Role of Caspar and Ter94 in Early *Drosophila* Development

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by

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INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

CERTIFICATE

This is to certify that this dissertation entitled 'Role of Caspar and Ter94 in Early *Drosophila* Development' towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Shruti Pawar at Indian Institute of Science Education and Research under the supervision of Prof. Girish Ratnaparkhi, Professor and Dean (Academics), Department of Biology, IISER Pune during the academic year 2022-2023.

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Committee: Biology

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DECLARATION

I hereby declare that the matter embodied in the report entitled 'Role of Caspar and Ter94 in Early *Drosophila* Development' are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Prof. Girish Ratnaparkhi and the same has not been submitted elsewhere for any other degree.

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Date: 1st April, 2023

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Caspar in *Drosophila* is an ortholog of mammalian Fas Associated Factor 1 (FAF1) and its domain structure is suggestive of the function of Casp protein being conserved between fruit flies and humans. It has been studied in the context of fly immunity; however, it has been shown in the lab to have a developmental role as well.

Casp expresses maternally. It interacts with ER-associated degradation protein Ter94 (Transitional endoplasmic reticulum 94). Casp and Ter94 are required for cell division in the early embryo and their loss leads to nuclear and cytoskeletal defects. Also, developmental progression of *casp*^{lof} embryos stalls before gastrulation which is an important step in *Drosophila* embryogenesis. Further, Casp and Ter94 have been shown to play a role in pole cell formation.

Based on its domain structure, we hypothesize that Casp might be functioning as an adapter for the ER-associated degradation pathway, helping to target unfolded proteins to the ubiquitin-proteasomal system, by connecting ubiquitinated proteins (bound by N-terminal UBA domain) to Ter94 (possibly bound by C-terminal UBX domain).

We choose Me31b (Maternal expression at 31B), a protein involved in the global repression of maternal mRNAs during the maternal to zygotic transition (MZT) in the early *Drosophila* embryo, as a target protein to check if Casp alone or in conjunction with Ter94 has a role in degrading proteins during the MZT. In our study, data is suggestive of Ter94 not playing a role in Me31b degradation and whether Casp plays a role in the same remains an open question.

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CONTRIBUTIONS

Contributor name	Contributor role
Shruti Pawar, Prof. Girish Ratnaparkhi and Dr. Sushmita Hegde	Conceptualization Ideas
Shruti Pawar, Prof. Girish Ratnaparkhi and Dr. Sushmita Hegde	Methodology
–	Software
–	Validation
Shruti Pawar	Formal analysis
Shruti Pawar	Investigation
Prof. Girish Ratnaparkhi and Dr. Sushmita Hegde	Resources
Shruti Pawar	Data Curation
Shruti Pawar	Writing - original draft preparation
Shruti Pawar and Prof. Girish Ratnaparkhi	Writing - review and editing
Shruti Pawar	Visualization
Prof. Girish Ratnaparkhi and Dr. Sushmita Hegde	Supervision
Prof. Girish Ratnaparkhi	Project administration
Prof. Girish Ratnaparkhi	Funding acquisition

CHAPTER 1 – INTRODUCTION

Development is a tightly regulated process in organisms which comprises of a series of linked events and changes in terms of cell number and morphology, morphogen gradients, cell fate specification and so on. Synchrony in these processes is brought about by both short range and long-range interactions between cells via extracellular molecules. *Drosophila* embryo development is initiated post fertilization with rapid free nuclear divisions resulting in the formation of a syncytium that develops into the blastula, which undergoes gastrulation (Adapted from Developmental Biology, 6th edition). In the first few hours of development, anteroposterior (A/P) and dorsoventral (D/V) axis are laid down by a combined patterning by three systems – A/P system, D/V system and terminal system. Cell fate specification is orchestrated by the activity of these systems during early development. It is interesting to note that the activation of these systems is under maternal control, i.e., it is under the control of events happening in the developing egg inside the mother's body, much before fertilization takes place (Weaver et al., 2018).

Setting up A/P and D/V Axes

During the maturation of oocytes in the ovaries of female flies, maternal dumping of huge reserves of mRNA, lipids, proteins and glycogen takes place. Such genes whose gene products like mRNAs or proteins are loaded into oocytes during their development by the nurse cells of the mother are called maternal effect genes (Pagan and Orr-Weaver, 2018). Cell polarity is determined during oogenesis itself by the localization of several of these dumped maternal mRNAs to specific sites within the oocyte: *bicoid* to the anterior, *oskar* to the posterior and *gurken* to the dorsal-anterior corner (Volhard et al., 1987). The D/V axis is a result of differential levels of Toll pathway activation creating a dorsoventral gradient in the nuclear uptake of the transcription factor dorsal (Moussian et al., 2005). The A/P axis is a result of gradients of Bicoid, Hunchback, Caudal and Nanos. These are involved in the regulation of transcription of gap genes and segmentation genes (Adapted from Developmental Biology, 6th edition). Terminal structures like the head and the tail are a result of terminal genes like *torso*, *tailless* etc. (Lu et al., 1993).

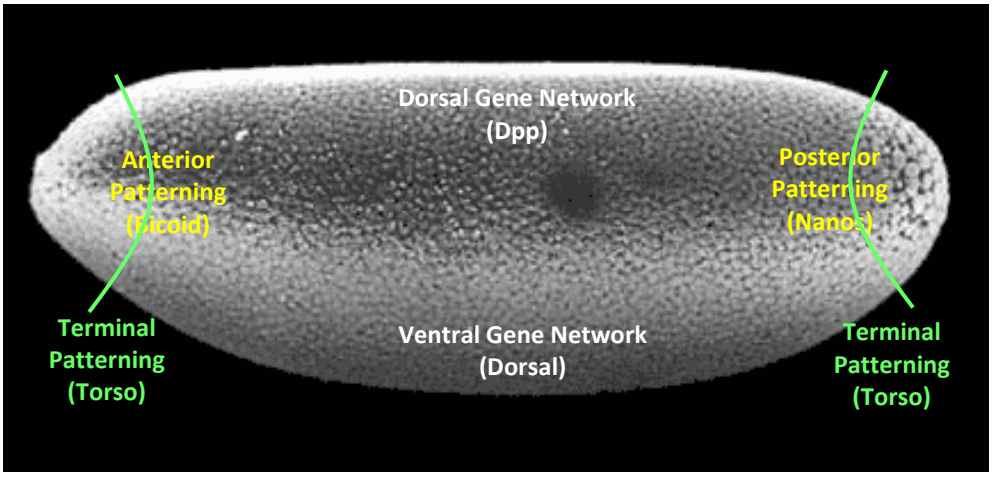


Figure 1: Setting up A/P and D/V axes. A Scanning Electron Micrograph of early *Drosophila* embryo showing players involved in A/P and D/V patterning.

Early Development and Maternal to Zygotic Transition (MZT)

Thirteen rapid synchronous free nuclear divisions result in the formation of a syncytial blastoderm (Fig. 2).

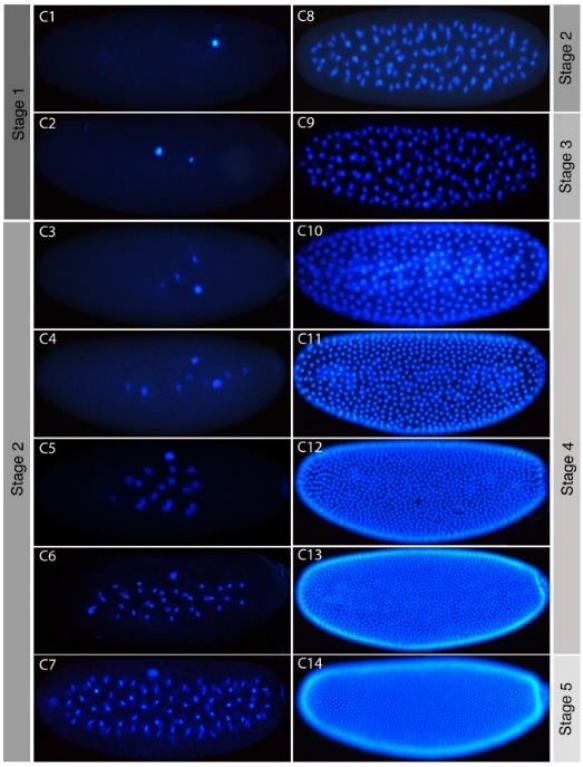


Figure 2: DAPI staining showing cleavage cycles (C1-C14) with formation of blastoderm at C14 (Adapted from Wotton et al. 2014).

This is followed by cellularization which involves the invagination of the plasma membrane of the syncytium to partition each of these nuclei into cells, leading to the formation of the cellular blastoderm (Turner et al., 1979). The subsequent four cell divisions are asynchronous and become progressively slower. Soon gastrulation takes place which ultimately leads to the segregation of the cells into three germ layers: ectoderm, endoderm and mesoderm. As mentioned earlier, the maternal factors (mRNAs and proteins) deposited into eggs during oogenesis determine the early nuclear divisions and events occurring in approximately the first 2-2.5 hours of development (Tadros et al., 2009).

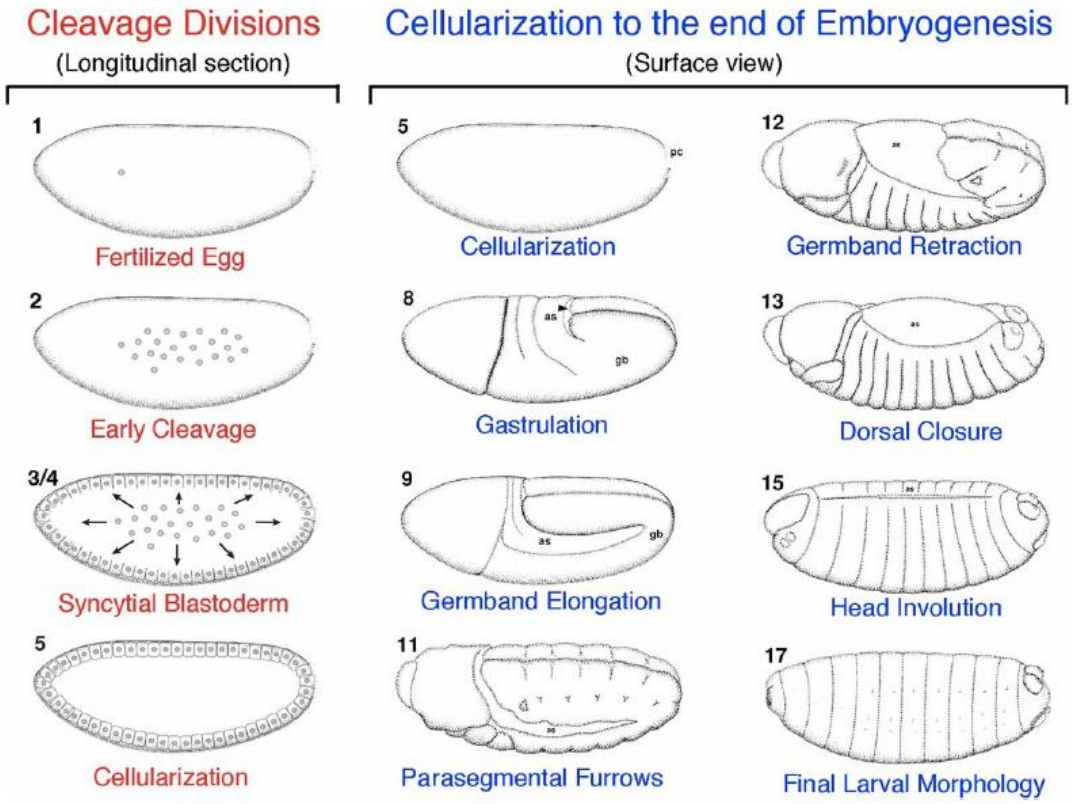


Figure 3: Stages in *Drosophila* embryo development. Illustration of all the stages in embryogenesis of *Drosophila melanogaster* – cleavage, cellularization, gastrulation, germband extension and retraction, dorsal closure etc. (Adapted from David M Roberts, 2015).

A major event in the developmental program of the *Drosophila* embryo is the maternal to zygotic transition (Fig. 4). This is mainly a result of two events: degradation of maternal mRNAs and proteins and the transcriptional activation of the zygotic genome. The latter produces proteins and micro RNAs (miRNAs) that enhance efficiency of the

former process. Transcription of the zygotic genome also sets up a positive feedback loop by the synthesis of transcription factors that enhance transcription of other zygotic genes.

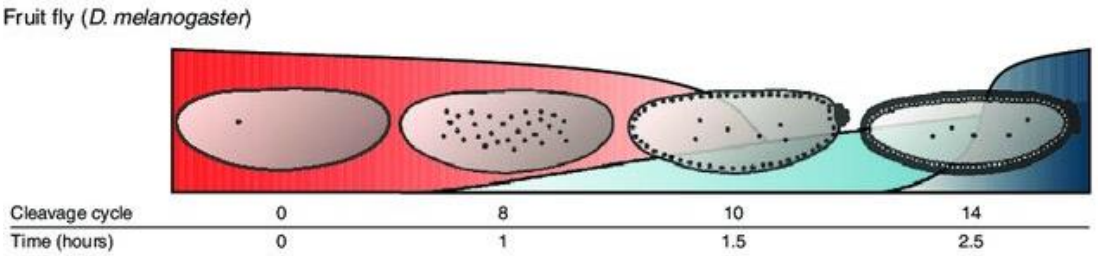


Figure 4: Maternal to Zygotic Transition in *Drosophila melanogaster*. Red marks the profile of mRNA degradation. Light and dark blue mark the waves of zygotic genome activation (minor and major waves respectively) (Adapted from Tadros and Lipshitz, 2009).

Determination of Maternal Effect Genes

Early development is governed by maternal factors like *oskar, dorsal, bicoid, hunchback, nanos and caudal,* to name a few. Mutants of such maternal effect genes do not show developmental defects because they receive wild-type copies from their mothers via mRNA and/or protein deposition into the eggs from which these embryos develop. In fact, the developmental defects are shown by their progeny because now the mutant mothers cannot deposit wild type copies of these genes into the eggs, thus affecting events occurring in early development (approx. 0 – 2.5 hours). Such defects cannot be rescued paternally since the zygotic and hence the paternal genome is not activated before MZT. Many of these genes were identified as part of the large-scale Heidelberg mutagenesis screen during 1970-80 (Volhard et al., 1979).

