMOylation of Casp modulates the protein functionality is our topic of interest. This subtle post-translational modification can provide insights into to the mechanism of action Casp.

Kinetics of REL cleavage upon *E.coli* infection.

Casp is known to act as a negative regulator of the IMD signaling pathway by blocking DREDD dependent cleavage of REL (Kim et al. 2006). To check whether Casp SUMOylation affects the IMD signaling pathway in a differential manner than non-SUMOylated Casp I used the SUMO deficient line *casp*^{K551R} to check the REL cleavage and kinetics upon *E.coli* infection. It is known that upon *E.coli* ,a gram-negative bacteria activate the IMD pathway. REL gets cleaved upon activation into two fragments: an N-terminus fragment REL-68 which gets translocated to the nucleus and activates transcription of anti-microbial peptides like diptericin and C-terminal REL-49 fragment which is cytoplasmic at is degraded (Kim et al. 2006). I did western blot analysis to check for the REL kinetics upon *E.coli* for *w*^{1118} and *casp*^{K551R}. In the two replicates done using DSHB (Anti-REL - C -21F3) antibody which detects the C-terminal fragment, so the antibody should detect the full-length REL and REL-49 fragment. I get prominent band below 75 kD and no band at 110 kD and 49 kD (upon infection) which is something surprising and unexpected. From this data to comment on the REL cleavage kinetics at this moment is not appropriate and possible with the data I have. In order to overcome this problem, I have developed a REL tagged GFP recombined with casp^{K551R} so that I can track GFP as a proxy for REL in the SUMO deficient Casp lines.

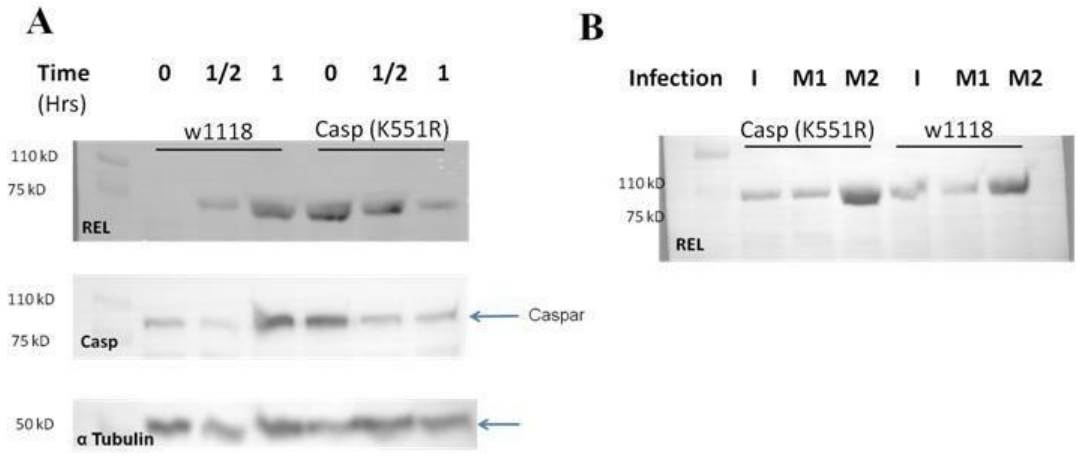

Fig. 21 REL Kinetics in w^{1118} and casp^{K551R} upon *E.coli* infection. (A) Time-dependent infection experiment for 3rd instar larvae of w^{1118} and casp^{K551R} for 0 minutes, 30 minutes and 1-hour post-*E.coli* infection. **(B)** Infection experiment for 3rd instar larvae *w*^{1118} and *casp*^{K551R} pricked with *E.coli* , M1 (pricking with distilled water) and M2 (pricking with just the needle) . Both blots show bands at ~ 75 kD which is not expected to show up as the antibody used was a C-terminal REL antibody.

Generation of REL:GFP, Casp^{K551R} recombinant line.

As the REL antibody did not work, I generated a REL:GFP, *casp^{K551R}* recombinant line to carry my immunity experiments (strategy shown in Fig. 22A) using *casp^{K551R}* lines and REL:GFP (BDSC 43956, w[1118]; PBac{y[+mDint2] w[+mC]=REL:GFP.FPTB}VK00037/SM5). A total of 250 lines were made for the screening of the REL:GFP, *casp^{K551R}* recombinant line, of which 86 were used to screen through PCR and restriction digestion using BssHII. Casp^{K551R} lines are in the *w^{1118}* background and REL:GFP gives an orange eye colour which was used to identify REL:GFP positive flies.

