A Maternal Role for Mon1

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled A Maternal role for Mon1 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 Anjana T at Indian Institute of Science Education and Research under the supervision of Dr. Girish Ratnaparkhi, Associate Professor, Department of Biology, during the academic year 2019-2020.

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This thesis is dedicated to flies, friends and family.

Declaration

I hereby declare that the matter embodied in the report entitled A Maternal Role for Mon 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 Dr. Girish Ratnaparkhi and co supervision of Dr. Anuradha Ratnaparkhi and the same has not been submitted elsewhere for any other degree.

Anjana T

Date: 29/03/2020

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Abstract

The complex of Mon1-Ccz1 functions as a GEF (Guanosine nucleoside exchange factor) for Rab7 and mediates the transition of Rab5 marked early endosomes to late endosomes. *Drosophila Mon1* mutants have a shortened lifespan, have motor defects and are sterile. The egg chambers fail to mature resulting in stalling of oocytes at the previtellogenic stages. These defects can be rescued by expression of *Dmon1* in the octopaminergic/tyraminergic neurons (DHIMAN *et al.* 2019). Eggs laid by neuronally-rescued $Dmon1^{lof}$ mutants fail to hatch into larvae indicating maternal roles for *Dmon1*. The cuticle pattern of these embryos shows absence of head structures and suggests a possible failure in axial patterning. We probed these embryos for spatiotemporal expression of A-P and D-V patterning genes using RNA *in-situ* hybridization. While D-V patterning genes, namely *twist*, *snail*, *short-gastrulation* and *decapentaplegic* show near normal patterns of expression, the A-P patterning genes *nanos* and *oskar* are mislocalised. This indicates that *Dmon1* has a critical role in localising the determinants of the A/P axis in the egg. We hypothesize that this role may be related to Mon1's function as a Rab converter.

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CHAPTER I

Introduction

The dynamic processes of membrane fission and fusion regulate the lipid and protein composition of cell membrane or membranous structures. The amount and distribution of membrane components are regulated by these events. Endocytosis is the mechanism by which internal membrane structures are formed de novo from the plasma membrane of cells. The process internalizes extracellular materials along with the lipid and proteins components of the membrane to the cytoplasm (DOHERTY AND MCMAHON 2009).

Endocytosis in vertebrates and *Drosophila* occurs via Clathrin dependent and Clathrin independent pathways (LE ROY AND WRANA 2005). In the former, accompanied by the adaptor proteins, clathrin molecules coat the invaginating membrane, and vesicles are formed with the help of scission by the GTPase Dynamin. Incoming vesicles can be directed towards different fates, after fusing with the early sorting endosome. They can be recycled back to the membrane after shunting to the recycling endosomes, or can be exocytosed or targeted to lysosomal degradation after being directed to become late endosomes (FISCHER *et al.* 2006).
Clathrin independent mechanism of endocytosis happens with the assistance of the protein molecule caveolin, leading to formation of caveolae, which are membrane invaginations. Caveolin is absent in *Drosophila,* but caveolae associated proteins are present in these organisms (FISCHER *et al.* 2006).

Internalization of the membranes and further sorting of these endosomes are mediated via several proteins and lipids in the cell. This includes conferring the identity of the vesicle by tagging the membrane with markers, along with assisting fusion of the membranes with target membrane bodies. Of these, the prominent ones are Rab proteins, which are small Ras-like GTPases, which associate with endosomal membranes, conferring its identity and assisting in fusion (ZERIAL AND MCBRIDE 2001). Rab proteins localize to membrane compartments upon activation, which is the Rab-GTP form. Rab5 marks early endosomes, Rab11 marks recycling endosomes, and Rab7 the late endosomes, targeting the lysosomal degradative pathway (Fig 1) (ENTCHEV *et al.* 2000).

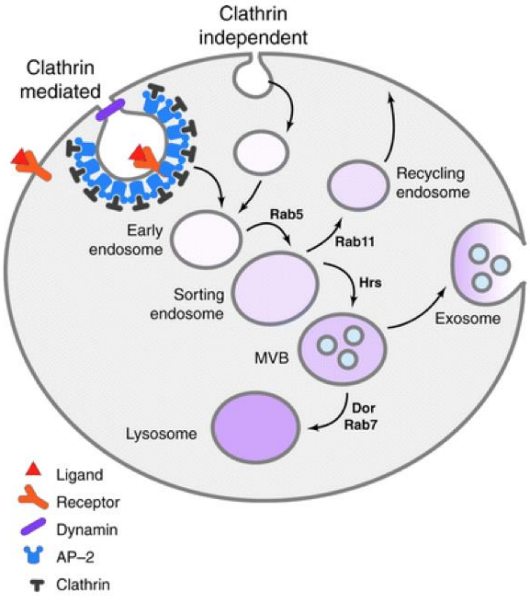

Fig 1: The endocytic pathway in cells. Endocytosis occurs predominantly via the Clathrin dependent and the Clathrin independent pathways. Invaginated vesicles from the plasma membrane fuse with Rab5 positive early endosomes. These vesicles are further channelled into the recycling endosomal pathway, as vesicles marked with Rab11 or towards the lysosomal degradative pathway, as vesicles marked with Rab7. Late endosomes marked by Rab7 are fused with the lysosomes or are exocytosed. (Adapted from FISCHER *et al.* 2006)

Endocytosis is implicated in various cellular processes other than membrane homeostasis. Several signalling pathways are mediated by endocytosis, which regulate receptor endocytosis, enrichment etc. Endocytic mechanisms are said to play a key role in development, via signalling through the receptors of morphogen gradients crucial for development at different stages of the organisms. Additionally endocytosis is one of the mechanisms that regulate gradient formation of different morphogens in development.

The formation of morphogen gradients of different molecules is critical to the patterning and development of an organism. Initial patterning events are orchestrated by morphogen gradients in embryos, such as that of *Bicoid*, *Oskar*, *Nanos*, *Dorsal*, *Dpp* in *Drosophila*. Although diffusion through the extracellular matrix guides gradient formation, fine tuning and shaping of these gradients require mechanisms involving tethering proteins, like HSPGs and endocytic pathways. This includes gradients of *Hedgehog*, *Wingless* and *Decapentaplegic* (*Dpp*). Receptor mediated endocytosis and resecretion of the morphogen is one mechanism proposed towards gradient formation (FISCHER *et al.* 2006). Additionally, endocytosis facilitates intracellular signalling via receptors of these ligands.

For instance, the *Sog* (*Short gastrulation*) gradient formation requires endocytosis. TGF-**β** signalling in vertebrates is facilitated by endosomes. *Dpp*, the *Drosophila* ortholog of TGF-**β** is also speculated to form a gradient in a similar fashion. Receptor mediated endocytosis shapes the *Hedgehog* and *Wingless* gradients in wing discs. The Notch-Delta signalling requires endocytosis of its ligands to activate signalling (FISCHER *et al.* 2006).

Mon1 in the endocytic pathway

The Monensin sensitivity 1 gene was identified for the first time in yeast in a screen for deletion mutations causing the cells to be defective in trafficking via the Cytoplasm to Vacuole (Cvt) and the Autophagy (Apg) pathways (WANG *et al.* 2002). Mon1 (Monensin sensitivity 1) and Ccz1 (Calcium Caffeine Zinc Sensitivity 1) mutants were defective in this trafficking. It was found that Mon1 and Ccz1 physically interact and function as a complex in different pathways delivering cargo to vacuoles (WANG *et al.* 2002). It was later found that homotypic vacuole fusion in yeast requires the function of the Mon1-Ccz1 complex (WANG *et al.* 2003).

Electron microscopy studies of the Mon1-Ccz1 complex (MC1) from *Chaetomium thermophilum* show that both the proteins form a globular heterotetramer (KIONTKE *et al.* 2017). The heterodimer formation between the individual molecules of Mon1 and Ccz1is mediated by the interaction between the Longin domains of the two proteins. The Longin domain also plays a role in mediating the enzymatic activity of the MC1 complex (Fig 2) (KIONTKE *et al.* 2017).

Rab GTPases switch between an active, GTP bound membrane localised state and an inactive GDP bound state (Kiontke *et al.* 2017). Active Rabs localise onto vesicle membranes conferring their identity and mediating fusion. Rab conversion describes the process of early to late endosome maturation (Wandinger-Ness and Zerial 2014). Mon1 and its homologues were identified to be involved in Rab conversion in the endocytic lysosomal degradation pathway. SAND1/Mon1 was identified in *C. elegans* as the switch that regulates early to late endosome transition, by recruitment of Rab7 onto Rab5 positive early endosomes.