Caspar is a Maternal Effect Gene Showing Developmental Defects

Caspar has been studied in the context of fly immunity and found to be a negative regulator of immune signaling. It encodes for a protein that inhibits the immune deficiency (Imd) pathway but not the Toll pathway by blocking cleavage and nuclear translocation of Relish, which a *Drosophila* NF-κB protein (Kim et al., 2006). However, it has been shown in the lab to be important in *Drosophila* development too (Hegde et

al., Unpublished). The mammalian ortholog of Casp is FAF1 (Fas-Associated Factor 1) and contains an N-terminal ubiquitin interaction domain (UBA), a ubiquitin-like self-association (UAS) and a C-terminal ubiquitin regulatory domain (UBX) (Kaduskar et al., 2020). There are just a couple of papers on Casp and the number of open questions abound.

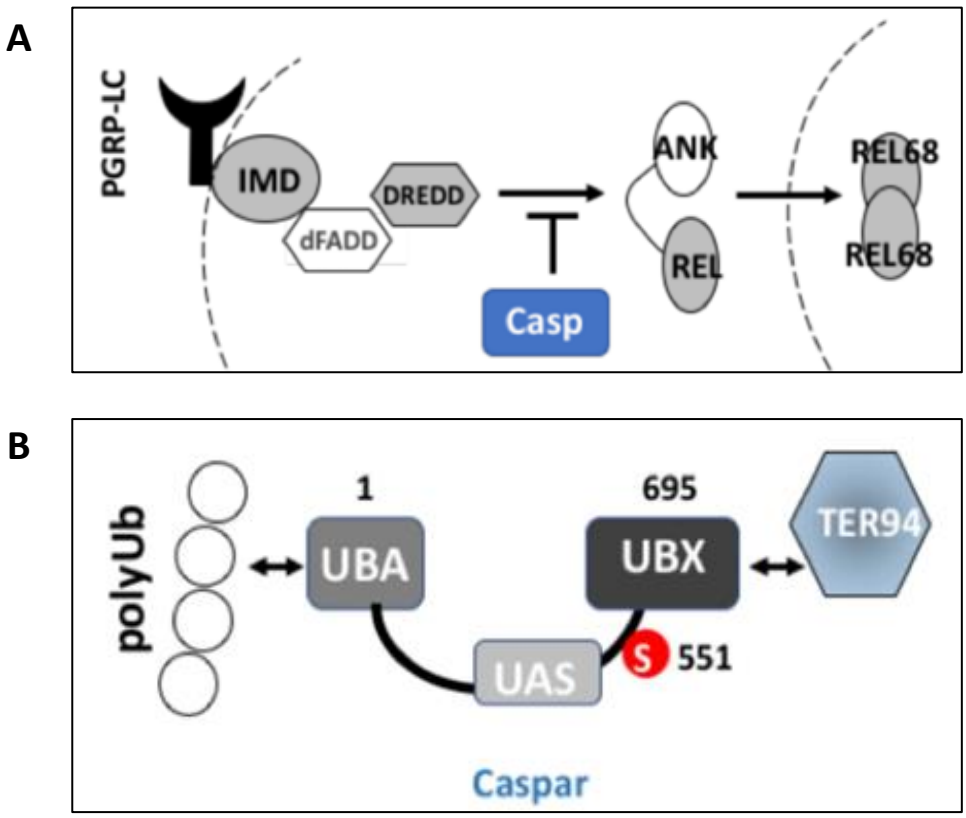


Figure 5: (A) Casp negatively regulates immune signalling by blocking the DREDD dependent cleavage and nuclear localisation of Relish. **(B)** Domain structure of Casp is conserved with its mammalian ortholog FAF1. It interacts with polyubiquitinated proteins via the UBA domain while the UBX domain facilitates its non-covalent interactions with other proteins (e.g., Ter94). (Adapted from Kaduskar et al., 2020).

It has been previously shown in the lab that Casp expresses maternally. It has also been shown to interact with ER-associated degradation protein Ter94 (Transitional endoplasmic reticulum 94). Casp and Ter94 are required for cell division in the early embryo and their loss leads to nuclear and cytoskeletal defects. Also, developmental progression of *casp*^{lof} embryos stalls before gastrulation which is an important step in *Drosophila* embryonic development. Further, Casp has been shown in the lab to regulate primordial germ cell number in the early *Drosophila* embryo. In addition, loss

of Ter94 has been linked to abnormal pole cell formation. Based on the its domain structure, we hypothesize that Caspar might be functioning as an adapter for the ER-associated degradation pathway, helping to target unfolded proteins to the ubiquitin-proteasomal system, by connecting ubiquitinated proteins (bound by N-terminal UBA domain) to Ter94 (possibly bound by C-terminal UBX domain).

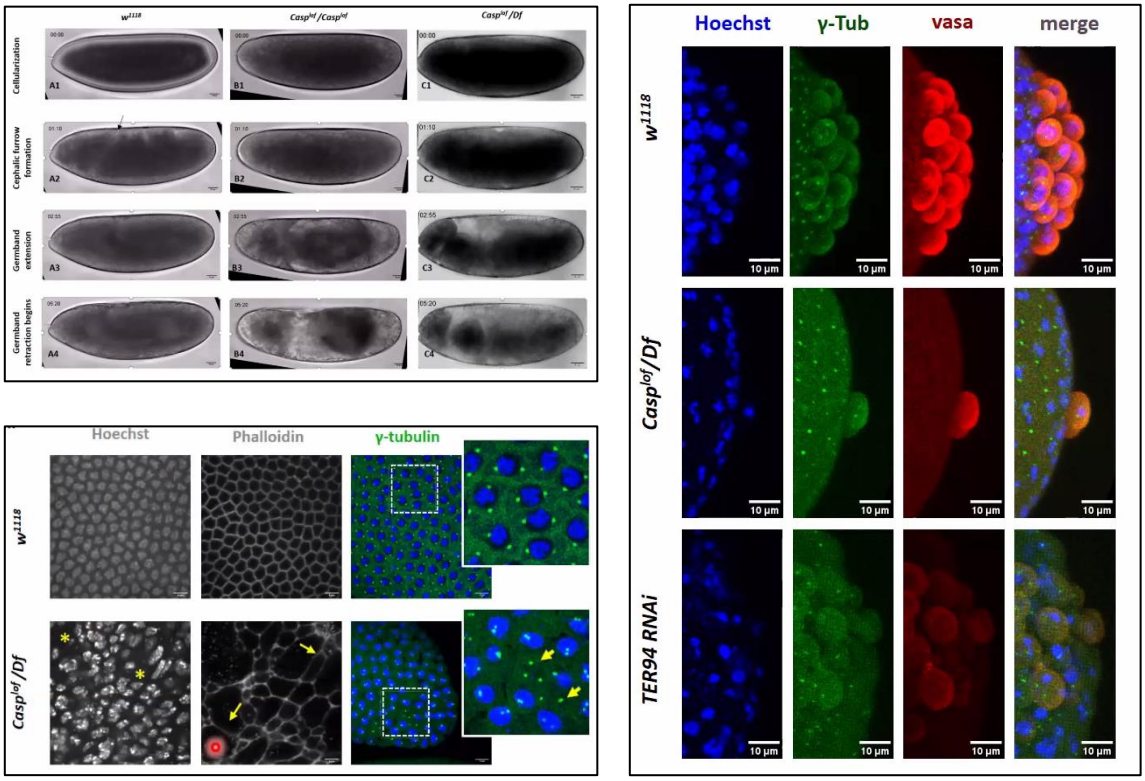


Figure 6: Developmental defects shown by loss of casp and/or Ter94. Developmental progression stalls before gastrulation in *casp^{lof}* mutant embryos. Pole cell formation is affected by loss of Casp and Ter94. (From Hegde et al., Unpublished).

So, the question then is: Is Casp (along with Ter94) involved in degrading proteins during maternal to zygotic transition (MZT) in a developing *Drosophila* embryo? Also, how does Caspar regulate pole cell formation independently or in conjunction with Ter94?

The objective of this project is to find an answer to the first question in the context of the hypothesis mentioned above. A target protein chosen to test the hypothesis is Me31b.

Why Me31b as a Target?

Maternal expression at 31b (Me31b) is involved in the global repression of maternal mRNAs during the maternal to zygotic transition (MZT) in the early *Drosophila* embryo (Wang et al., 2017). It is a maternal effect gene. There is a 10-fold decrease in Me31b levels during MZT (Fig. 7).

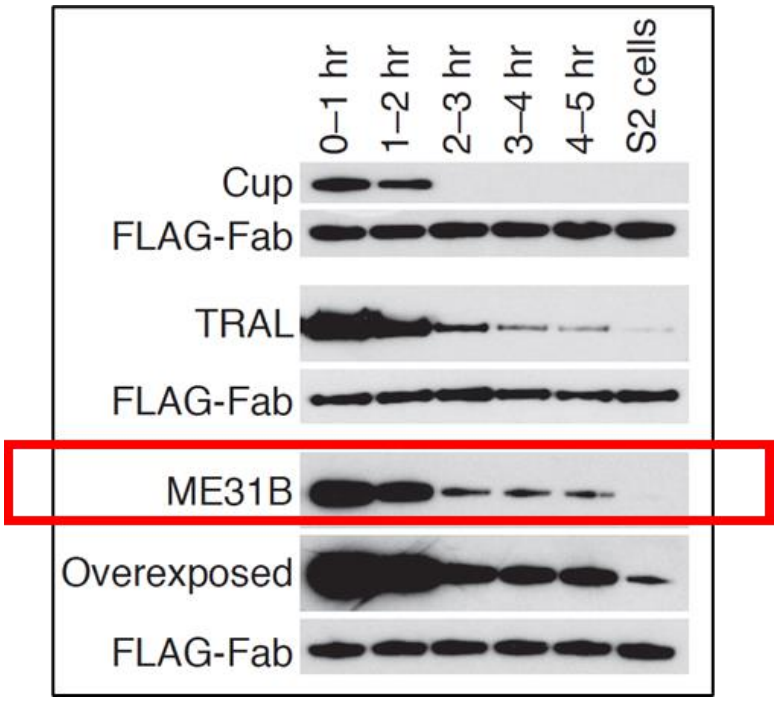


Figure 7: Western Blot showing degradation kinetics of Me31b (highlighted in red) during *Drosophila* embryogenesis. Levels of Me31b protein rapidly decrease after the first two hours of embryo development (Adapted from Wang et al., 2017).

A protein named *smg* is involved in the degradation of maternal mRNAs of *me31b*, however, the proteins and the pathway involved in the degradation of Me31b protein remain elusive (Fig. 8).

Figure 8: SMG is involved in degrading maternal mRNAs of *me31b* but mechanisms of Me31b protein degradation remain elusive.

Me31b has been picked up as an interactor of Casp (Hegde et al., Unpublished). Further, Me31b has been linked to Ter94 in an interactome analysis (Thomson et al., 2008). Thus, it serves as a good target protein to test if Casp and/Ter94 are involved in degrading proteins during MZT.

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

B

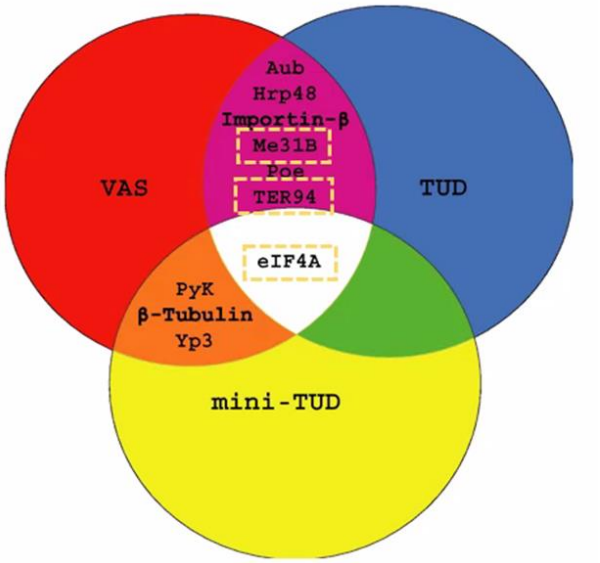


Figure 9: (A) Me31b and Ter94 have been picked up as interactors of Casp in the lab (Hegde et al., Unpublished). **(B)** Me31b has been linked to Ter94 in an interactome analysis (Adapted from Thomson et al., 2008).

From our study, we suggest that Ter94 is not involved in Me31b degradation. Also, whether Casp plays a role in Me31b degradation remains an open question. Thus, there is a need to look for other target proteins to test our hypothesis and provide conclusive evidence for (or against it).

Objective

The objective of this study is to check if the loss of Casp and/or Ter94 affects the degradation kinetics of Me31b. For this, specific aims include the following:

Aim 1: To standardize the measurement of the degradation kinetics of Me31b using western blotting.

Aim 2: To measure the degradation kinetics of Me31b in *caspRNAi* background and compare with the degradation kinetics in wild-type control embryos.

Aim 3: To measure the degradation kinetics of Me31b in *Ter94RNAi* background and compare with the degradation kinetics in wild-type control embryos.

CHAPTER 2 – MATERIALS AND METHODS

Fly Lines and Genetics: All the fly lines were maintained in bottles containing standard yeast cornmeal media (6% agar) at 25^{o}C. Fly stocks were flipped every 3 days during expansion and every 15 days during maintenance. For cross set-up, virgin females were collected thrice a day (morning, afternoon and evening) and put with males in vials or bottles containing standard fly media (6% agar). The ratio of females:males taken for cross set-up was 3:1. Fly lines were either obtained from BDSC, Indiana University or made in the lab. For maternal knockdown of *casp*, a line expressing dsRNA for RNAi of *casp* under UAS control of VALIUM20 vector (BDSC 44027) was crossed to a line maternally expressing GAL4-VP16 fusion protein under the alphaTub67C promoter which is loaded into eggs (BDSC 7062). For maternal knockdown of *Ter94*, a line expressing dsRNA for RNAi of *Ter94* under UAS control of VALIUM20 vector (BDSC 32869) was crossed to a line maternally expressing GAL4-VP16 fusion protein under the alphaTub67C promoter which is loaded into eggs (BDSC 7063). Other lines along with these obtained from BDSC or VDRC to generate desired fly lines in the lab have been listed in Table 1 below.