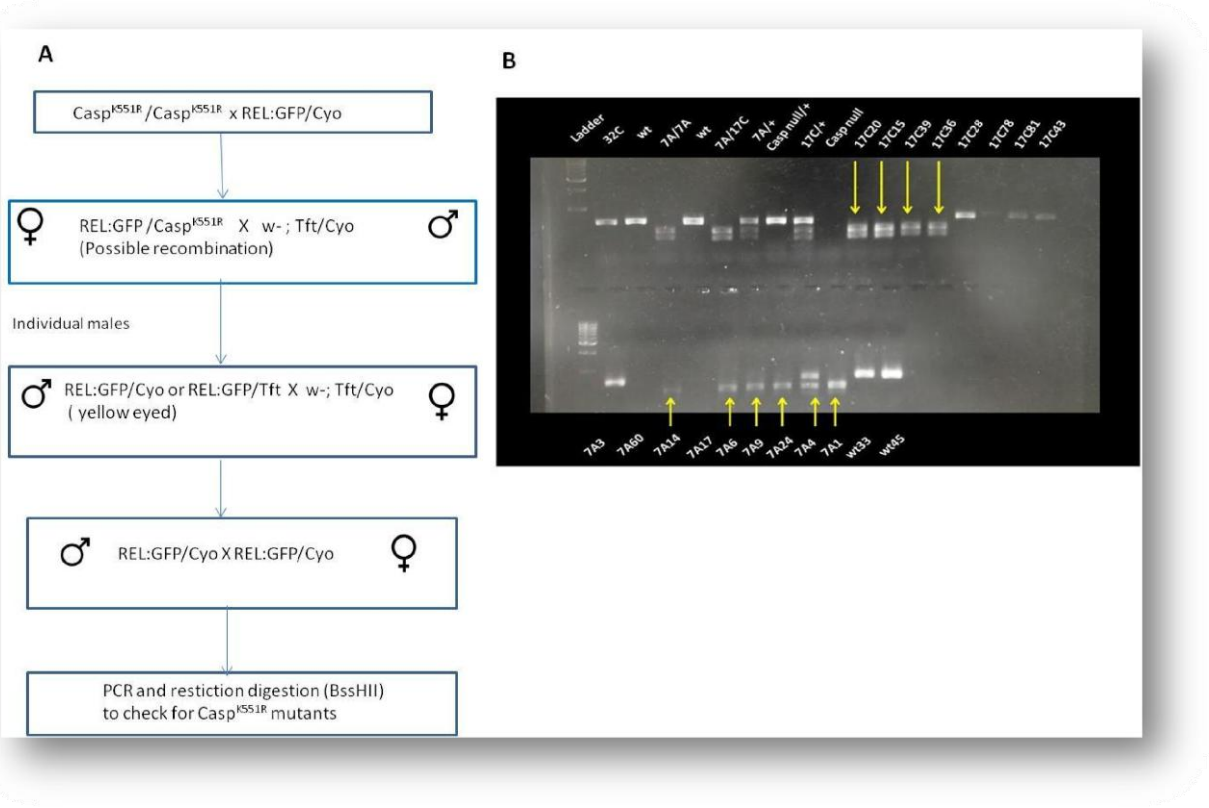

Fig. 22 Generation of the recombinant lines (A) Strategy for making REL:GFP, *casp^{K551R}* recombinant line. Homozygous *casp^{K551R}* lines were crossed to a 2nd balanced REL:GFP (Table 2), whose progeny were balanced by crossing to a 2nd chromosome balancer. Individual males from F1 of these crosses were again balanced using a 2nd chromosome balancer. The balanced REL:GFP were selfed and homozygous REL:GFP

were used for PCR screen to identify lines with $casp^{K551R}$ allele. **(B) Screening for REL:GFP, $casp^{K551R}$ recombinant line.** An agarose gel for restriction digested using BssHII of PCR amplified *casp* locus . Blue arrows show the positive lines which have $casp^{K551R}$ mutation. 32C denotes CRISPR control for $casp^{K551R}$, wt is w^{1118}, 7A is $casp^{K551R}$ line 1 and , 17C is $casp^{K551R}$ line 2.

The substitution K551R incorporated the unique restriction site which can be identified by the restriction enzyme BssHII. $casp^{K551R}$ upon PCR amplification and restriction digestion gives 2 bands; one at 400bp and other at 350 bp on the agarose gel, whereas Casp wt gives a band at 750 bp. Using this screening strategy 11 recombinant lines out of the 86 were obtained. The identified lines are 17C20, 17C15, 17C29, 17C36, 7A14, 7A17, 7A9, 7A6, 7A24, 7A4, 7A1 (Fig. 22B). All the lines are pure population arising from single parents. These line will be used for further experiments to study the kinetics and dynamic of REL cleavage using GFP probes. Getting such a large number of lines allows for complementation analysis and reassure that the phenotypes arise from our target gene and not any off-target effects.

Contributions: Bhagyashree Kaduskar for SUMO resistant $casp^{K551R}$ lines using CRISPR/Cas9 technique.

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