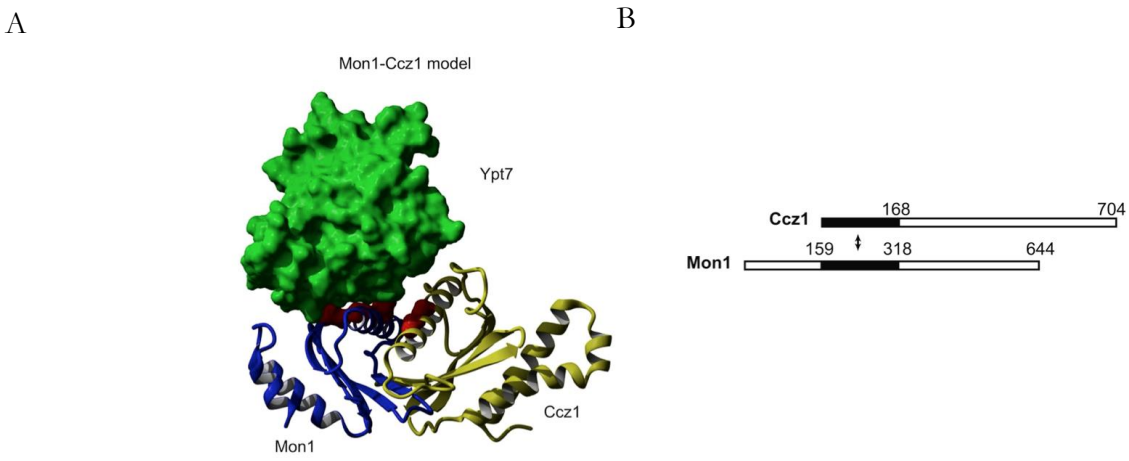

Fig 2: Predicted structure of the Mon1-Ccz1 complex. Mon1 functions as a complex with Ccz1 in cells. The interaction of Mon1 to Ccz1 and to the yeast protein Ypt7, which is a homolog of the mammalian Rab7, is predicted to be mediated via the Longin domains present in both of the proteins.
(A) Predicted structure of the interaction between the Mon1-Ccz1 complex and Ypt7 through their Longin domains. **(B)** Representation of the amino acid sequence of Mon1 and Ccz1, with the Longin domain region essential for interaction and enzymatic activity marked in black.
(Adapted from CABRERA *et al.* 2014 and NORDMANN *et al.* 2010)

The Rab7 recruitment by SAND1 is assumed to be taking place via an interaction with the HOPS complex, along with interrupting Rab5 activation (POTERYAEV *et al.* 2010). The Mon1-Ccz1 complex acts as a GEF (Guanine nucleotide Exchange Factor) for Rab7. Existing models suggest that the MC1 complex recruitment to early endosomes is mediated by Rab5, PI3P (phosphoinositol-3-phosphate) and other factors (CABRERA *et al.* 2014). The GEF activity of MC1 directs Rab7 to localise to the membrane. It is also assumed that the complex facilitates the removal of Rab5 from these endosomes by aiding the recruitment of Rab5 GAP molecules (POTERYAEV *et al.* 2010). This model suggests the recruitment of Rab7 to early endosomes by replacing Rab5, to facilitate endosomal maturation (Fig 3).

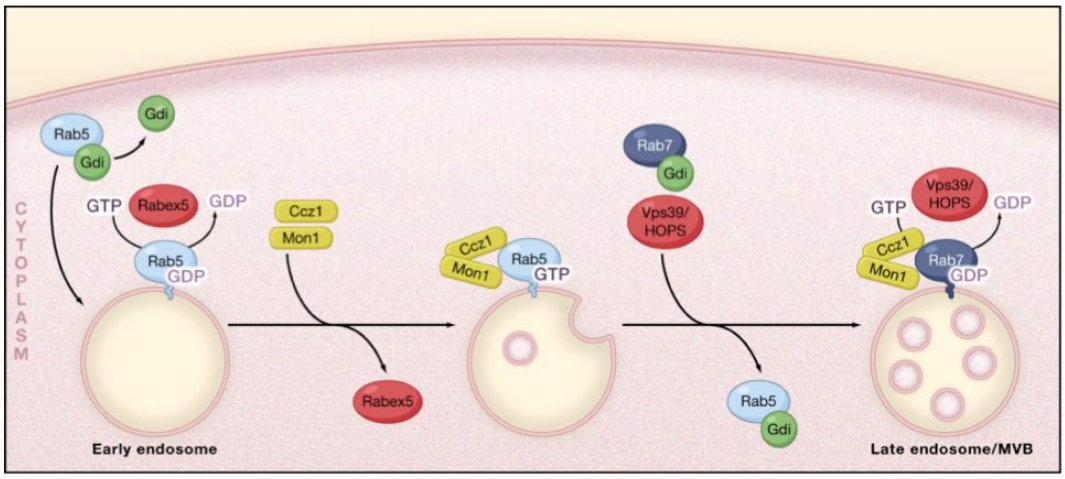

Fig 3: Rab conversion by the Mon1-Ccz1 complex. The Mon1-Ccz1 complex functions in as a Guanosine nucleotide exchange factor (GEF) which activates and facilitates the membrane localisation of Rab7. The model suggests that Rab5 along with membrane components like phophatidyl-inositol-3-phosphate facilitates the recruitment of the MC1 complex. The MC1 complex facilitates the displacement of the Rab5 GEF, Rabex-5, responsible for the activation and membrane localisation of Rab5. Rab7 is recruited onto the membrane by the Mon1-Ccz1 complex, via an interaction with the HOPS complex, which facilitates the activation of Rab7. (Adapted from CABRERA AND UNGERMANN 2010)

Mon1 is also implied to have roles in autophagy in cells. Autophagy, which is the digestion of cellular materials by the cell itself, is involved in protein and organelle degradation, which has implications in several diseases (MIZUSHIMA *et al.* 2008). Both autophagy and endocytosis converge at the level of lysosomes, where cargo is delivered for degradation. The Ccz1-Mon1-Rab7 module was found to be required for degradation by fusion of the autophagosome to the lysosome (HEGEDUS *et al.* 2016). The study suggests that Rab7 recruitment on autophagosomes was dependent on the functioning of the Ccz1-Mon1 complex suggesting a role for Mon1 in autophagy.

Dmon1 in *Drosophila*

The loss of function mutation of the *Drosophila* Mon1 gene caused an increase in endosomal vesicle size, and also impairment in their association with Rab7 (YOUSEFIAN *et al.* 2013). Transmembrane proteins like Notch were present in the cargo inside these enlarged vesicles. Loss of function of Dccz1 also rendered similar phenotypes of accumulation of enlarged maturing endosomes, suggesting their function as a complex in metazoan cells (YOUSEFIAN *et al.* 2013).

The *Drosophila* Mon1 mutants $Dmon1^{\Delta 181}$ (Δ181) and $Dmon1^{\Delta 129}$ were generated by P-element excisions upstream of the gene *pog* (DEIVASIGAMANI *et al.* 2015). The $Dmon1^{\Delta 181}$ was used for further experiments. The $Dmon1^{\Delta 181}$ allele produces a truncated transcript, with the deletion spanning from the second intron of the gene to just upstream of the neighbouring *pog* gene (DEIVASIGAMANI *et al.* 2015). Flies homozygous for this mutation showed lethality throughout all stages of development and during eclosion. Very few flies eclose, and the ones which eclose die within 7 days. These flies are late emerging, were found to be weak, showing motor defects, in addition to being sterile. The lifespan and motor defects were rescued by pan-neuronal expression of *Dmon1* (Fig 4).

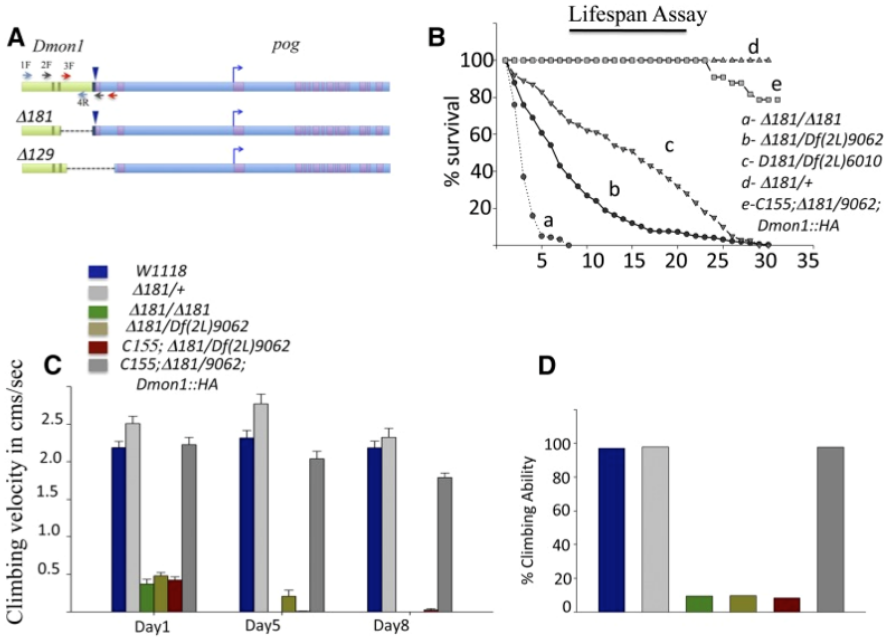

Fig 4: Characterisation of *Dmon1* mutants. The $Dmon1^{-\Delta 181}$ (Δ181) allele shows various phenotypes of decreased lifespan and motor defects
(A) Genomic position of the *Dmon1* gene and the Δ181 and Δ129 deletion mutation alleles in *Drosophila*. **(B)** Lifespan assay conducted on $Dmon1^{-\Delta 181}$ (Δ181) flies. Δ181 homozygotes show a drastic reduction in their lifespans, showing lethality at all stages of development. Very few flies eclose, and the ones which do die in 7 days. **(C)** The climbing velocity for Δ181 homozygotes with respect to wild type controls. It can be seen that the Δ181/ Δ181 genotypes show the lowest velocity compared to the other genotypes. **(B)** Percentage climbing ability of the $Dmon1^{-\Delta 181}$ flies. $Dmon1^{-\Delta 181}$ flies show severe motor defects in both climbing velocity and speed of climbing. The homozygotes can be seen to express a very low index of climbing ability. (Adapted from DEIVASIGAMANI *et al.* 2015)

Consistent with the known functions of Mon1, the mutant flies showed accumulation of Rab5 positive early endosomes in their cells. The localisation of Rab7 onto these early endosomes was impaired. At the synaptic junctions, the morphology of boutons was altered, where they were found to appear larger in *Dmon1*$^{\Delta 181}$ homozygotes. The mutants had a reduced neurotransmitter vesicle size. A decrease in the levels of the protein Bruchpilot, involved in assembling active zones in the presynaptic region was observed. The levels of post- synaptic ionotropic glutamate receptor subunit GlurIIRA were higher and that of the subunit GluIIRB were reduced in these mutants. This synaptic phenotype was rescued upon pre and post- synaptic expression of Dmon1. The presynaptic knockdown of *Dmon1* is sufficient to cause the GluIIRA phenotype, suggesting a presynaptic regulation of glutamate receptor levels by *Dmon1*, in the larval NMJ. Mon1 is also suggested to be secreted in a membrane bound form or otherwise for functioning (DEIVASIGAMANI *et al.* 2015).