Table 1: List of Fly Lines Used

Sr. No.	Obtained From	Genotype of Flies	Chromosome Expressing Construct(s)
1.	BDSC (11373)	*casp^{lof}/Cyo*	II
2.	BDSC (23691)	*Casp^{Df}/Cyo*	II
3.	BDSC (51530)	*me31b-GFP/SM6a*	II
4.	BDSC (44027)	*caspRNAi/caspRNAi*	III
5.	BDSC (7063)	*Matgal4/Matgal4*	III

6.	BDSC (4087)	*Ter94$^{46Db-26}$/SM6a*	II
7.	BDSC (9871)	*Ter94$^{22-30}$/Cyo*	II
8.	VDRC (318894)	*caspGFP/caspGFP*	III
9.	BDSC	*If/Cyo; MKRS/Ser*	II, III
10.	BDSC (32869)	*Ter94RNAi/Ter94RNAi*	III

Embryo Collection: To look at maternal effect of a gene, virgin females of the respective genotypes were mated with *w1118* males and 0–3-hour embryos of such females were collected by setting up cages with sugar-agar plates (3% sugar; 2.5% agar) and a little yeast paste as food for the flies. Fresh plates were supplied every 24 hours during acclimatization of flies to the cage (2 days for small cages and 4 days for large cages). Before starting embryo collection, flies were synchronized by changing plates after every 1 hour at least twice. For 0-3 hour collections, the plates were changed every 2.5 hours at least 3 times a day and collected embryos were stored at 4^{o}C for downstream processing and applications. For studying degradation kinetics of Me31b, plates were changed every hour 4 times and these 0-1 hour plates were incubated at testing temperature (25^{o}C or 29^{o}C) for 3, 2, 1 and 0 hours respectively to obtain 3-4 hour, 2-3 hour, 1-2 hour and 0-1 hour embryos respectively.

Immunostaining: All embryos collected in a single day were subjected to dechorionation (using 100% bleach), fixation (using equal volumes of heptane and 4% PFA in 1X PBS) and devitellinization (using methanol) using standard protocols and either stored at -30^{o}C in methanol or immediately processed further for immunostaining. During immunostaining, the embryos were permeabilized with 0.3% PBST (0.3% Triton-X in PBS). The blocking solution used was 2% BSA in 0.3% PBST. The primary and secondary antibodies were added in appropriate dilutions. All intermediate washing steps were performed 3 times (10 minutes each) using 0.3% PBST. Approximately 200 such immunostained embryos were mounted per slide per

genotype. All embryos were visualized using Leica Sp8 confocal microscope in the Microscopy Facility, IISER Pune.

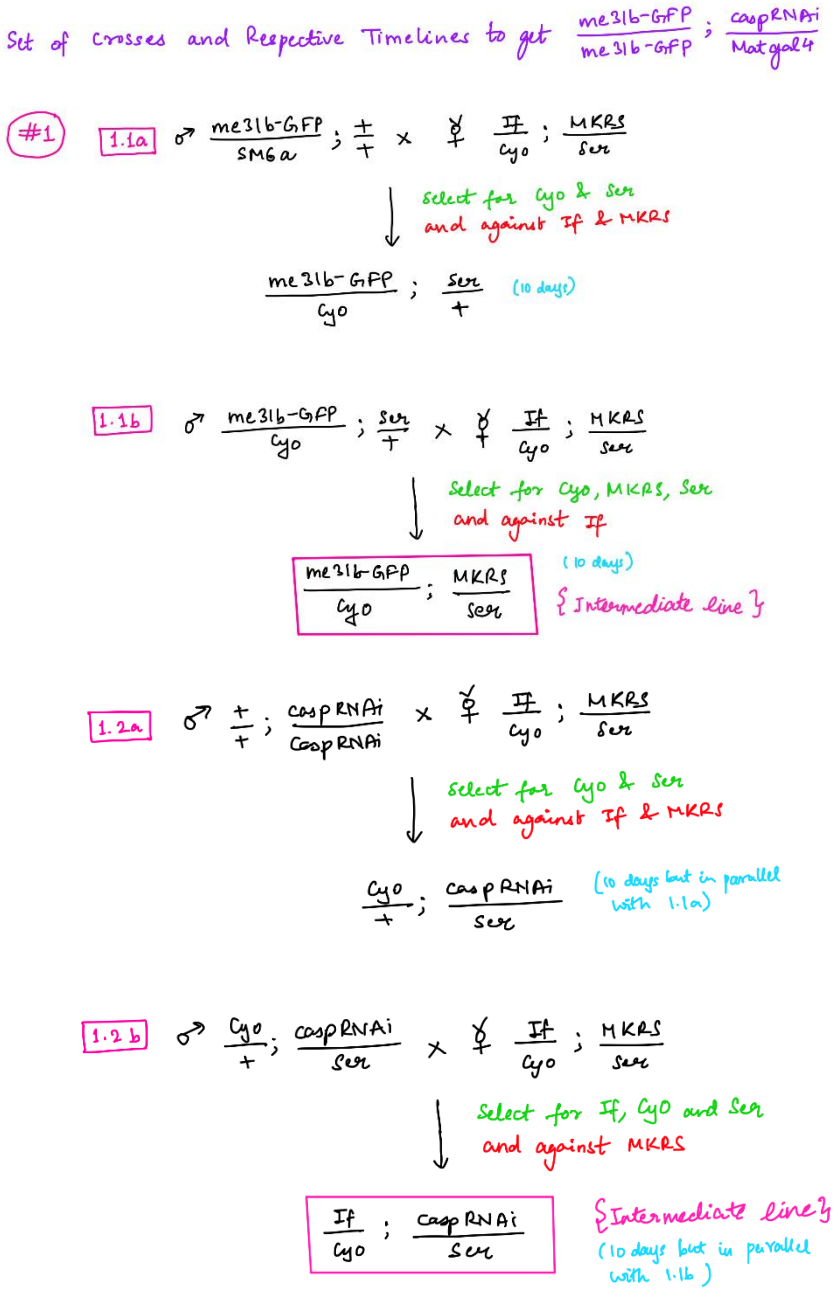
Western blotting: All embryos collected in a single day were subjected to dechorionation (using bleach), washing (using 1X PBS) followed by snap-freezing (using liquid N2) and stored at -80^{o}C. Embryos were lysed in RIPA-PIC mixture and centrifuged at 4^{o}C, 16.9 rcf for 30 mins. The protein layer from the supernatant was isolated and subjected to protein estimation for all samples using the BCA Assay. Calculations were made to load 50 µg of total protein into each well. All lysates were then boiled in 1X loading dye and equal amounts (50 µg) were loaded in SDS PAGE gel (5% stacking and 10% resolving), which was then run at 90V in stacking and 120V in resolving after adding running buffer. Bio-Rad pre-stained protein ladder was also loaded (4-5 µL) in each gel. Transfer to blotting paper was carried out at 90V, 4^{o}C for 1.5 hours after adding transfer buffer. Ponceau staining was performed to check equal loading followed by destaining using 0.1% TBST (0.1% Tween-20 in TBS) washes. The destained blots were subjected to blocking using 5% milk in 0.1% TBST for 1 hour on slow mutator. This was followed by incubation of blots in 1^{o} antibody (Roche Mouse GFP – 1:5000 dilution in blocking) 4^{o}C mutator for 12-16 hours. Post incubation, the blots were washed using 0.1% TBST (4 times, 15 mins each) on mutator. The primary antibody was stored for reuse in 0.05% sodium azide. This was followed by the incubation of blots in HRP-conjugated 2^{o} antibody (α Mouse HRP – 1:10,000 dilution in blocking) for 2 hours followed by washing with 0.1% TBST (4 times, 15 mins each) on mutator. Finally, the blots were visualized using SynGene via chemiluminescence by the addition of a mixture of equal volumes of HRP substrate and luminol reagent on the blot.

Embryonic lethality assay: Flies were allowed to lay eggs in cages for 12 hours followed by spotting of eggs onto fresh sugar-agar plates, which were kept for 24 hours before counting the number of hatched and unhatched eggs. The ratio of unhatched eggs to the total eggs was calculated to obtain the percentage of embryonic lethality. The counting and calculation of the ratio was repeated after keeping the plates spotted with eggs for 24 hours more to distinguish between lethality v/s sterility and also account for delayed hatching.

CHAPTER 3 – RESULTS AND DISCUSSION

Creation and Validation of Fly Lines

Two fly lines were made (*me31b-GFP/Cyo; caspRNAi/Ser* and *me31b-GFP/Cyo; Matgal4/Ser*) using a series of crosses (Fig. 10 A) to accomplish the objective of this study. The validation of these lines was carried out using a combination of embryonic lethality assay, western blotting and immunostaining followed by confocal microscopy.



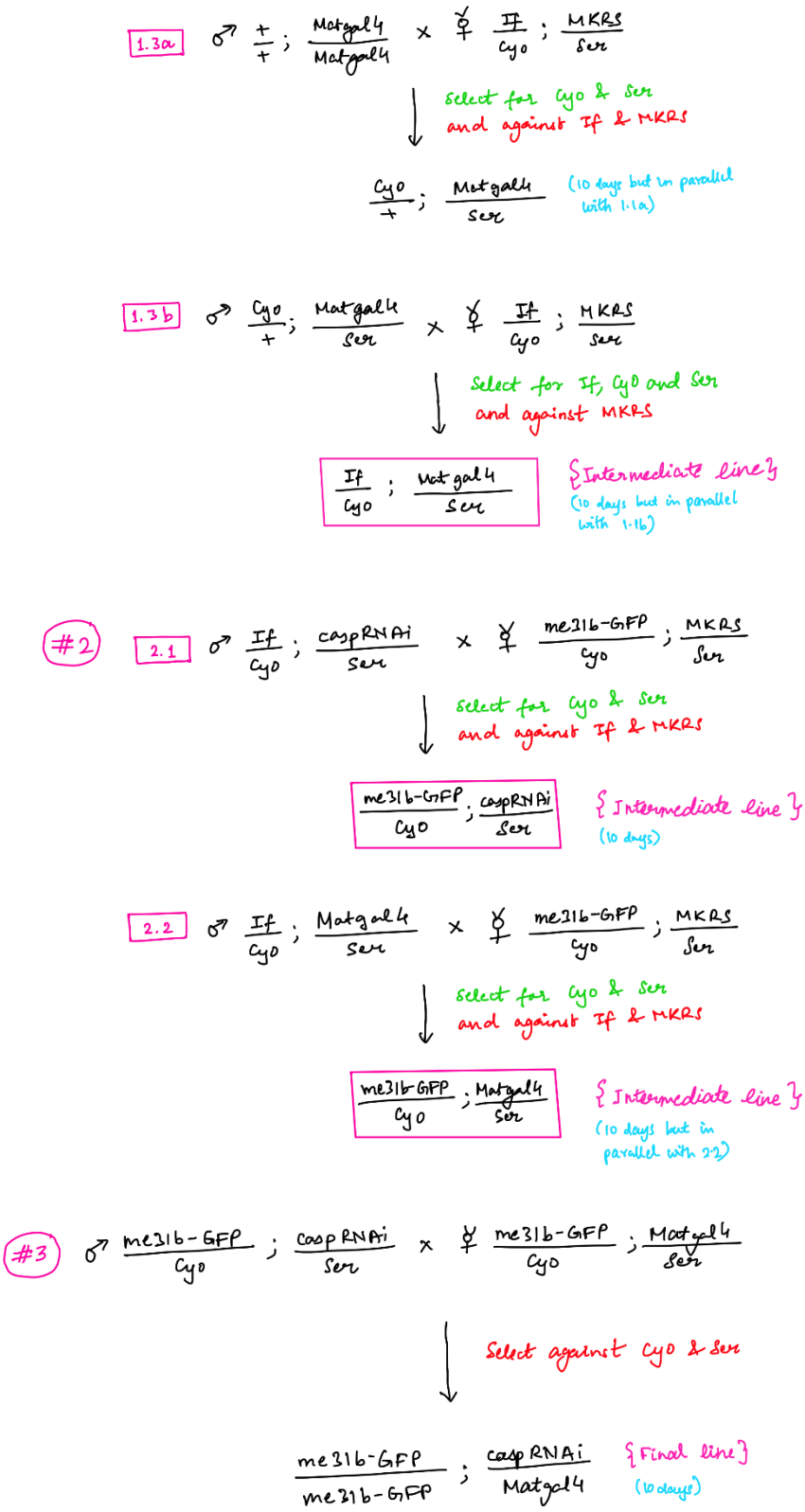


Figure 10: (A) Set of crosses to make *me31b-GFP/Cyo; caspRNAi/Ser* and *me31b-GFP/Cyo; Matgal4/Ser.*

Set of crosses and Respective Timelines to get $\frac{casp^{df}}{casp^{lof}}$; $\frac{cagp\ GFP}{casp\ GFP}$

#1

1.1a ♂ $\frac{+}{+}$; $\frac{casp\ GFP}{casp\ GFP}$ × ♀ $\frac{If}{Cyo}$; $\frac{MKRS}{Ser}$

↓ select for Cyo & Ser and against If & MKRS

$\frac{Cyo}{+}$; $\frac{casp\ GFP}{Ser}$ (10 days)

1.1b ♂ $\frac{Cyo}{+}$; $\frac{casp\ GFP}{Ser}$ × ♀ $\frac{If}{Cyo}$; $\frac{MKRS}{Ser}$

↓ Select for If, Cyo and Ser and against MKRS

$\frac{If}{Cyo}$; $\frac{Casp\ GFP}{Ser}$ (10 days) {Intermediate line}

1.2a ♂ $\frac{Casp^{lof}}{Cyo}$; $\frac{+}{+}$ × ♀ $\frac{If}{Cyo}$; $\frac{MKRS}{Ser}$

↓ select for Cyo & Ser and against If & MKRS

$\frac{Casp^{lof}}{Cyo}$; $\frac{Ser}{+}$ (10 days but in parallel with 1.1a)

1.2b ♂ $\frac{casp^{lof}}{cyo}$; $\frac{Ser}{+}$ × ♀ $\frac{If}{Cyo}$; $\frac{MKRS}{Ser}$

↓ Select for Cyo, MKRS and Ser and against If

$\frac{casp^{lof}}{Cyo}$; $\frac{MKRS}{Ser}$ {Intermediate line}

(10 days but in parallel with 1.1b)

1.3a ♂ $\frac{Casp^{df}}{Cyo}$; $\frac{+}{+}$ × ♀ $\frac{If}{Cyo}$; $\frac{MKRS}{Ser}$

↓ select for Cyo & Ser and against If & MKRS

$\frac{Casp^{df}}{Cyo}$; $\frac{Ser}{+}$ (10 days but in parallel with 1.1a)

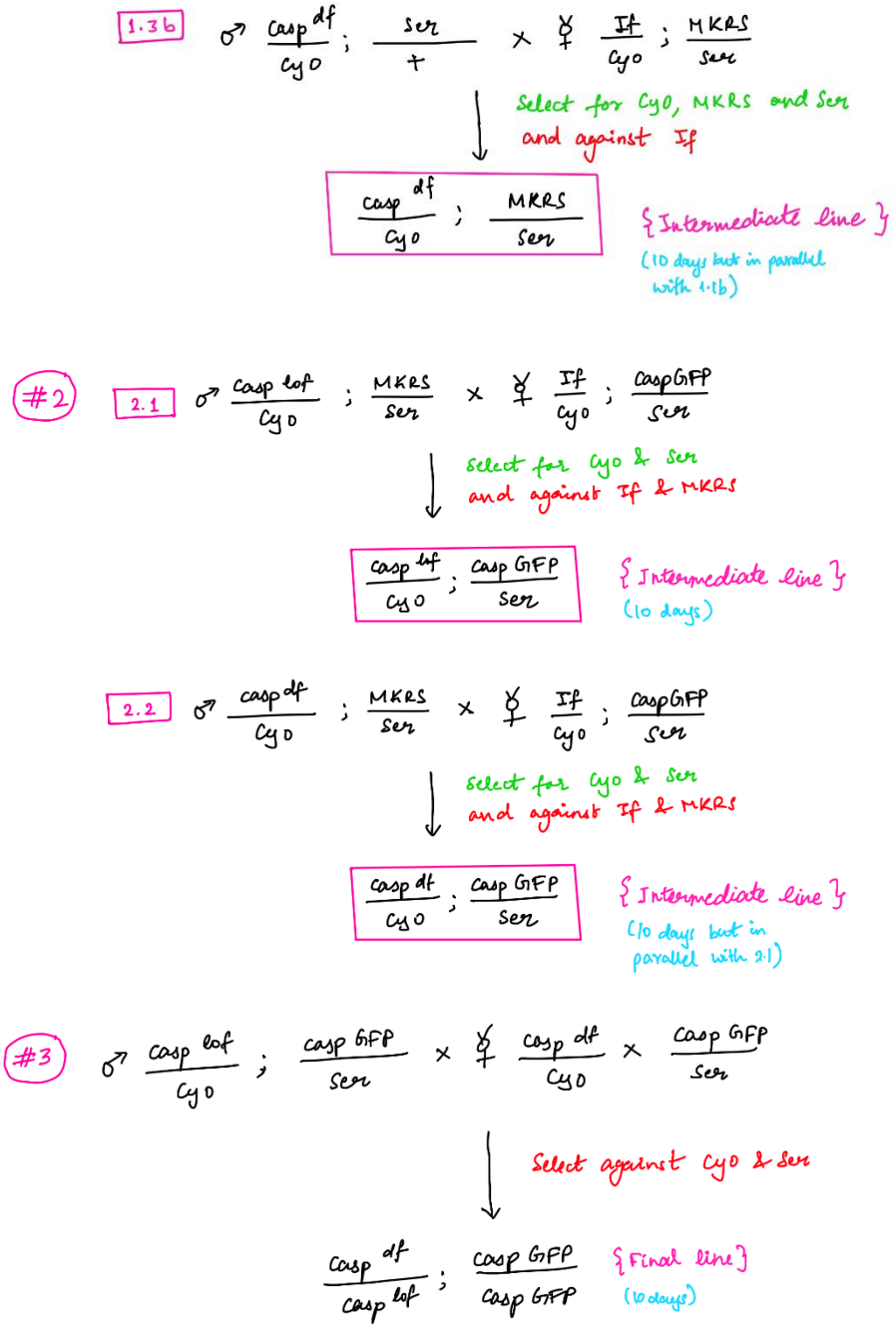


Figure 10: (B) Set of crosses to make *casp^{lof}/Cyo; caspGFP/Ser* and *casp^{Df}/Cyo; caspGFP/Ser*.

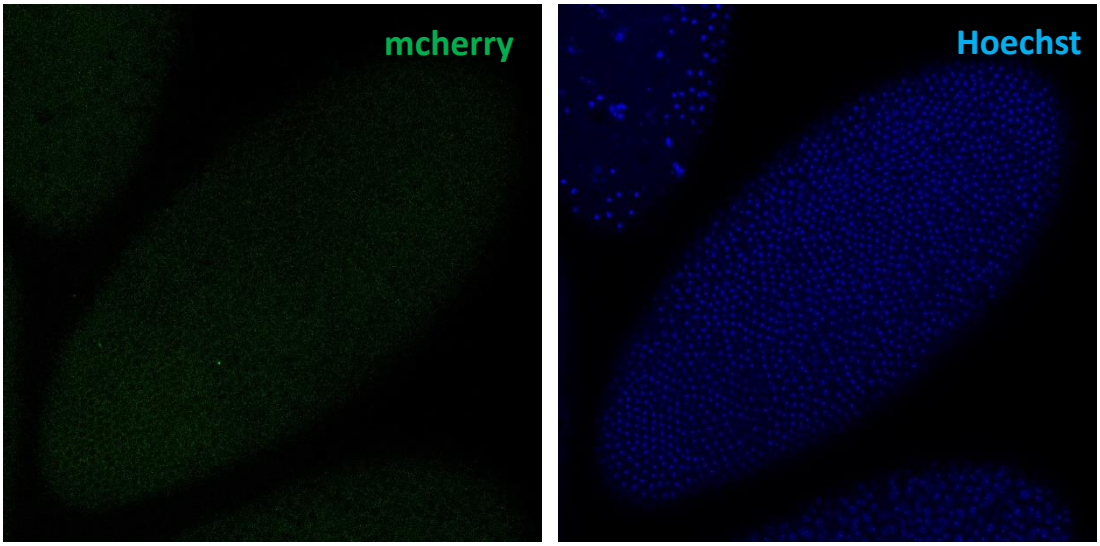
To validate the successful integration of the *caspRNAi* construct in the first line (*me31b-GFP/Cyo; caspRNAi/Ser*) using embryonic lethality assay, virgin females of

this genotype (BDSC 44027) were crossed to *Matgal4* (BDSC 7063) males and incubated at both 25^{o}C (permissive temperature) and 29^{o}C (condition of heat stress). F1 virgin females of this cross (*me31bGFP/+; Matgal4/caspRNAi*) were collected and mated with *w1118* (wild-type control) males; embryos of these females were then used to perform embryonic lethality assay. It was found that the embryonic lethality was 18% at 25^{o}C and 37% at 29^{o}C (Table 2). This is similar to the lethality of embryos of *Matgal4>caspRNAi* females at 25^{o}C and 29^{o}C respectively shown previously in the lab (Hegde et al., Unpublished), thus confirming the successful integration of *caspRNAi* construct on the third chromosome.

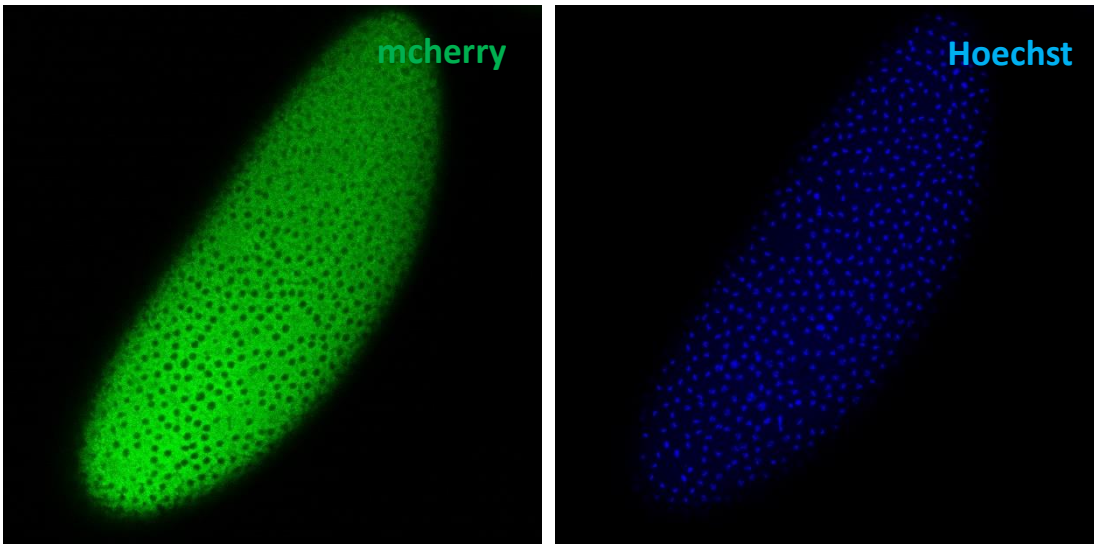
Table 2: Summary of Embryonic Lethality Assay Results for Various Genotypes

S. No.	Genotype of Fly Line	Embryonic Lethality (%)
1.	*w1118*	8%
2.	*me31b-GFP*	12%
3.	*caspGFP*	14%
4.	*casp^{lof}/Cyo; caspGFP/Ser*	56%
5.	*casp^{df}/Cyo; caspGFP/Ser*	58%
6.	*me31b-GFP; caspRNAi/matgal4 (25^{o}C)*	18%
7.	*me31b-GFP; caspRNAi/matgal4 (29^{o}C)*	37%

To validate the successful integration and expression of the *Matgal4* construct in the second line (*me31b-GFP/Cyo; Matgal4/Ser*) using immunostaining, virgin females of this genotype were crossed to *pUASp-mCherry* males. Virgins of F1 females of this cross (*me31b-GFP/+; UAS-mCherry/Matgal4*) were crossed to *w1118* males; embryos of these females were then collected, fixed and stored. Immunostaining and confocal imaging results validate the successful incorporation of *Matgal4* in this line (Fig. 11).



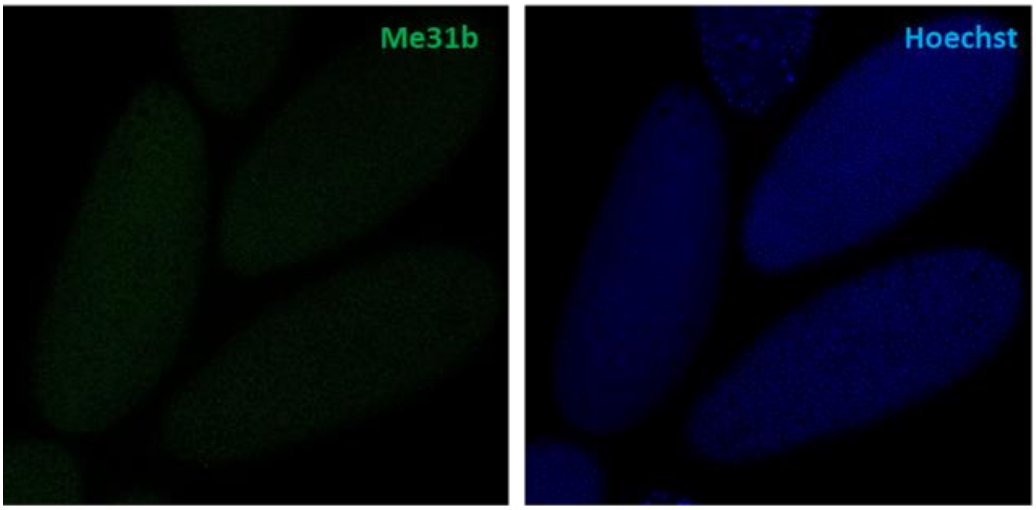
(A) *w1118* (control)



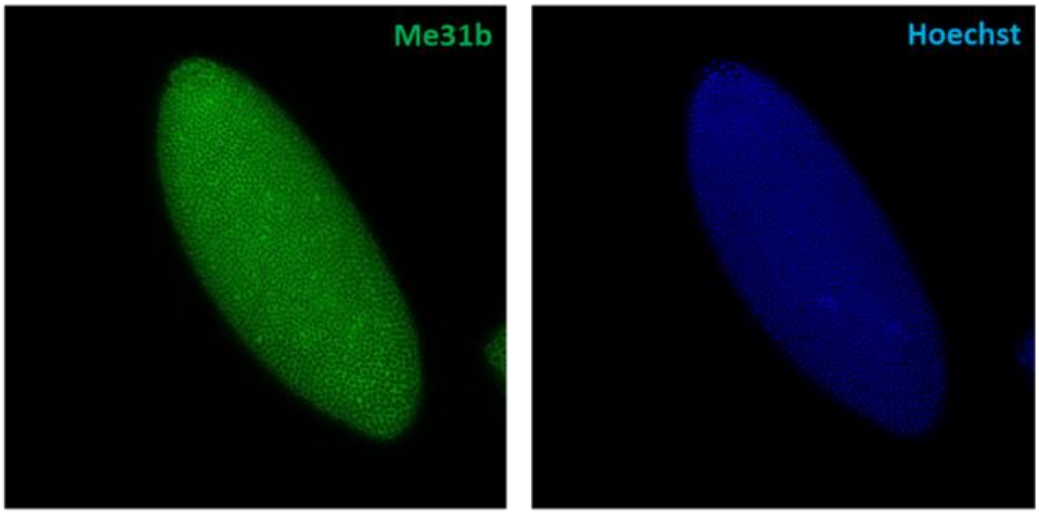
(B) *Me31b-GFP/Cyo; Matgal4/Ser*

Figure 11: Validation of *me31b-GFP/Cyo; Matgal4/Ser* for *Matgal4* incorporation using confocal imaging of immunostained embryos. (A) No mcherry expression observed in wild-type control embryos. **(B)** Bright mcherry expression driven by Matgal4 observed in me31b-GFP/Cyo; Matgal4/Ser embryos.