The *Dmon1*$^{\Delta 181}$ flies are sterile- the sterility arises from the fact that females have very small ovaries and oogenesis stalls at stage 8, in the previtellogenic stages (Fig 5) (DHIMAN *et al.* 2019).

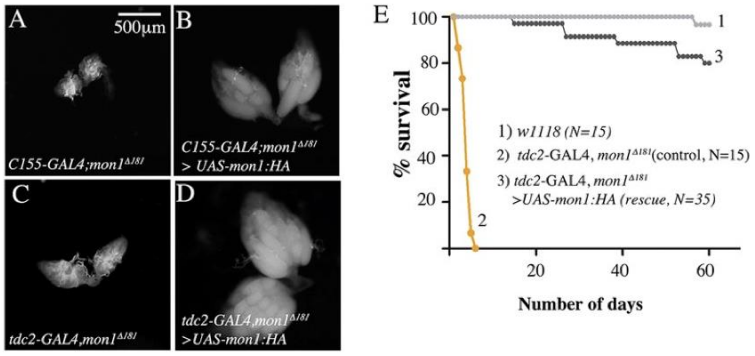

Fig 5: Neuronal rescue of sterility and lifespan of *Dmon1*$^{\Delta 181}$ homozygotes. The Δ181 homozygotes show sterility, exhibiting stalling of oogenesis by stage 8 of oocyte development. This phenotype was rescued by the pan neuronal expression of *Dmon1* in the flies and an OPN neuronal specific expression of *Dmon1*. **(A)** Gal4 control for the pan neuronal expression of *Dmon1* in Δ181 homozygotes, showing very small ovaries. **(B)** Pan neuronal expression of *Dmon1* using the c155 pan neuronal driver in Δ181 homozygotes. The ovary size can be seen to have improved. **(C)** Tdc2-Gal4 control for the OPN neuronal specific expression of *Dmon1* in Δ181 homozygotes, showing ovaries of reduced size. **(D)** OPN neuronal specific expression of *Dmon1* in Δ181 homozygotes improves the ovary size and functioning, phenocopying the pan neuronal expression of *Dmon1*. **(E)** Survival plot for the OPN neuron specific *Dmon1* rescue flies. It can be seen that the expression significantly increases the survival of the flies, making it close to the

wild type survival plot, as compared to the Δ181 homozygotes. (Adapted from DHIMAN *et al.* 2019)

This phenotype of sterility and lifespan defects, along with motor defects were rescued by the pan neuronal expression of *Dmon1*. An Octopaminergic and Tyraminergic (OPN) neuronal specific knockdown of Mon1 partially mimicked this phenotype of arrested ovariole development. OPN neurons innervate the peritoneal sheath muscles of ovaries in *Drosophila*. Pan neuronal expression of *Dmon1* led to the completion of oogenesis in these homozygous flies. It was found that expression of *Dmon1* in OPNs alone using the Tdc-2-Gal4 driver phenocopied the pan neuronal expression of *Dmon1* (Fig 5) (DHIMAN *et al.* 2019).

The Insulin pathway in *Drosophila* has roles in the physiology of the animal. It is established that oogenesis gets affected by mutations of genes falling in this pathway (LA FEVER and DRUMMOND- BARBOSA 2005). The phenotype of stalled oogenesis is similar to mutants of genes in the insulin pathway. Upon further investigation, several *Drosophila* Insulin- like peptides, DILPs, were found to be downregulated in *Dmon1* mutants. The ILPs DILP5 and DILP3 were seen to be downregulated in the Insulin producing cells (IPCs) in the Δ181 homozygous mutants, and the expression of these molecules in the IPCs was found to improve oogenesis in the mutants. While a previously existing model suggests the regulation of oogenesis via signalling to the IPCs from the fat body, after sensing nutrient levels in the body, through a JAK/STAT ligand Upd2, the current model places *Dmon1* between the IPCs and OPN neurons, in the regulation and completion of oogenesis. It is assumed that the activity of Mon1 in the OPNs influence the levels of DILPs in IPCs, particularly that of DILP5, effecting a regulation of oogenesis via the insulin signalling pathway (DHIMAN *et al.* 2019).
A model of signalling between the OPN neurons and the insulin-producing cells, which produce the *Drosophila* insulin-like peptides, DILPs, that signal to regulate oogenesis in the organisms, has been proposed to explain the role of *Dmon1* in the regulation of oogenesis (Fig 6) (DHIMAN *et al.* 2019).

The current model suggests a function for *Dmon1* in the inter organ signalling between the brain and the ovaries. It puts *Dmon1* in the pathway of DILP signalling from IPCs, in a different axis, which is alternatively regulated by nutritional cues, detected by the fat body. The current study reveals a regulation where the OPN neurons communicate to the IPCs via an unknown mechanism, which involves *Dmon1* (DHIMAN *et al.* 2019).

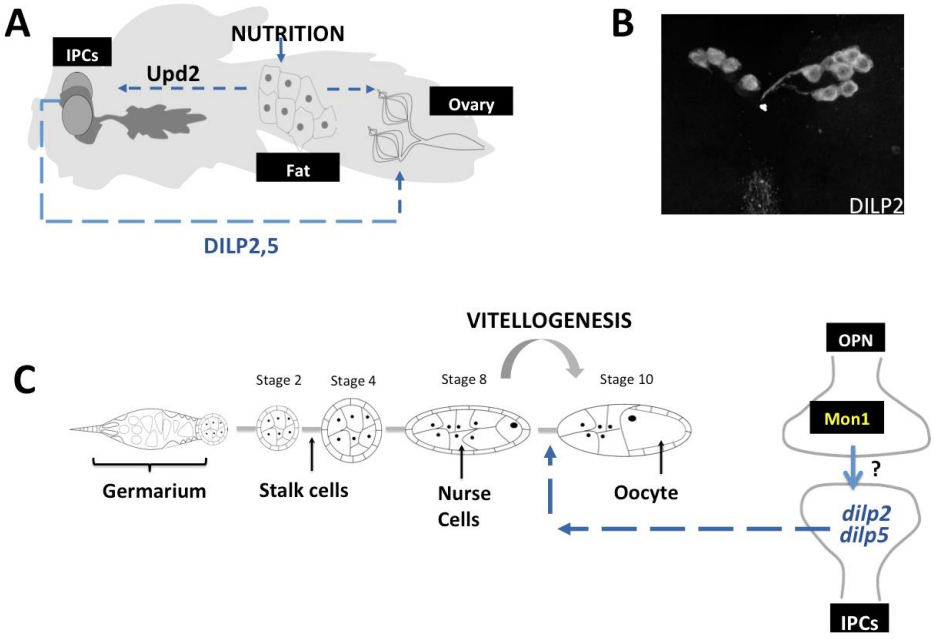

Fig 6: OPN specific expression of *Dmon1* restores oogenesis in the mutants through Insulin pathway signalling. OPN (Octopaminergic and Tyraminergic neurons) specific expression of *Dmon1* using the Tdc2-Gal4 driver has been shown to rescue the oogenesis in the Δ181 homozygotes. The stalled oocyte development at stage 8 was observed to be proceeding to completion in these female flies.
(A) Previous model of regulation of oogenesis in *Drosophila*. Nutritional levels in the body are sensed by the fat body of the animal. The JAK/STAT ligand Upd2 signals the Insulin producing cells (IPCs) in the *Drosophila* brain in response to this. Downstream of this, the IPCs signal the ovaries via DILP2 and DILP5 to regulate oogenesis. **(B)** DILP2 expressing cells in the *Drosophila* brain, among the IPCs. **(C)** Model of signalling involving *Dmon1* in the regulation and rescue of oogenesis. In the Δ181 homozygotes, oogenesis stalls at stage 8. This stalling is relieved by an expression of *Dmon1* in the OPN neurons. *Dmon1* in OPN neurons, via an unknown mechanism, signals the IPCs and induces downstream Insulin signalling via DILP2 and DILP5. A restored DILP signalling in the Δ181 homozygotes regulates and promotes oogenesis and allows the completion of the development of the egg chambers. This model places *Dmon1* in the axis of signalling between the brain and the ovaries in its physiological regulation. (Adapted from DHIMAN *et al.* 2019)

A functional role for Mon1 in dendritic arborisation (da) of type II, Class IV da neurons of the peripheral nervous system in *Drosophila* larvae has been identified (HARISH *et al.* 2019). Apart from its role in the endosomal degradative pathway, the role for the Mon1-Ccz1 complex in this scenario has been attributed to its possible modulation of the endosomal recycling pathway. Rab 5 and Rab 11 have been identified to have roles in modulating dendritic arborisation. Rab 11 is a part of the recycling endosomal pathway. Loss of *Dmon1* leads to an increase, and an overexpression of *Dmon1* causes a decrease in arborisation and complexity of the neurons. The phenotypes of increased or decreased dendritic arborisation upon genetic modulation of Rab 11 were

found to be of similar extents but in an opposing manner as exhibited by *Dmon1* mutants.

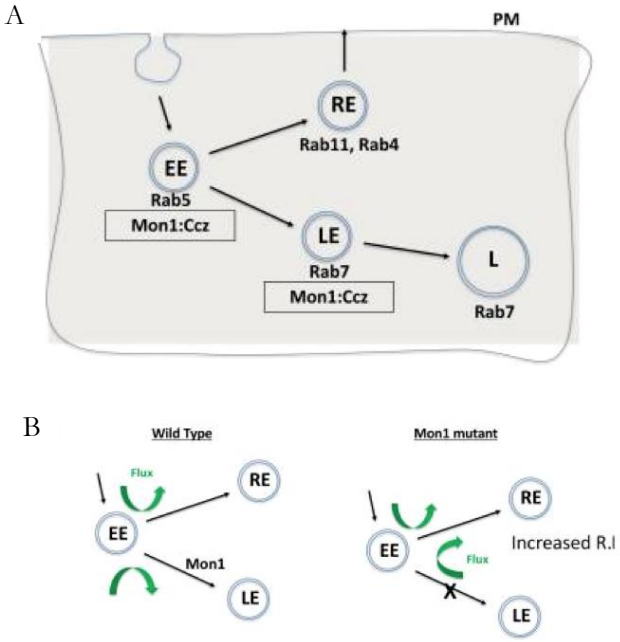

Fig 7: *Dmon1* mediates dendritic arborisation by regulating vesicular flux between the recycling endosomal and lyso-endosomal pathway. The degree of dendritic arborisation (da) of type II, Class IV da neurons of the peripheral nervous system in *Drosophila* larvae was shown to be regulated by *Dmon1*. **(A)** The model of the endosomal recycling and lysosomal degradative pathways marked by Rab 11 and Rab 7. Incoming early endosomes marked by Rab 5 are channelled to the recycling endosomal pathway marked by Rab 11 and Rab 4, or the lyso-endosomal pathway, marked by Rab 7 vesicles. **(B)** Model of dendritic arborisation regulation by *Dmon1*. The flux between the RE and LE pathways are regulated in wild type condition, while in a *Dmon1* mutant, the flux towards the RE pathway increases as vesicle maturation in the LE pathway is disrupted, leading to an increase in the Rab 11 mediated vesicular recycling and dendritic arborisation, shown by an increase in the Ramification Index (R.I.) indicating the extend of arborisation of the neurons. Adapted from HARISH *et al.* 2019.

The paper suggests a possible arborisation regulation where *Dmon1* modulates the vesicular flux of Rab 11 mediated recycling endosomal (RE) pathway, where the RE pathway is identified as the key regulator of the process. While *Dmon1* functions are attributed until now to the endo-lysosomal pathway, the pathway is not identified as a determinant in da. The model suggests that a loss of *Dmon1* shunts the accumulated Rab 5 positive early endosomes into the RE pathway, while an increase in *Dmon1* increases the Rab 5 to Rab 7 conversion of endosomes, which in turn reduces the flux of RE vesicles, reducing arborisation (Fig 7). It is suggested that an improved flux of the RE

vesicles will cause increased exocytosis, which is correlated to an increased arborisation, which is seen in the context of larval CIVda neurons (HARISH *et al.* 2019).

Maternal effect genes

The physiology of an organism is traditionally considered to reside in the genetic make-up and gene expression patterns of cells. Apart from the influence of the genetic identity of the organisms, initial signalling events that dictate the body axis of an organism and orchestrate patterning and early development come from the different gene products that are deposited in the egg by the mother, which is in turn influenced by the genetic make-up of the same. The term "Maternal effect" refers to the genotypic or phenotypic influence of the mother on the offspring (WOLF AND WADE 2009). Maternal effect genes are the ones whose transcripts or gene products are deposited by the mother into the egg, which later goes on to direct initial patterning events in the embryo. This is influenced by the mother's genome and physiology. This mechanism is conserved across different phyla. In *Drosophila*, a series of incomplete mitotic and meiotic cells divisions render 16 cells that are connected to each other cytoplasmically, of which one of them with the maximum number of connections to the neighbouring cells is specified as the oocyte. Following this, the remaining 15 cells endocycle and function as nurse cells which deposit gene products, in the form of RNA or protein into the oocyte, during oogenesis (BASTOCK AND ST JOHNSTON 2008) (Fig 8). The deposition is mediated by transport via microtubules inside the egg chambers. Different genes such as bicaudal-D, orb and egalitarian determine oocyte specification and microtubule organisation into the predetermined oocyte (GONZALEZ-REYES *et al.* 1997).

The gene products in the form of mRNA or proteins are deposited to form gradients, which set up the body axis of the organism, even before fertilisation. Initial patterning events upto a certain duration are dictated by these maternally deposited morphogens. The zygotic genome later kick-starts its activity, after this time point, in response to the maternal gene products. Maternal effect genes are dominant from 0-2.5 hrs in *Drosophila* and transition to zygotic expression takes place later (Fig 8) (TADROS AND LIPSHITZ 2009).

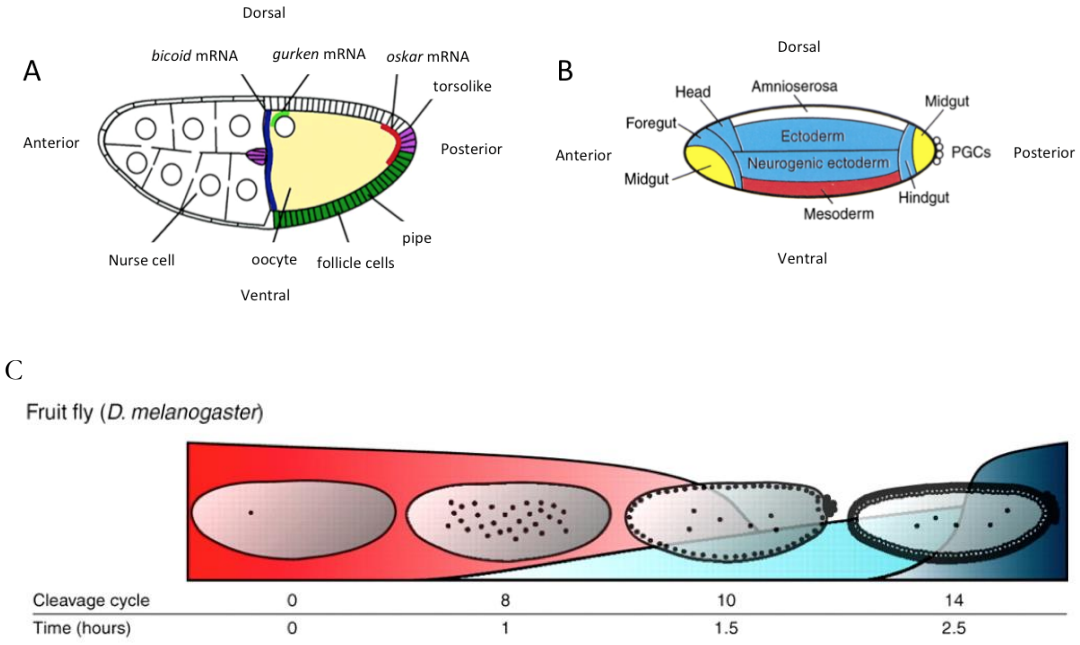

Fig 8: Maternal effect genes and maternal to zygotic transition in *Drosophila*. Gene products in the form of RNA or protein are deposited in the oocyte by the nurse cells in *Drosophila*, contributing to the maternal effect. **(A)** *Drosophila* stage 10 egg chamber with nurse cells and developing oocyte. Different maternal gene products are deposited into the oocytes, such as that of *gurken*, *oskar* and *bicoid*, by the nurse cells, which sets up oocyte polarity. **(B)** A developing blastoderm embryo of *Drosophila*. The initial polarity set up by the maternal genes is later translated into the zygotic dorso-ventral and antero-posterior axes. Early development is orchestrated by these maternally deposited factors. **(C)** Maternal to zygotic transition in *Drosophila* oocytes. Initial patterning events in the embryo are mediated by the activity of maternally deposited factors or the maternal genes, shown in red. The activity of the zygotic genes kick-starts at a later stage in response to maternal genes, called the maternal to zygotic transition. In *Drosophila*, zygotic genes are activated and take over maternal effect gene activity by 2.5 hours after fertilisation of the oocyte. The process is conserved among different phyla. Adapted from/ source: A- St Johnston Lab Home page. B-. STAINIER 2002, C- TADROS AND LIPSHITZ 2009.