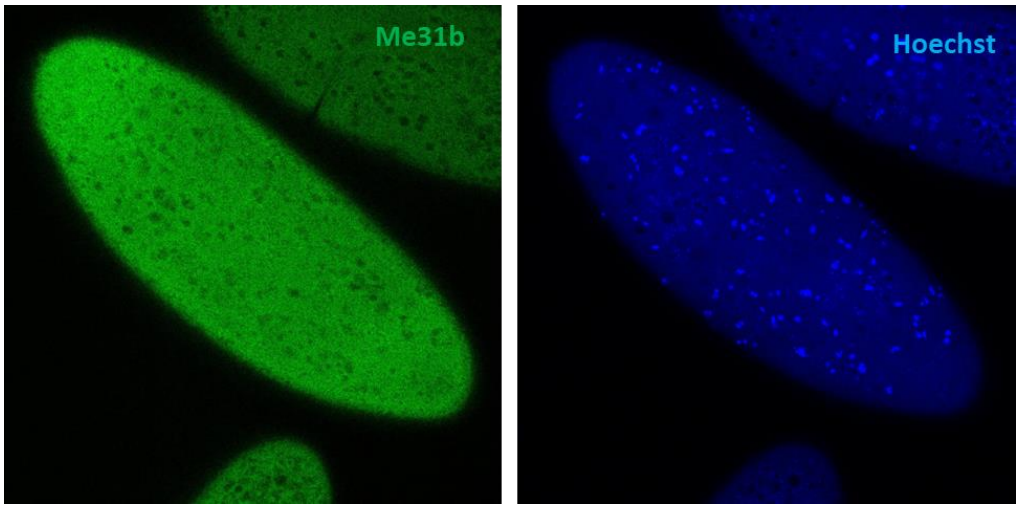
To validate the successful integration of *me31b-GFP* construct in both the lines (*me31b-GFP/Cyo; caspRNAi/Ser* and *me31b-GFP/Cyo; Matgal4/Ser*), females of both these genotypes as well as those of the parent line from which these lines were made (BDSC 51530) were caged and embryos were collected and processed both for immunostaining (Fig. 12) and western blotting (Fig. 13). It was successfully shown via both methods that *me31b-GFP* construct has been incorporated on the second chromosome in both the lines created.



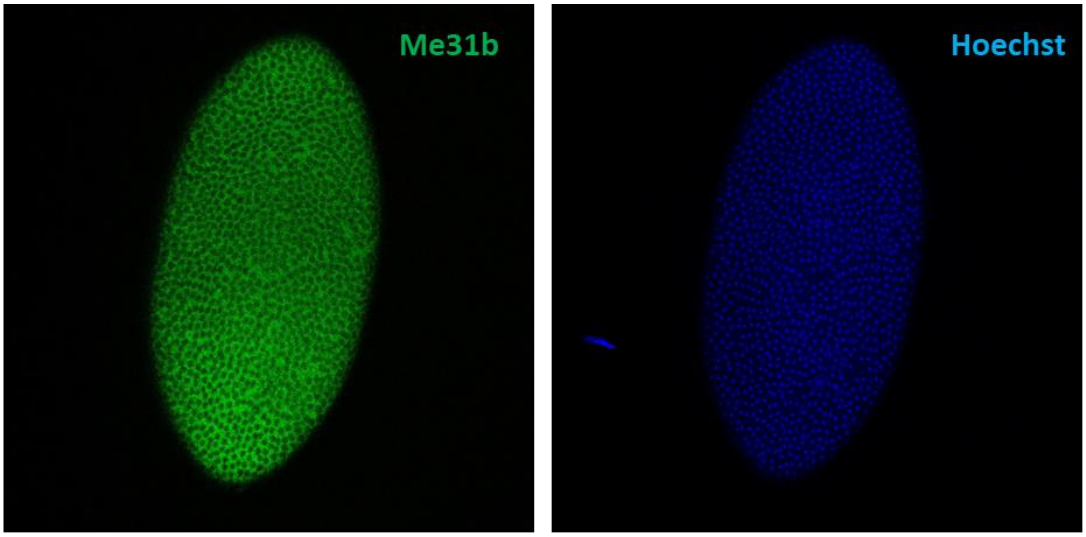
(A) w1118 (control)



(B) me31b-GFP/Cyo; caspRNAi/Ser



(C) me31b-GFP/Cyo; Matgal4/Ser



(D) me31b-GFP

Figure 12: Validation of *me31b-GFP/Cyo; Matgal4/Ser, me31b-GFP/Cyo; caspRNAi/Ser* and *me31b-GFP* parent line for expression of Me31b-GFP fusion protein using confocal imaging of immunostained embryos (anti-GFP antibody probe). (A) No GFP expression observed in wild-type control embryos. **(B)** Bright GFP expression in *me31b-GFP/Cyo; caspRNAi/Ser* embryos. **(C)** Bright GFP expression in *me31b-GFP/Cyo; Matgal4/Ser* embryos. **(D)** Bright GFP expression in *me31b-GFP* parent line embryos.

Further, two additional lines were made (*$casp^{Df}$/Cyo; caspGFP/Ser* and *$casp^{lof}$/Cyo; caspGFP/Ser*) to serve as reagents for live imaging of Caspar and rescue lethality and other defects caused by *casp null* flies (*$casp^{Df}$/$casp^{lof}$*) using a series of crosses (Fig. 10 B). The validation of these lines was also carried out using a combination of embryonic lethality assay, western blotting and immunostaining followed by confocal microscopy.

To validate the successful integration of the *$casp^{Df}$* construct in the third line (*$casp^{Df}$/Cyo; caspGFP/Ser*) and the *$casp^{lof}$* construct in the fourth line (*$casp^{lof}$/Cyo; caspGFP/Ser*), virgin females of each were separately crossed with *w1118* males. Embryos of these females were used to perform embryonic lethality assay. It was found that the embryonic lethality was 58% for *$casp^{Df}$/Cyo; caspGFP/Ser* and 56% for *$casp^{lof}$/Cyo; caspGFP/Ser*, thus validating the successful incorporation of these constructs in both the lines (Table 2).

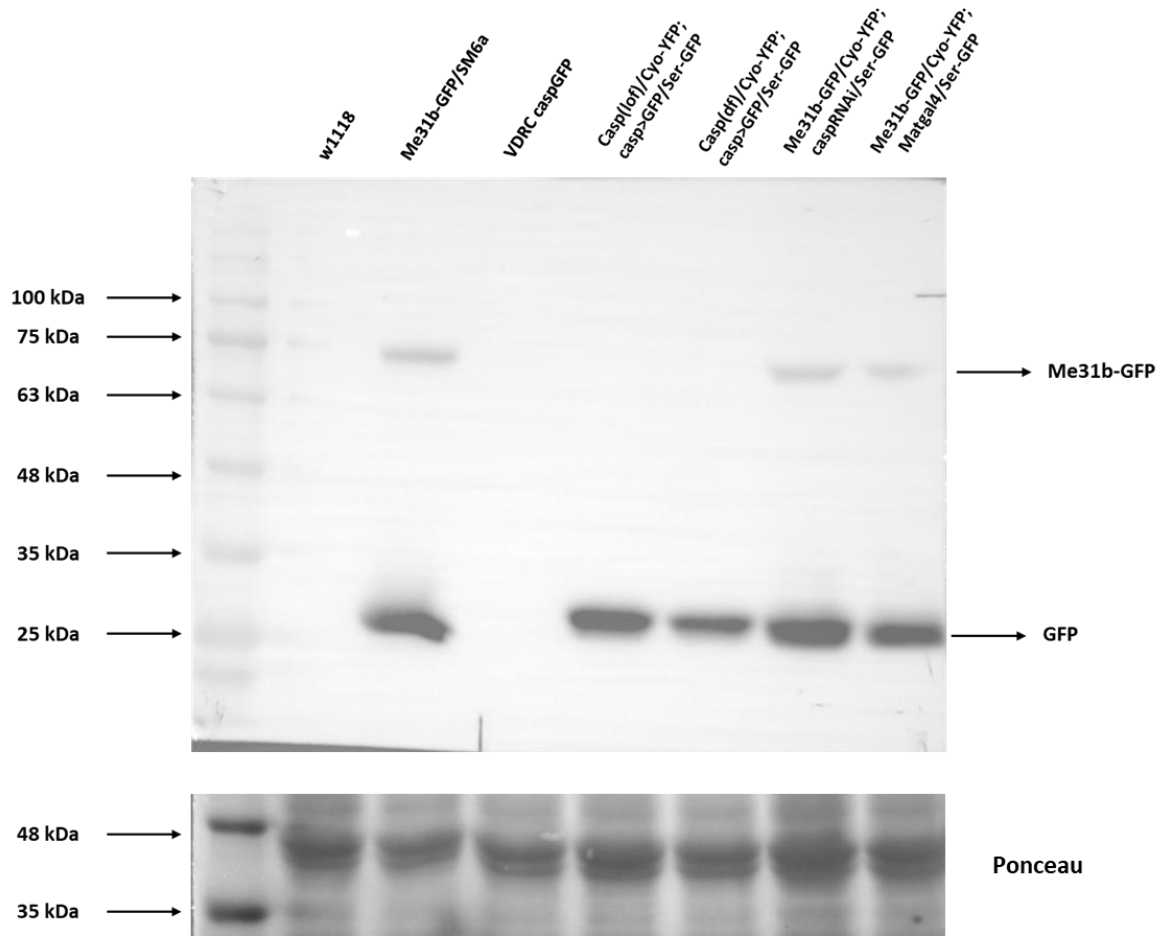


Figure 13: Validation of fly lines created using western blotting (anti-GFP antibody probe). All lanes contain 0-3 hour embryo lysates obtained from females of corresponding genotypes. Me31b-GFP fusion protein is expressing successfully in embryos of females having genotypes *me31b-GFP/Cyo; caspRNAi/Ser* (Lane 7), *me31b-GFP/Cyo; Matgal4/Ser* (Lane 8) corresponding to the first two fly lines created. Me31b-GFP fusion protein is also expressing in the parent line from which these lines were created (Lane 3). The parent line (VDRC *caspGFP*) from which the other two lines were created (*casp^{Df}/Cyo; caspGFP/Ser* and *casp^{lof}/Cyo; caspGFP/Ser*) itself does not express CaspGFP fusion protein and these two lines too don't express it hence (Lanes 4,5 and 6); these therefore cannot be used further.

To validate the successful integration of *caspGFP* construct in both these lines (*casp^{Df}/Cyo; caspGFP/Ser* and *casp^{lof}/Cyo; caspGFP/Ser*), western blotting was used to probe for GFP in these lines as well as in the parent line from which the construct was incorporated (VDRC 318894). There are no bands at 105 kDa (expected molecular weight of CaspGFP fusion protein) in either of the lines created or the parent line itself (Fig. 13). Thus, it was concluded that the parent line itself was not working and hence the new lines created from it cannot be used as reagents in the lab.

Standardizing Measurement of Me31b Degradation Kinetics

A major success has been the standardization of the degradation of Me31b during MZT using western blotting. As mentioned earlier, according to Wang et al., 2017, there is a 10-fold decrease in the levels of Me31b during the maternal to zygotic transition (MZT) during the early development of *Drosophila* embryo and that there is a steady decrease in Me31b levels every hour during early development. It would be interesting to see if this degradation kinetics is affected by the loss of Casp and/or Ter94 in the background (using lines created above), which would uncover a role of casp in the degradation of Me31b in early development of *Drosophila* embryo. To achieve this, the standardization of time-dependent embryo collections and western blotting protocol to show degradation kinetics was carried out using anti-GFP antibody.

Initially, 0-1 hour, 1-2 hour, 2-3 hour and 3-4 hour embryo collections were attempted using medium sized cages (1 cage per genotype with 100 flies each), however, the 1-hour time window was too small to collect enough number if eggs with this standard number of flies used for 0-3 hour collections. Hence, the collections were scaled up to using 2 cages per genotype with 200 flies each. But this too didn't suffice. Thus, a need for a major scaling up was realized and 2 large cages (approx. 1L) were used per genotype containing 700-800 flies each. Finally, enough number of eggs could be collected for each 1-hour time window and were used for downstream processing for western blotting.

The embryos thus collected were lysed and protein was extracted and quantified for 0-1 hour, 1-2 hour, 2-3 hour and 3-4 hour time windows for *w1118* (wild-type control flies) and *me31b-GFP* line expressing me31b-GFP fusion protein (BDSC 51530). The lysates were loaded into SDS-PAGE gel for molecular weight-based separation followed by immunoblotting. After a couple of trials, illustration of Me31b degradation kinetics was standardized using western blotting (Fig. 14). It can be seen that there is a rapid decrease in Me31b levels after the first two hours of *Drosophila* embryo development and there is no detectable protein beyond the MZT (maternal to zygotic transition). This can now be used as a reference to compare Me31b degradation kinetics in the background of Casp or Ter94 knocked down to comment on their roles in degrading proteins during MZT.