The ModENCODE data from Flybase suggests that *Dmon1* transcripts are present in moderately high levels in 0-2 hour embryos, which will be deposited by the mother into the oocyte (Fig 9). This suggests that there might be a potential maternal role for Dmon1.

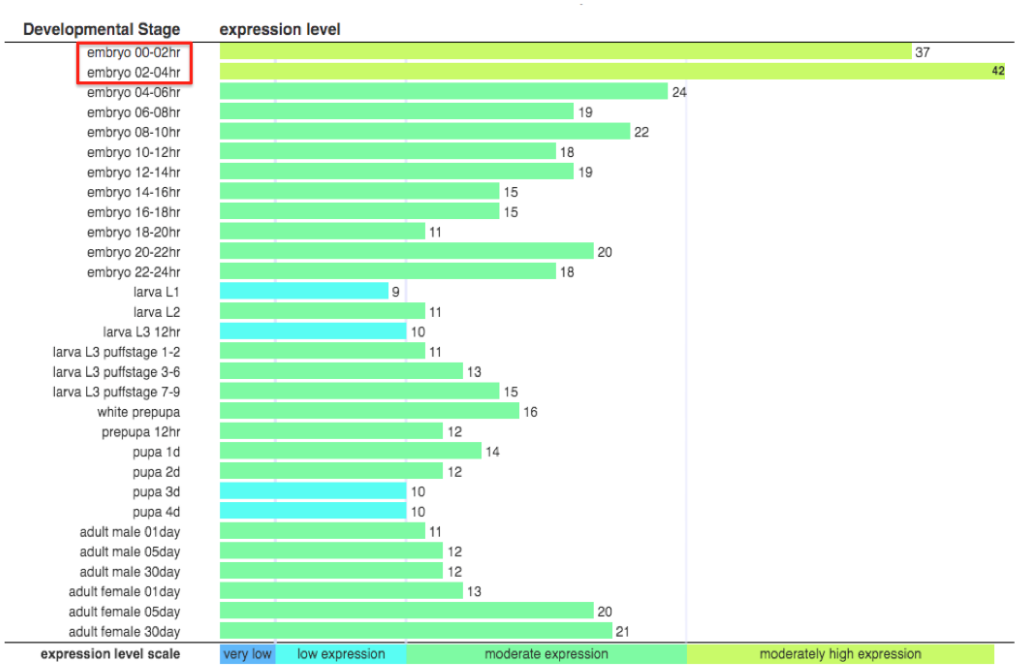

Fig 9: *Dmon1* shows moderately high expression in early embryos. ModENCODE data of transcript levels of *Dmon1* from Flybase. 0-2 hour and 2-4 hour embryos show a moderately high expression of transcript levels of *Dmon1*. Since the maternal effect genes are of predominant activity at this time interval, these transcripts are likely deposited by the nurse cells in the oocytes from the mother, and leads us to the fact that there can be a maternal function for the gene

Interestingly, it was observed that mutant females that are rescued for fertility and lifespan through expression of *mon1* in all neurons (pan-neuronal) or just in the octopaminergic/tyraminergic neurons (OPNs) alone, laid eggs that developed cuticle but failed to hatch (Kumari Shweta and Anuradha Ratnaparkhi., personal communication). This indicated a possible maternal role for the gene since the rescue is restricted to the soma and not the germline. Preliminary experiments also showed that these embryos have patterning defects (Sushmita Hegde, Girish Ratnaparkhi, Anuradha Ratnaparkhi., personal communication). The above preliminary observations, together with the ModENCODE expression data indicated a maternal role for Mon1 in embryonic development.

Taking advantage of the fact that *Dmon1* homozygous mutants can be rescued by OPN expression of the gene, one can thus study the outcomes in development and patterning of the embryo brought about by the absence of *Dmon1* in the germline. This project aims at identifying and characterising the role of *Dmon1* as a maternal effect gene in *Drosophila*.

CHAPTER II

Approach

In order to study the maternal effect of a gene, embryos that lack any maternal transcript or other gene products of the particular gene of interest should be obtained. One can achieve this if the animals null for the gene are fertile and lay eggs that can undergo fertilization. In such animals, which are somatic and germline loss of function mutants for the gene of interest, the fertilised embryos will be devoid of any maternally deposited factor of the gene. In the case of *Dmon1* mutants, the animals are short-lived and sterile due to stalling of oogenesis. Both these phenotypes are rescued upon expression of the gene in neurons (DHIMAN *et al.* 2019). These 'rescue' females are still mutant for the germline which includes the nurse cells that dump maternal mRNAs into the oocyte. Consequently the eggs laid by these 'rescue' mothers would lack maternal mon1. To obtain maternal mon1 mutant embryos we used 'rescue' females which are somatic, and germline mutants, except in the OPN neurons.

Strategy to obtain Mon1 maternal null embryos

As mentioned above, the study on the role of *Dmon1* in the context of oogenesis reveals a regulatory mechanism in the *Drosophila* brain. *Dmon1* homozygous mutants are weak, sterile and have motor defects, with a severely reduced lifespan. These phenotypes of lifespan of the organism, along with the egg lay and motor defects are rescued by the pan-neuronal expression of *Dmon1*. In addition, expression in the OPN neurons alone is sufficient to rescue these defects (DHIMAN *et al.* 2019).

These neuronal 'rescue' females are mutant for all other somatic tissues including the germline. This means that the nurse cells which produce and dump the maternal effect genes in the oocyte are also mutants for *Dmon1* in these animals.

These animals were obtained by conducting genetic crosses between different stable lines, mentioned in the materials and methods section (Fig 10).

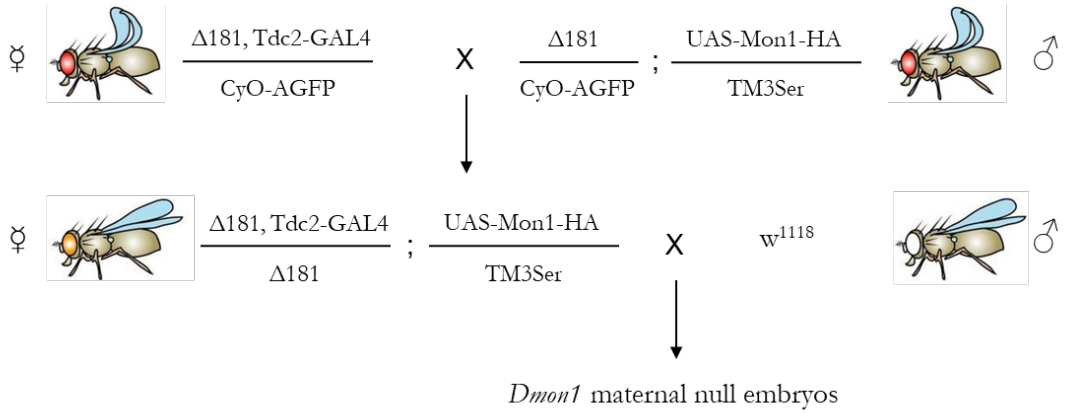

Fig 10: Schematic representation of the crosses set up to obtain *Dmon1* rescued females, used to obtain *Dmon1* maternal null embryos. Δ181, Tdc2-GAL4/CyO-AGFP virgin females were collected and kept at 18 ^{0}C before setting up a cross with Δ181/CyO-AGFP; UAS-Mon1-HA/TM3 Ser males. The crosses were set in 6 % fly media bottles, and kept at 25 ^{0}C. The straight winged, orange eyed, virgin female progenies, which are of the *Dmon1* rescued phenotype, Δ181, Tdc2-GAL4/ Δ181; UAS-Mon1-HA/ + genotype, were collected to obtain *Dmon1* maternal null embryos, after setting up a cross with w^{1118} males.

For all future experiments, the *Dmon1* neuronal rescue animals and the maternal null embryos from the above cross were used.

CHAPTER III

Results

Characterisation of the *Dmon1*$^{\Delta 181}$ fly lines

The parent fly lines used to set up the cross to obtain *Dmon1* maternal null flies were subject to genotyping prior to their use in the experiments. Primers used to conduct genomic PCR are mentioned in the materials and methods section. Genomic PCR was done to DNA extracted from homozygous larvae and flies to confirm the presence of the Δ181 deletion (Fig 11).

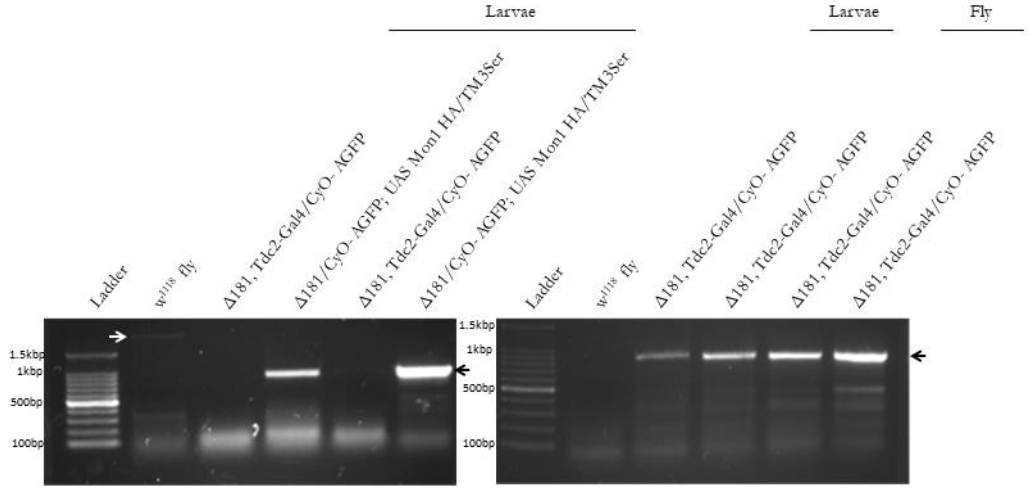

Fig 11: PCR products for amplification of Genomic DNA using *Dmon1* specific primers. PCR amplification of the genome was done using *Dmon1* specific primers. For wild type flies, full gene product for *Dmon1* can be seen at a molecular weight of above 1.5kbp (white arrow). An 850 bp deletion product can be observed in case of Δ181 homozygous lines, both larvae and adult flies, marked by black arrows.