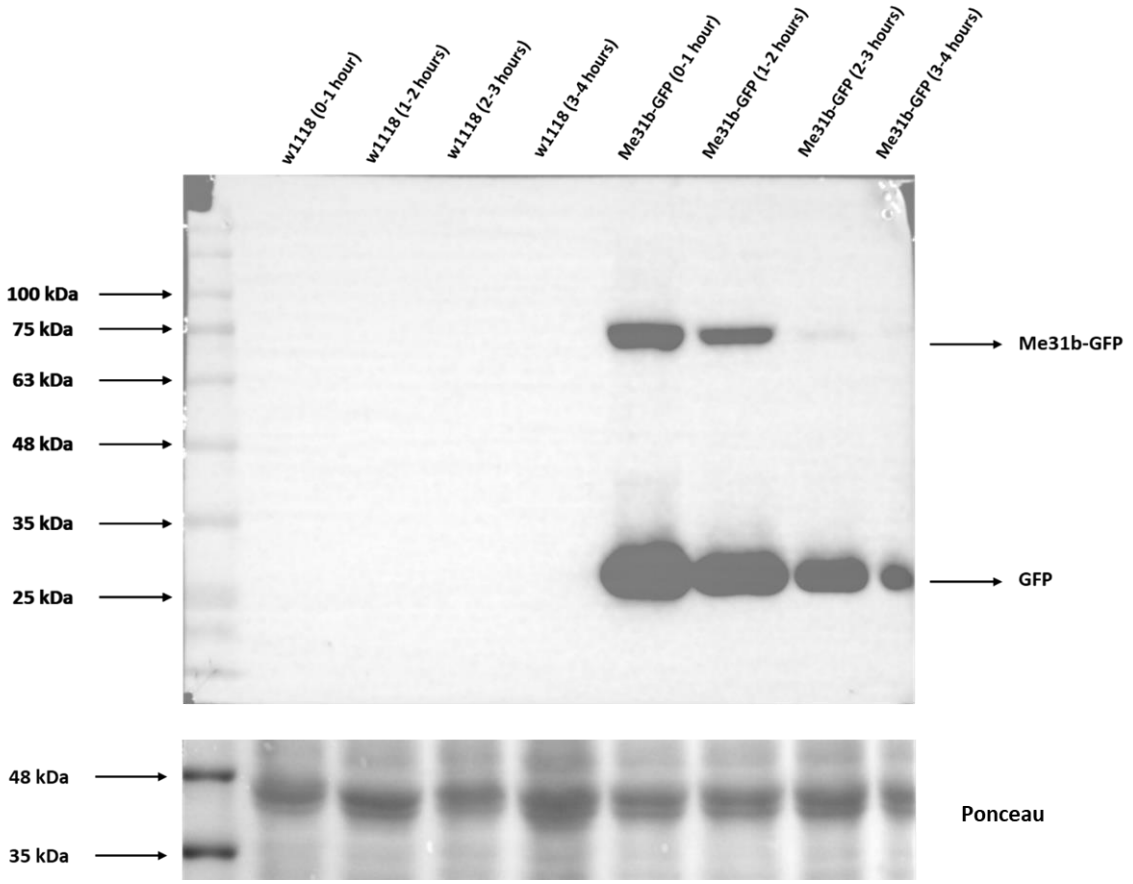


Figure 14: Successful standardization of the measurement of Me31b degradation kinetics using western blotting. As shown in Wang et al., 2017, Me31b protein levels rapidly decrease over the first few hours of embryogenesis, especially after the first two hours. Ponceau staining below confirms equal loading.

Ter94 is not involved in Me31b Degradation

Ter94 was maternally knocked down using RNAi conducted at 25^{o}C (permissive temperature). A line expressing dsRNA for RNAi of *Ter94* under UAS control of VALIUM20 vector (BDSC 32869) was crossed to the line made at the beginning using a series of crosses (*me31b-GFP/Cyo; Matgal4/Ser*) that stably expresses Me31b-GFP fusion protein (on the second chromosome) and maternally expresses GAL4-VP16 fusion protein under the alphaTub67C promoter which is loaded into eggs (on the third chromosome).

Success of the RNAi knockdown was validated using embryonic lethality assay of eggs laid by F1 females of this cross (*me31b-GFP/me31b-GFP; matgal4/ter94RNAi*).

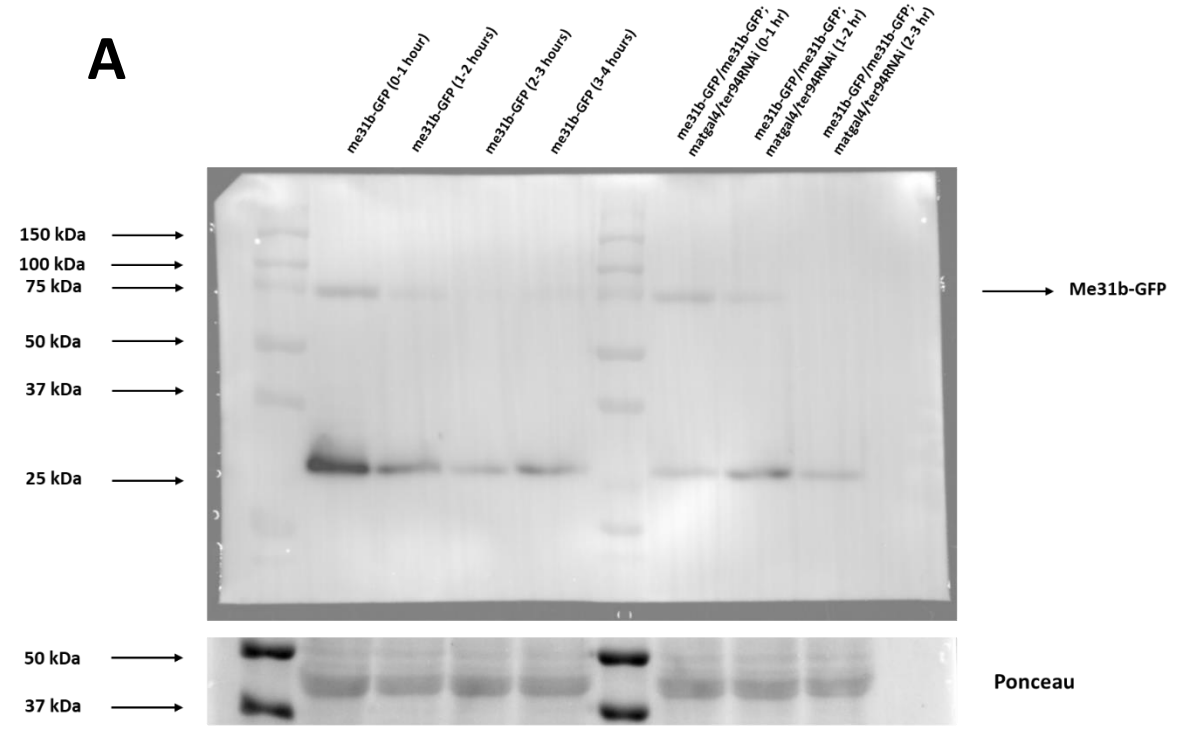
It was found that 97% of the eggs did not hatch which is a lethality found generally for *Ter94RNAi*, thus confirming successful knockdown (Table 3).

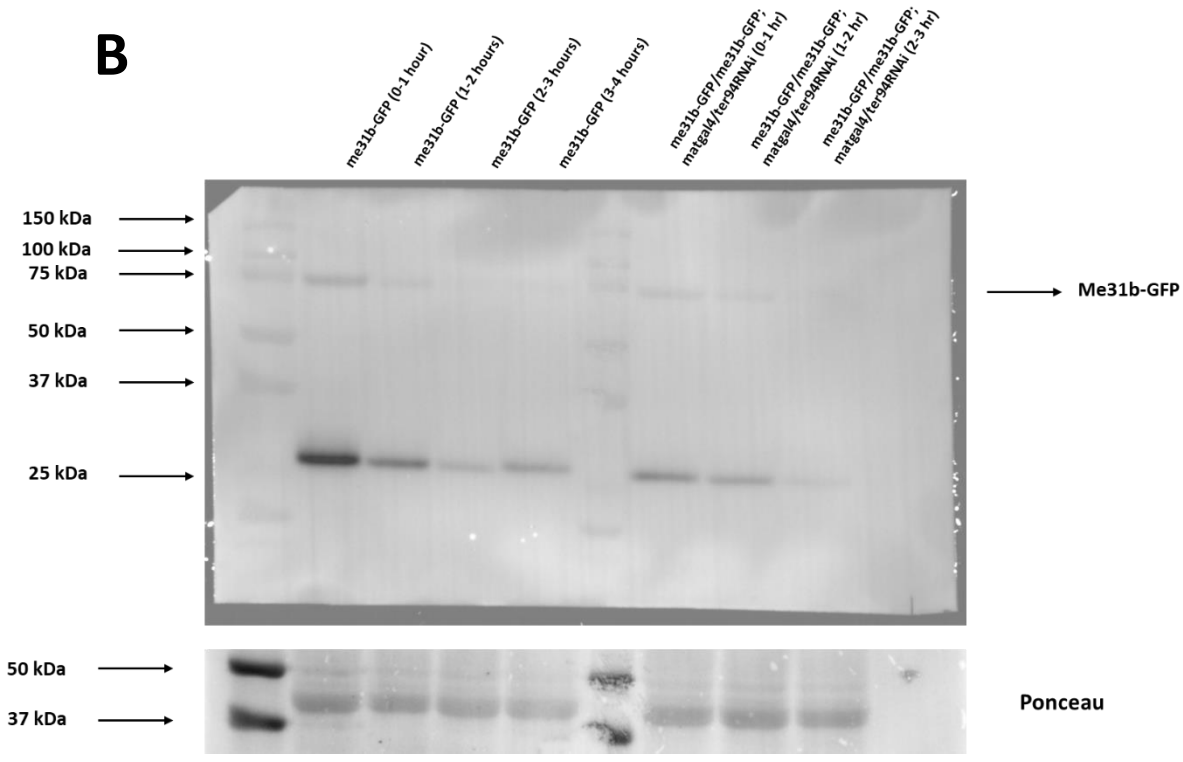
Table 3: Embryonic Lethality Assay for Validation of Knockdown of *Casp* and *Ter94* using Fly Lines Created

S. No.	Genotype of Female Fly	Embryonic Lethality (%)
1.	*w1118*	5%
2.	*me31b-GFP/me31b-GFP; matgal4/ter94RNAi*	97%
3.	*me31b-GFP/me31b-GFP; matgal4/caspRNAi*	18%

Eggs of F1 females (*me31b-GFP/me31b-GFP; matgal4/ter94RNAi*) along with eggs of me31b-GFP control flies (BDSC 51530) were collected for the following time windows: 0-1 hour, 1-2 hour, 2-3 hour and 3-4 hour using large (approx. 1L) cages containing 700-800 flies per genotype. As mentioned earlier during standardization of Me31b degradation kinetics, at least 2 cages per genotype each containing 700-800 flies is required to get enough embryos for western blotting application. However, when the cross was set up in 4 bottles each with 30 virgin females & 10 males, only 300-400 flies of required genotype were obtained which were not enough for large-scale embryo collections within the small 1-hour time windows. Thus, the crosses were scaled up to 8 bottles each with 30 virgin females & 10 males which finally produced 700-800 flies enough for 1 large cage.

The embryos thus collected were lysed and protein was extracted and quantified for 0-1 hour, 1-2 hour, 2-3 hour and 3-4 hour time windows for *me31b-GFP/me31b-GFP; matgal4/ter94RNAi* and *me31b-GFP* genotypes. The lysates were loaded into SDS-PAGE gel for molecular weight-based separation followed by immunoblotting. This was replicated four times (Fig. 15). It can be seen from the quantification of blots (Fig. 16) that Me31b degradation kinetics in *Ter94RNAi* background resembles that for *me31b-GFP* control embryos. Thus, the data is indicative of no involvement of Ter94 in the degradation of Me31b protein during MZT.


A
me31b-GFP (0-1 hour)
me31b-GFP (1-2 hours)
me31b-GFP (2-3 hours)
me31b-GFP (3-4 hours)
me31b-GFP/me31b-GFP; matgal4/ter94RNAi (0-1 hr)
me31b-GFP/me31b-GFP; matgal4/ter94RNAi (1-2 hr)
me31b-GFP/me31b-GFP; matgal4/ter94RNAi (2-3 hr)
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
Me31b-GFP
50 kDa
37 kDa
Ponceau


B
me31b-GFP (0-1 hour)
me31b-GFP (1-2 hours)
me31b-GFP (2-3 hours)
me31b-GFP (3-4 hours)
me31b-GFP/me31b-GFP; matgal4/ter94RNAi (0-1 hr)
me31b-GFP/me31b-GFP; matgal4/ter94RNAi (1-2 hr)
me31b-GFP/me31b-GFP; matgal4/ter94RNAi (2-3 hr)
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
Me31b-GFP
50 kDa
37 kDa
Ponceau