Testing the Δ181, 9313/CyO AGFP line for a functional Tdc2-Gal4 construct

A cross was set up between the Δ181, Tdc2-Gal4/ CyO AGFP line and a UAS CD4 GFP/ CyO lines to confirm that the Tdc2-Gal4 construct was functional. The larvae showed expression of GFP in the Tdc2 neurons in the brain and ventral nerve cord (Fig 12).

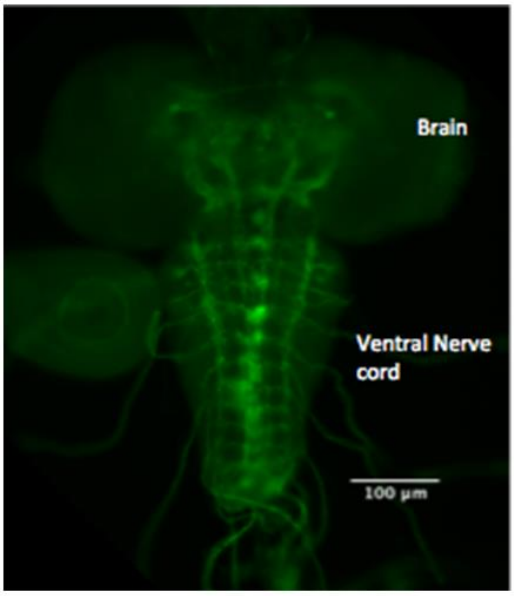

Fig 12: Δ181, Tdc2-Gal4/ UAS CD4 GFP Larval brain and ventral nerve cord showing Tdc2 neurons immunostained against GFP. The GFP fluorescence can be seen restricted to the 2 columns of the Octopaminergic and Tyraminergic (OPN) neurons of the larval brain.

Dmon1 Maternal nulls are embryonic lethal

Preliminary observations (Kumari Shweta and Anuradha Ratnaparkhi, personal communication) had indicated that embryos lacking maternal *mon1* develop to late stages but most of them fail to hatch. As a first step towards understanding the maternal role for *mon1*, a detailed analysis of the lethality was conducted.

Dmon1 maternal null embryos displayed a high rate of early lethality (Fig 13). More than 90 percent of Mon1 embryos failed to hatch after 24 hours. Very few hatch and proceed to form pupae and eclose (<5 in 200). This preliminary experiment convinced us that a lack of maternal *Dmon1* has adverse effects on the development of the organism, even though a zygotic copy of the gene from the male parent was present.

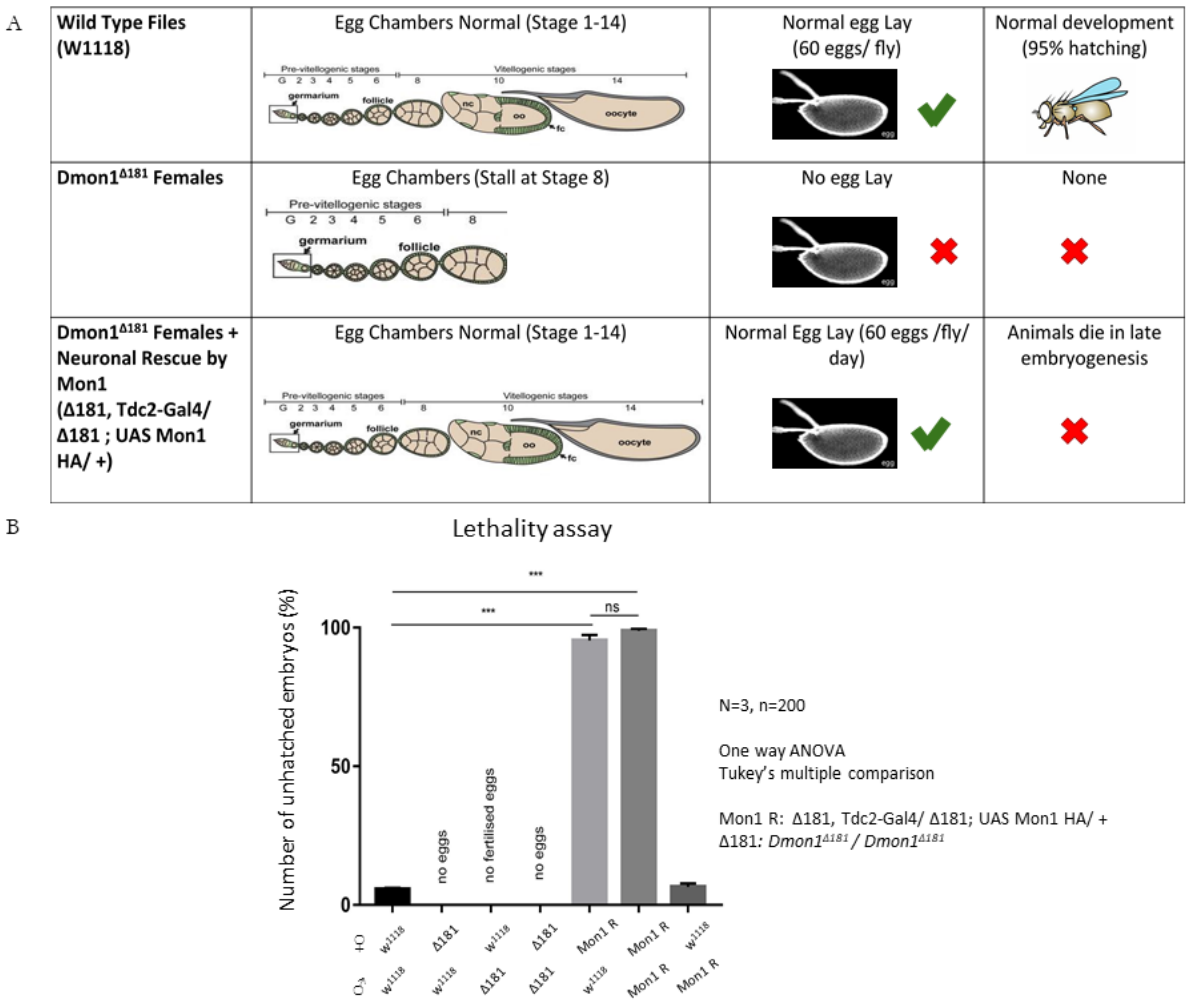

Fig 13: *Dmon1* maternal null embryos show high rate of early lethality. Δ181 homozygous flies with OPN specific expression of *Dmon1* were used to obtain *Dmon1* maternal null embryos. Lethality test was conducted to see if the embryos hatched normally. **(A)** Neuronal rescue of Δ181 homozygotes. In wild type flies, oogenesis goes through 14 stages of oocyte or egg chamber development. They have normal egg lay and 95% of the embryos hatch and develop into adults. In Δ181 homozygotes, the oogenesis stalls by stage 8 and the flies lay no fertilised eggs. In Δ181 homozygotes with OPN specific expression of *Dmon1*, oogenesis is restored and the flies lay eggs in a number comparable to wild type flies. Even though the embryos are fertilized, they die in late embryogenesis and fail to hatch. **(B)** Lethality assay conducted to quantify the embryonic lethality expressed by *Dmon1* maternal null embryos. 200 embryos were spotted on a sugar agar plate and aged for 24 hours and number of unhatched embryos was scored for. Wild type embryos show very less lethality rates, Δ181 homozygous females lay no eggs whether mated with a wild type or a Δ181 homozygous male. Δ181 homozygous males fail to fertilise wild type oocytes. *Dmon1* rescued females lay eggs that show high rate of lethality irrespective of the genotype of the male parent, that is, a wild type or a *Dmon1* rescued male. This is confirmatory of the fact that the lethality arises due to the genotype of the female and not of the male, and that the maternal effect of the gene is causing the phenotype. The function of the gene is essential in the maternal context, as a wild type copy of the gene from the male parent, as in the case of a *Dmon1* rescued female mating with a wild type male, was not sufficient to rescue the lethality phenotype.

The data was statistically analysed using the Graphpad-Prism software. One way ANOVA and Tukey's multiple comparisons test were performed. p value < 0.05 (*), <0.01 (**), <0.001 (***)

Dmon1 Maternal nulls show Antero-posterior and Dorso-ventral (A-P and D-V) patterning defects

Preliminary observations (Sushmita Hegde, Girish Ratnaparkhi., personal communication) seemed to indicate patterning defects in *mon1* maternal mutant embryos. Cuticle preparations serve as a useful technique to gain a broad insight into the patterning phenotypes caused by the specific genetic make-up of the embryo. It provides information regarding patterning defects which would lead to the perturbations of developmental mechanisms and the lethality of the embryos.

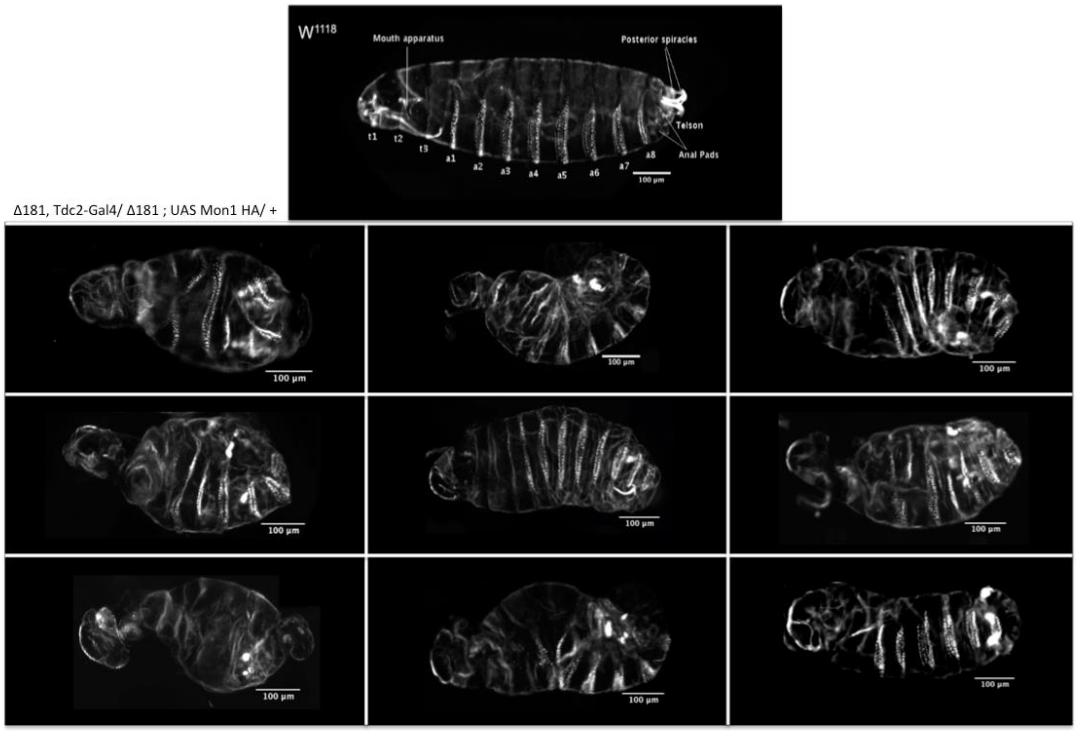

Fig 14: *Dmon1* Maternal null embryos show patterning defects. Cuticle preparation of *Dmon1* maternal null embryos shows a range of patterning defects in these organisms. Top panel shows a wild type cuticle with normal patterning. The denticle bands can be observed, along with proper head and tail structures (terminal structures) in these cuticles. Bottom panel shows *Dmon1* Maternal null cuticles with patterning defects. Both antero-posterior and dorso-ventral patterning defects can be seen in these embryos. The phenotypes are of varying intensities. The embryos lack head structure. Some of them lack tail structures, too. The denticle bands of these embryos are perturbed from their normal pattern, with bands missing, or bands seen to fuse together. Some of the embryos show a twisted phenotype, reminiscent of the phenotype of *twist* embryos. Some of the cuticles resemble a phenotype exhibited by *dpp* mutants.

(Embryos imaged anterior left, dorsal up orientation, in 10X magnification, Dark-field imaging using Apotome microscope).

A range of phenotypes was observed in the cuticles of the Mon1 maternal null embryos. Absence of head structures was a noted feature of these embryos. The denticle bands of these embryos were perturbed, the antero-posterior and dorso-ventral alignment of the bands were disrupted and some of them were fused. Some of these embryos also lacked terminal structures, and few of them had appeared to show a twisted morphology. Such developmental defects of varying severity would have led to the early lethality of these embryos.

The results show a clear defect in the A-P and D-V patterning of the embryos. Some of the mutant cuticles resemble that of *twist*, *dpp* mutants, while some of them (<10 in 100) are more comparable to wild type cuticles, indicating an incomplete penetrance of the phenotype in the population.

Mon1 Maternal nulls show a distinct nuclear phenotype

Since the *Dmon1* maternal null embryos show lethality and a range of patterning defects, the next step was to narrow down and look into the patterning, cellular and physiological aspects that were getting perturbed in the embryos. This included looking at patterning genes and their localisation, along with characteristics of the defects in the embryonic development throughout different stages. Antibody and DAPI staining in early embryos revealed an interesting phenotype in *Dmon1* maternal null embryos. A number of 0-3 hour *Dmon1* maternal null embryos showed an irregular distribution of the nucleus on their periphery. The nuclei in these embryos showed a patchy distribution as compared to the wild type. Dividing nuclei showed asymmetry in the division of the nuclear material. These were patches with no nuclei. In cellularised embryos, the distribution of the nuclei seemed uneven (Fig 15).

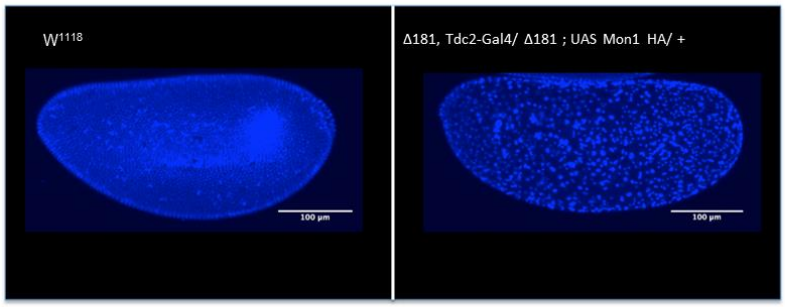

Fig 15: *Dmon1* maternal null embryos show nuclear defects. Irregularities in the size and distribution of the nuclei in the periphery of the 0-3 hour embryos were found in the case of *Dmon1* maternal nulls. The DAPI nuclear staining of embryos gives a picture on the nuclear distribution of these embryos. In a w^{1118} embryo, the nuclei can be seen distributed evenly throughout the periphery of the embryo. In the *Dmon1* maternal null embryo, patchy distribution of the nuclei can be observed. Patches with no nuclei can be seen. The dividing nuclei show an asymmetric distribution of the genetic material. Nuclear distribution throughout the periphery of the embryo looks uneven.
(Embryos imaged anterior left, dorsal up orientation, in 20X magnification, Confocal imaging using Leica SP8 microscope).

Oskar and Nanos *In-situ* experiments show mislocalisation of pole-plasm

As the cuticles of the maternal nulls showed a range of patterning defects, both antero-posterior and dorso-ventral, one could trace back and investigate the underlying molecular mechanisms that are perturbed in these embryos during patterning. Since patterning genes are expressed early on in the embryos, we looked at the localisation of candidate A-P and D-V patterning genes in early embryos where maternal genes dictate patterning.

One can visualise this by either antibody or RNA staining. *In-situ* hybridization is a powerful technique to identify the localisation of mRNA in tissues. It gives us qualitative data on the position or extent of morphogen gradients which are in the form of mRNA expression or deposition. Since polarity specification has been known to be directed by deposition of mRNA of the different patterning genes, we first approached the problem using this method.

From the *in-situ* experiments conducted, a visible disruption in the localisation of the pole-plasm components was observed in the Mon1 maternal null embryos. *Osk* (Fig 16) and n*os* (Fig 18) staining shows a mislocalization, diffused localisation and for some embryos, no localisation at all in the posterior of the embryo.

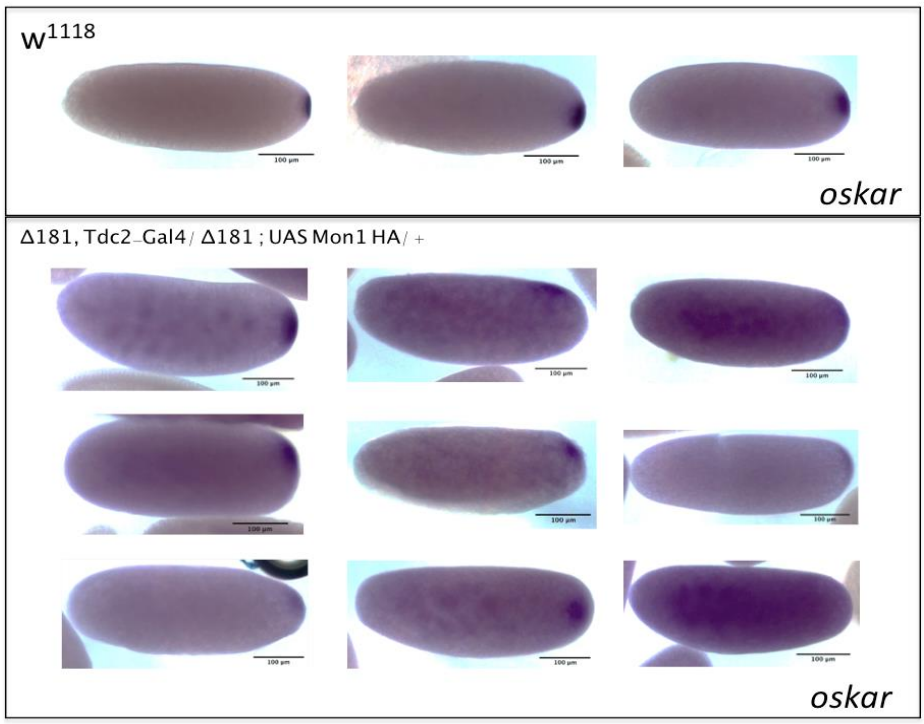

Fig 16: *Oskar* mRNA is mislocalised in *Dmon1* maternal nulls. *In-situ* hybridization against *oskar* in 0-3 hour *Dmon1* maternal null embryos shows a mislocalisation of the pole plasm from the posterior. Top panel shows 3 wild type embryos with a normal localization of *oskar* mRNA in the posterior. The mRNA can be seen confined to the posterior end of the embryo. Bottom panel shows *Dmon1* maternal null embryos showing a mislocalisation of the mRNA, away from the posterior tip as compared to a wild type embryo. Different phenotypes such as a lack of posterior localization or diffused localisation were observed. (Embryos imaged anterior left, dorsal up, in 10X magnification, Bright-field imaging on Apotome microscope).