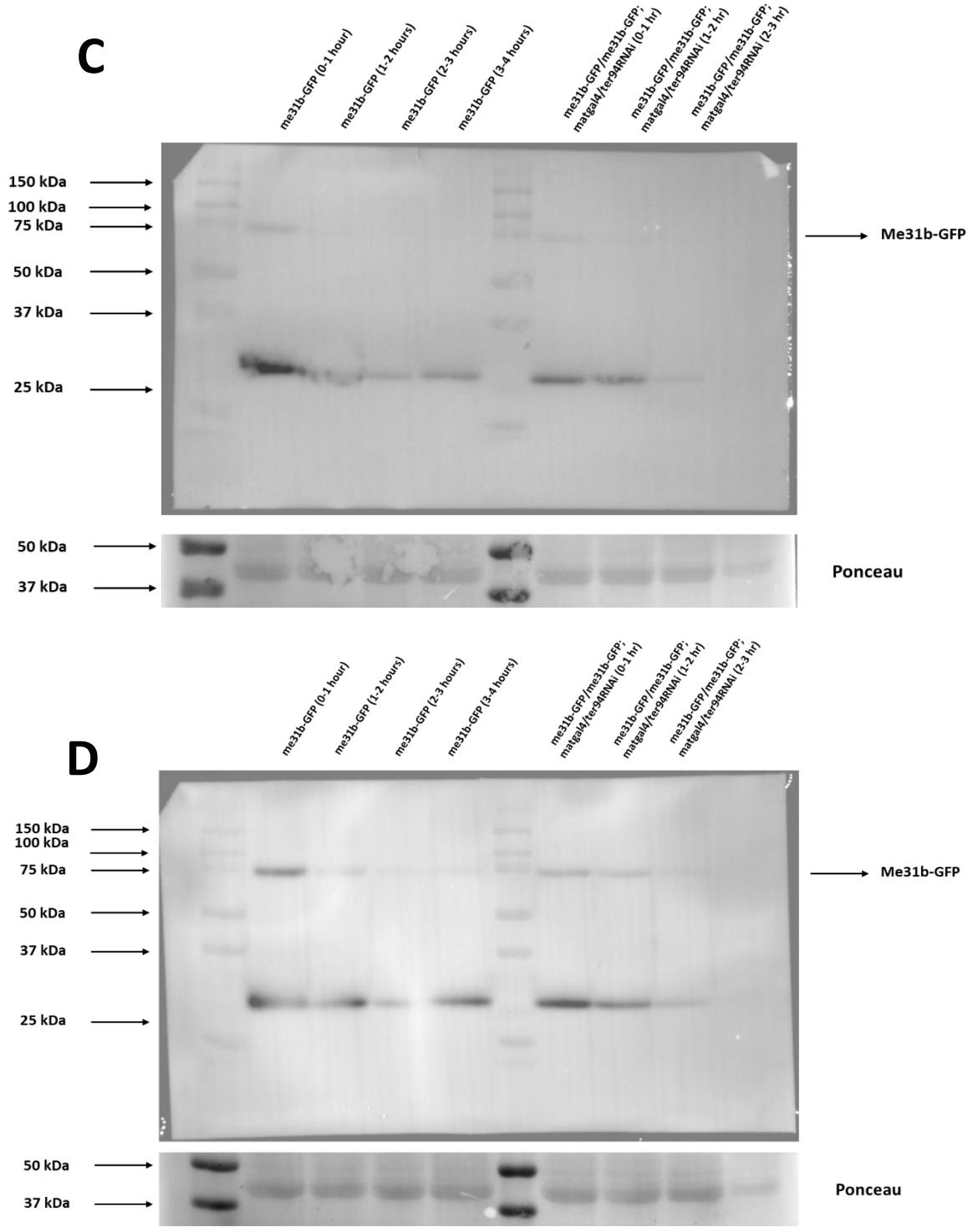
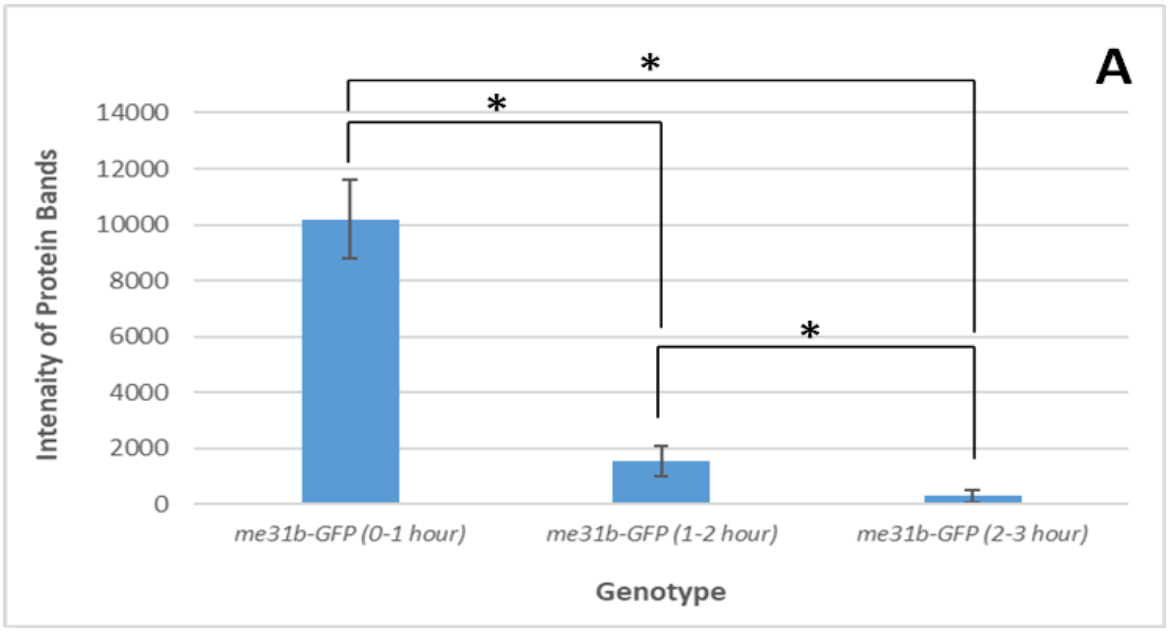


Figure 15: Western Blots for Measurement of Me31b Degradation Kinetics in *Ter94RNAi* Background. (A) Replicate 1 **(B)** Replicate 2 **(C)** Replicate 3 **(D)** Replicate 4. Lanes 2 – 5 contain embryo lysates of *me31b-GFP* females corresponding to the time windows 0-1, 1-2, 2-3 and 3-4 hour respectively. Lanes 7 – 9 contain embryo lysates of *me31b-GFP/me31b-GFP; matgal4/ter94RNAi* females corresponding to the time windows 0-1, 1-2 and 2-3 hour respectively. Enough embryos were not collected for 3-4 hour window. *Ter94 RNAi* does not affect Me31b degradation kinetics. Anti-GFP antibody probe was used to detect Me31b-GFP fusion protein. Ponceau staining shows equal loading.



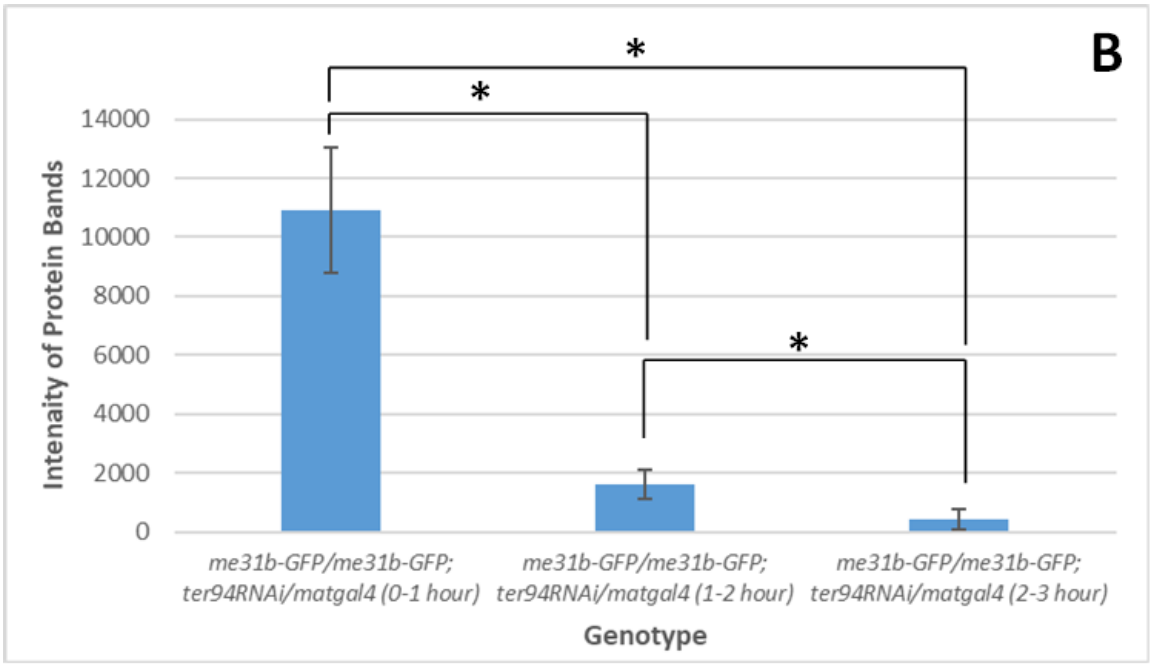


Figure 16: Quantification of Western Blots from Fig. 15. (A) Quantification of Me31b band intensities for lanes 2 – 4 containing embryo lysates of females of genotype me31b-GFP (control) at 25ºC. There is a significant decrease in Me31b levels from 0-1 hour to 1-2 hour, 0-1 hour to 2-3 hour and 1-2 hour to 2-3 hour time windows. **(B)** Quantification of Me31b band intensities for lanes 7 – 9 containing embryo lysates of females of genotype *me31b-GFP/me31b-GFP; matgal4/ter94RNAi at 25ºC*. There is a significant decrease in Me31b levels from 0-1 hour to 1-2 hour and 0-1 hour to 2-3 hour time windows. Band intensities were quantified using ImageJ software. Plotting of graphs was done using MS Excel. Pair-wise Students' T-test (two-tailed) was used to calculate p-values. '*' indicates pairs where $p < 0.5$.

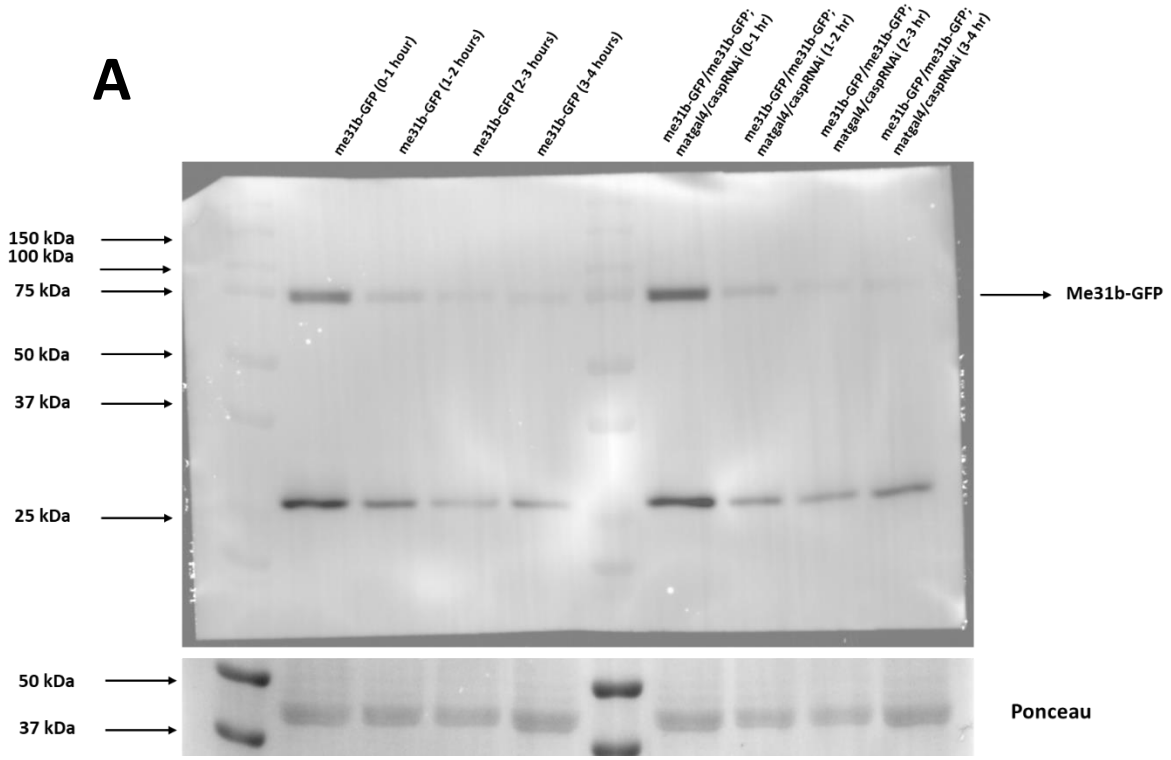
Involvement of Casp in Me31b Degradation Remains Elusive

Casp was maternally knocked down using RNAi conducted at 29^{o}C (heat stressed condition) because the RNAi at 25^{o}C (permissive temperature) is very weak using the available lines. The lines made at the beginning (*me31b-GFP/Cyo; Matgal4/Ser* and *me31b-GFP/Cyo; caspRNAi/Ser*) were crossed. The F1 progeny (*me31b-GFP/me31b-GFP; matgal4/caspRNAi*) stably expresses Me31b-GFP fusion protein (on the second chromosome) and dsRNA for RNAi of *casp* under UAS control of VALIUM20 vector along with maternal expression of GAL4-VP16 fusion protein under the alphaTub67C promoter which is loaded into eggs (on the third chromosome).

Success of the RNAi knockdown was validated using embryonic lethality assay of eggs laid by F1 females of this cross (*me31b-GFP/me31b-GFP; matgal4/caspRNAi*). It was found that 36% of the eggs did not hatch which is a lethality found generally for *caspRNAi* at 29^{o}C, thus confirming successful knockdown (Table 3).

Eggs of F1 females (*me31b-GFP/me31b-GFP; matgal4/tcaspRNAi*) along with eggs of *me31b-GFP* control flies (BDSC 51530) were collected for the following time windows: 0-1 hour, 1-2 hour, 2-3 hour and 3-4 hour using large (approx. 1L) cages containing 700-800 flies per genotype. During cross set up at 29^{o}C, the media became very dry and hence very few flies emerged. To compensate for this, the number of cross bottles were doubled and a petri plate containing water was kept in the vicinity of the bottles in the incubator. This maintained moisture in the microenvironment thus reducing the water loss from the fly media. Together this led to a significant increase in the number of F1 flies obtained.

The embryos thus collected were lysed and protein was extracted and quantified for 0-1 hour, 1-2 hour, 2-3 hour and 3-4 hour time windows for *me31b-GFP/me31b-GFP; matgal4/caspRNAi* and *me31b-GFP* genotypes. The lysates were loaded into SDS-PAGE gel for molecular weight-based separation followed by immunoblotting. This was replicated four times (Fig. 17). It can be seen from the quantification of blots (Fig. 18) that Me31b degradation kinetics in *caspRNAi* background resembles that for *me31b-GFP* control embryos. However, it should be noted that the RNAi is very weak even at 29^{o}C (as inferred from embryonic lethality assay). Thus, it is difficult to comment whether Casp is not involved in Me31b degradation or the RNAi being weak masks the actual degradation kinetics.


A
me31b-GFP (0-1 hour)
me31b-GFP (1-2 hours)
me31b-GFP (2-3 hours)
me31b-GFP (3-4 hours)
me31b-GFP/me31b-GFP; matgal4/caspRNAi (0-1 hr)
me31b-GFP/me31b-GFP; matgal4/caspRNAi (1-2 hr)
me31b-GFP/me31b-GFP; matgal4/caspRNAi (2-3 hr)
me31b-GFP/me31b-GFP; matgal4/caspRNAi (3-4 hr)
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100 kDa
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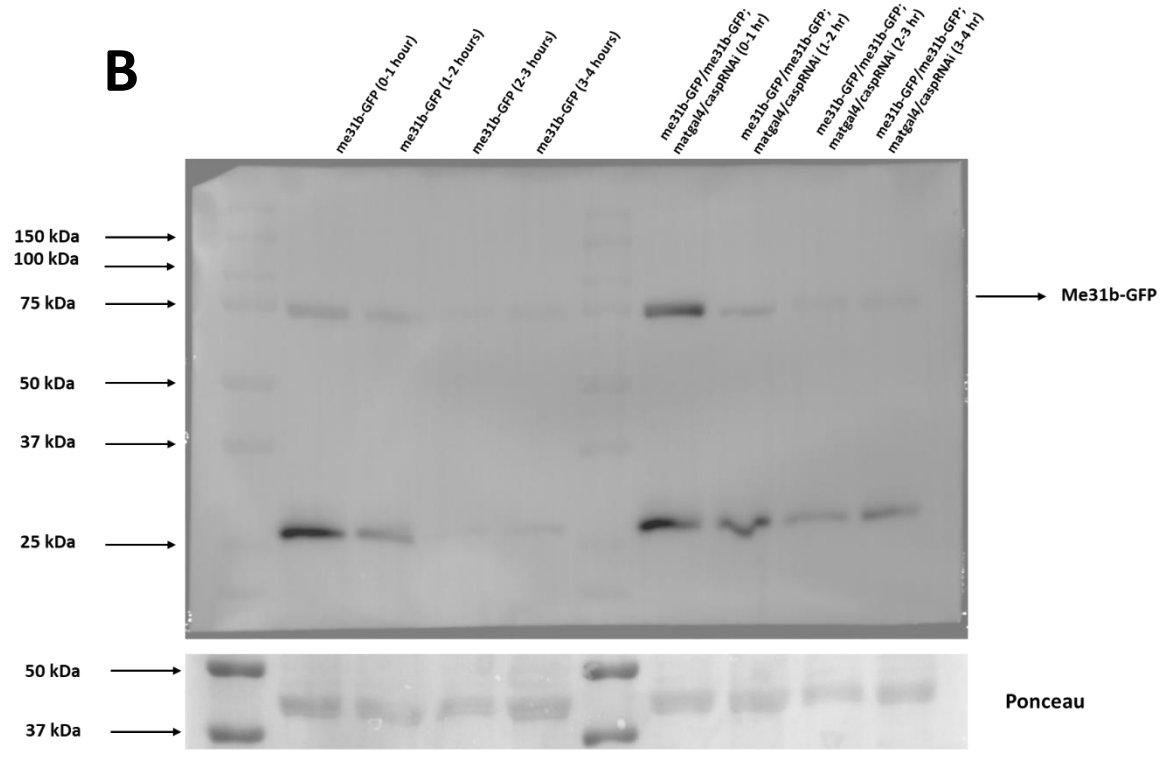
50 kDa
37 kDa
25 kDa
Me31b-GFP
50 kDa
37 kDa
Ponceau


B
me31b-GFP (0-1 hour)
me31b-GFP (1-2 hours)
me31b-GFP (2-3 hours)
me31b-GFP (3-4 hours)
me31b-GFP/me31b-GFP; matgal4/caspRNAi (0-1 hr)
me31b-GFP/me31b-GFP; matgal4/caspRNAi (1-2 hr)
me31b-GFP/me31b-GFP; matgal4/caspRNAi (2-3 hr)
me31b-GFP/me31b-GFP; matgal4/caspRNAi (3-4 hr)
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50 kDa
37 kDa
Ponceau

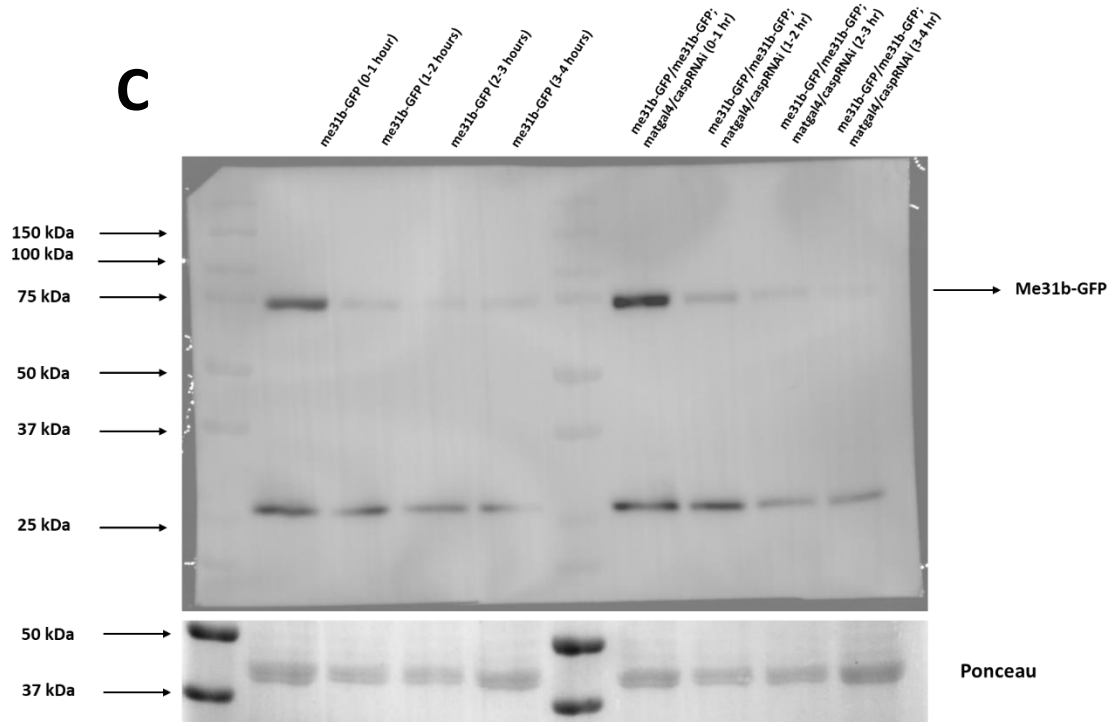
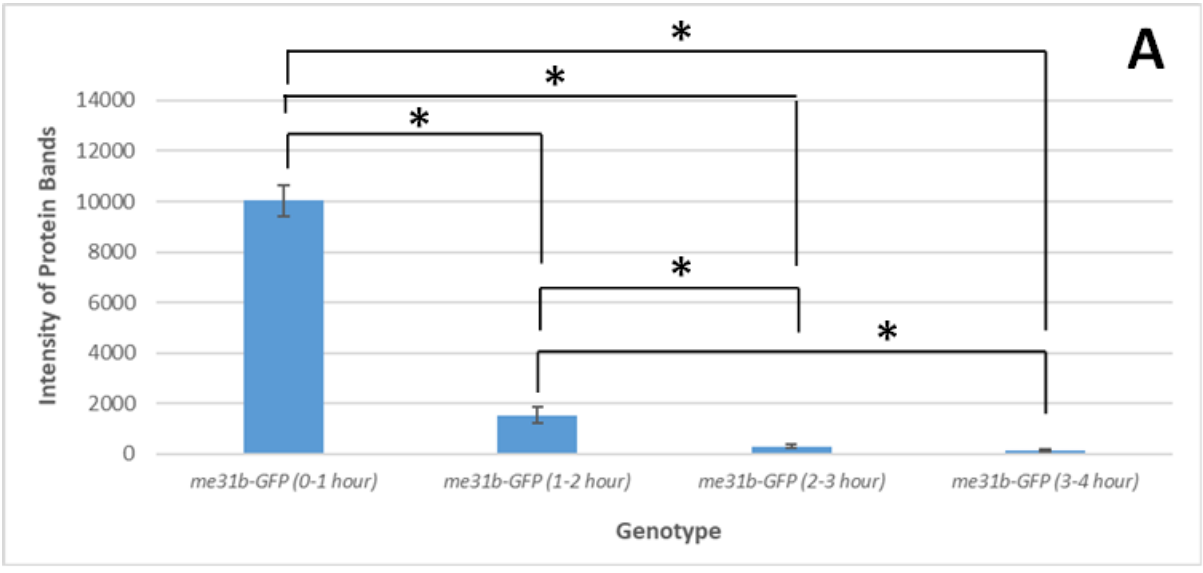


Figure 17: Western Blots for Measurement of Me31b Degradation Kinetics in *CaspRNAi* Background. (A) Replicate 1 **(B)** Replicate 2 **(C)** Replicate 3. Lanes 2 – 5 contain embryo lysates of *me31b-GFP* females corresponding to the time windows 0-1, 1-2, 2-3 and 3-4 hour respectively at 29ºC. Lanes 7 – 10 contain embryo lysates of *me31b-GFP/me31b-GFP; matgal4/caspRNAi* females corresponding to the time windows 0-1, 1-2, 2-3 and 3-4 hour respectively at 29ºC. *CaspRNAi* was very weak even at 29ºC and Me31b degradation kinetics is not affected; hence the role of Casp in Me31b degradation remains elusive. Anti-GFP antibody probe was used to detect Me31b-GFP fusion protein. Ponceau staining shows equal loading.



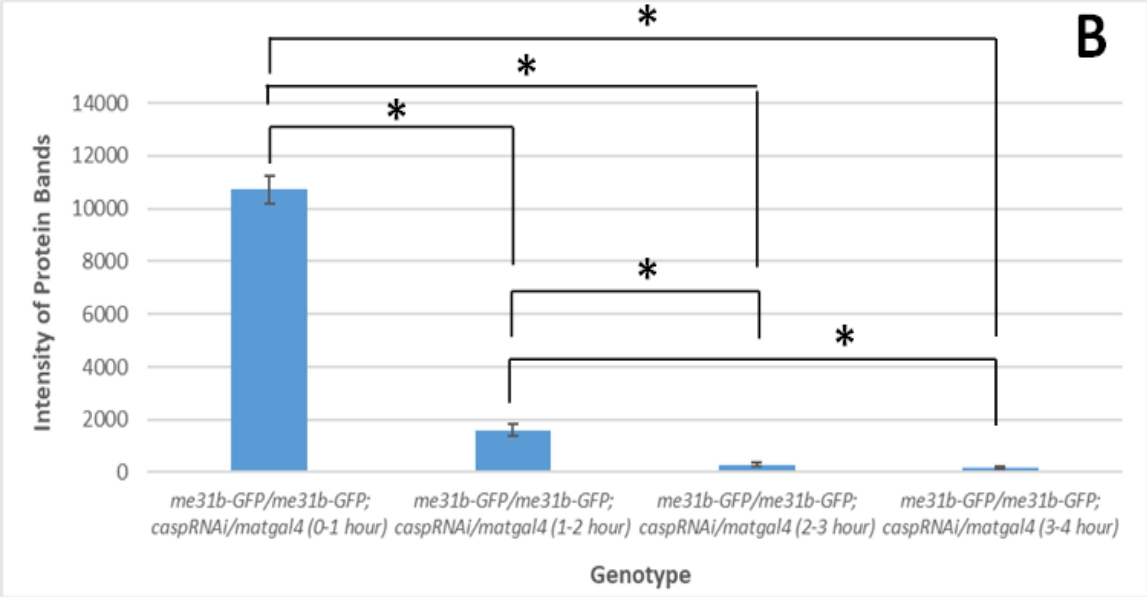


Figure 18: Quantification of Western Blots from Fig. 17. (A) Quantification of Me31b band intensities for lanes 2 – 5 containing embryo lysates of females of genotype *me31b-GFP* (control) *at 29^{o}C*. There is a significant decrease in Me31b levels from 0-1 hour to 1-2 hour, 0-1 hour to 2-3 hour and 0-1 hour to 3-4 hour time windows. **(B)** Quantification of Me31b band intensities for lanes 7 – 10 (containing embryo lysates of females of genotype *me31b-GFP/me31b-GFP; matgal4/caspRNAi at 29^{o}C*. There is a significant decrease in Me31b levels from 0-1 hour to 1-2 hour, 0-1 hour to 2-3 hour, 0-1 hour to 3-4 hour, 1-2 hour to 2-3 hour and 1-2 hour to 3-4 hour time windows. Band intensities were quantified using ImageJ software. Plotting of graphs was done using MS Excel. Pair-wise Students' T-test (two-tailed) was used to calculate p-values. '*' indicates pairs where $p < 0.5$.

Conclusion

We hypothesized that Casp acts as an adaptor molecule which on one hand binds to polyubiquitinated proteins and on the other hand binds to Ter94, thus targeting the protein for degradation. And in this way, Casp and Ter94 might have a role in degrading maternal proteins during MZT. Me31b was chosen as a target protein to test this hypothesis for the purpose of this study.

The results are indicative of Ter94 not playing a role in the degradation of Me31b and whether Casp is involved in Me31b degradation remains an open question. To conclusively state if Casp is involved in Me31b degradation, *casp^{lof}* mutants can be used to replace the weak knockdown carried out using RNAi.

However, to make a fly line expressing both Me31b-GFP fusion protein and *casp^{lof}* construct, regular sets of crosses as carried out to make the stable line *me31b-GFP/Cyo; caspRNAi/Ser* will not yield the desired mutant since both of the constructs are on the second chromosome. Hence, recombination scheme is necessary to combine *casp^{lof}* and *me31b-GFP* onto the same chromosome.

Another strategy could be to use anti-me31b antibody for probing Me31b protein on western blots instead of using anti-GFP antibody to probe for Me31b-GFP fusion protein. This would make the presence of Me31b-GFP dispensable and *casp^{lof}* mutants can directly be used for immunoblotting using anti-me31b antibody probe. However, the me31b antibody currently available in the lab gives noisy results (Hegde et al., Unpublished) and hence this strategy could not be employed.

It is important to emphasize that even though the data suggests that Ter94 does not have a role in Me31b degradation and whether Casp has a role is debatable, this is just one of the many maternal proteins degraded during MZT. Other target proteins need to be employed to check our hypothesis and gather conclusive evidence in favor of or against it.

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