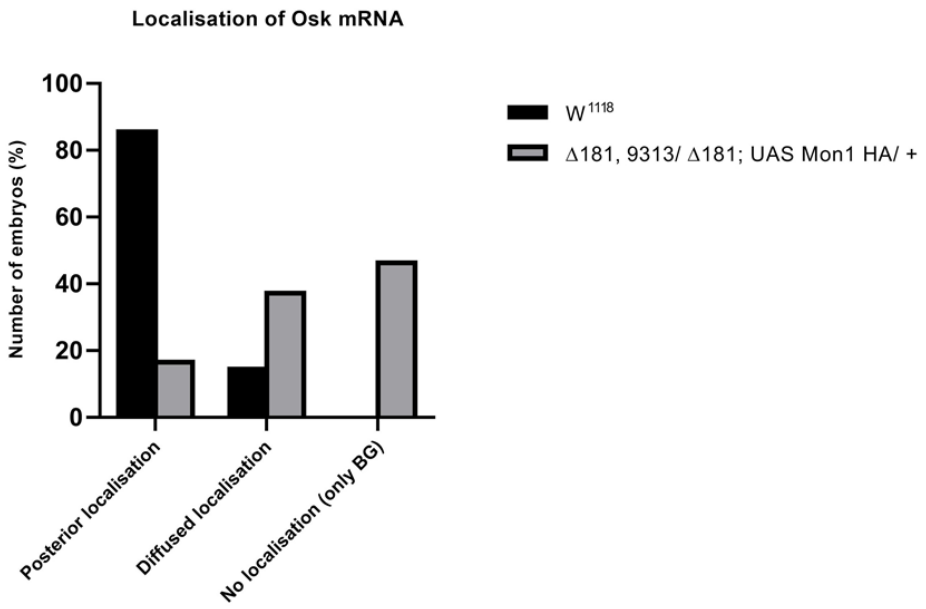

Fig 17: Categorisation of embryos showing different *oskar* localisation phenotypes. Different *Dmon1* maternal null embryos showed different phenotypes of *oskar* localisation. The number of embryos showing proper posterior localisation was less. Many of the embryos showed a diffused localisation of the mRNA compared to a wild type embryo. Many of them showed an absence of posteriorly localised mRNA staining, and instead showed only background (BG) staining. N=1.

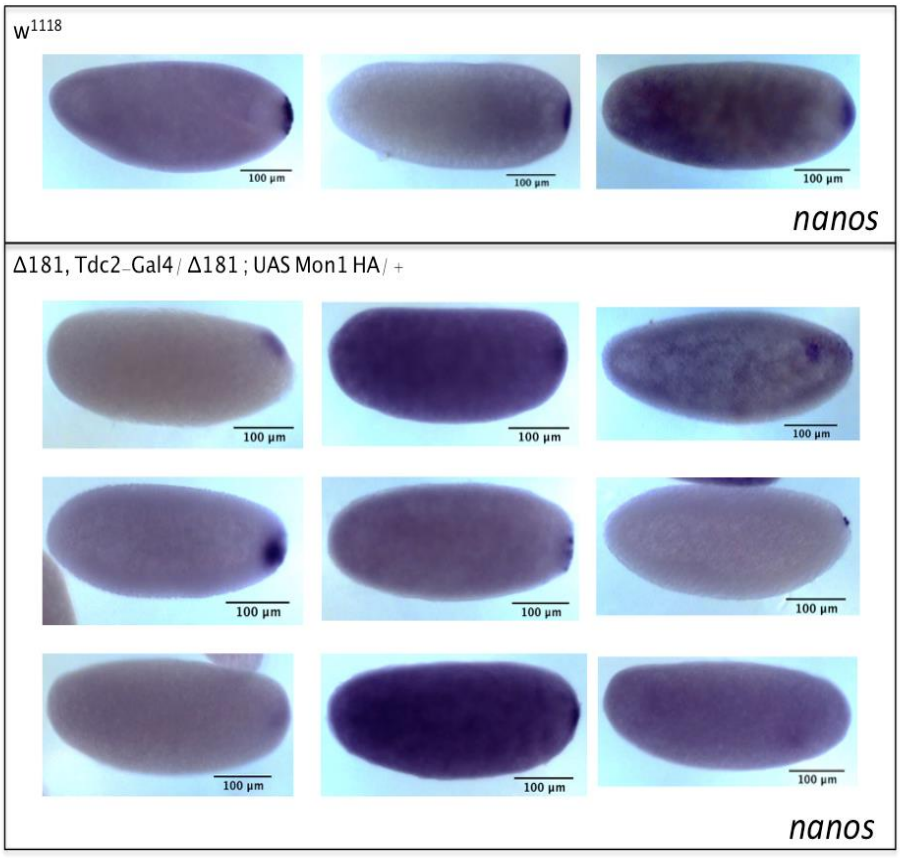

Fig 18: *Nanos* localization is perturbed in *Dmon1* maternal nulls. *In-situ* hybridization against *nanos* in 0-3 hour *Dmon1* maternal null embryos shows a mislocalisation of the pole plasm from the posterior, compared to wild type. Top panel shows 3 wild type embryos with a normal

localization of *nanos* mRNA in the posterior. The mRNA can be seen confined to the posterior end of the embryo. Bottom panel shows *Dmon1* maternal null embryos showing a mislocalisation of the mRNA, away from the posterior tip or with lesser staining, as compared to a wild type embryo. Different phenotypes such as a lack of posterior localization or diffused localisation were observed.
(Embryos imaged anterior left, dorsal up, in 10X magnification, Bright-field imaging on Apotome microscope).

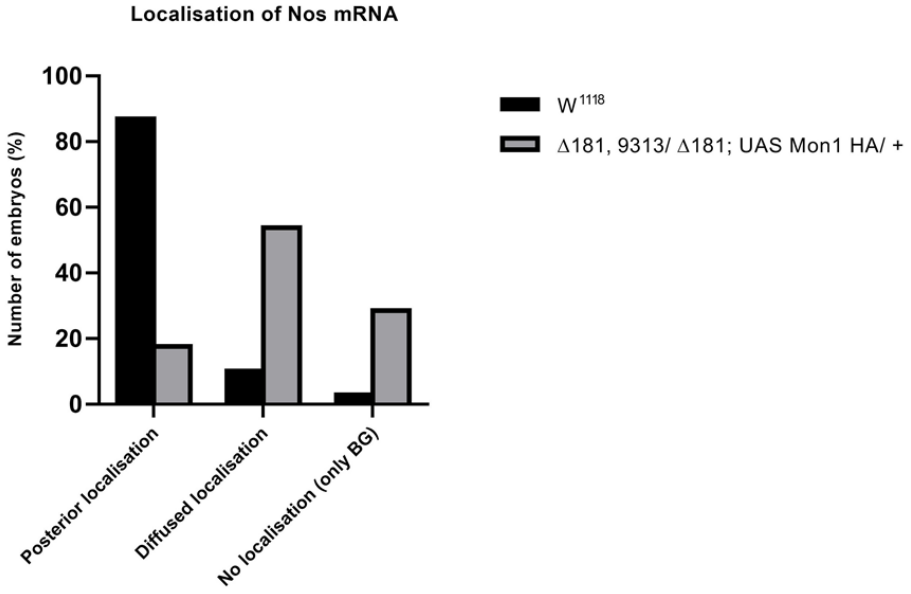

Fig 19: Categorisation of embryos showing different *nanos* localisation phenotypes. Different *Dmon1* maternal null embryos showed different phenotypes of nanos localisation. The number of embryos showing proper posterior localisation was less. Many of the embryos showed a diffused localisation of the mRNA compared to a wild type embryo. Many of them showed an absence of posteriorly localised mRNA staining, and instead showed only background (BG) staining. N=1.

D-V patterning genes are expressed normally in *Dmon1* maternal null embryos

In-situ experiments conducted on D-V patterning genes such as *twist*, *sog*, *rhomboid* showed no perturbation in the localisation of these mRNAs in the *Dmon1* maternal null embryos.

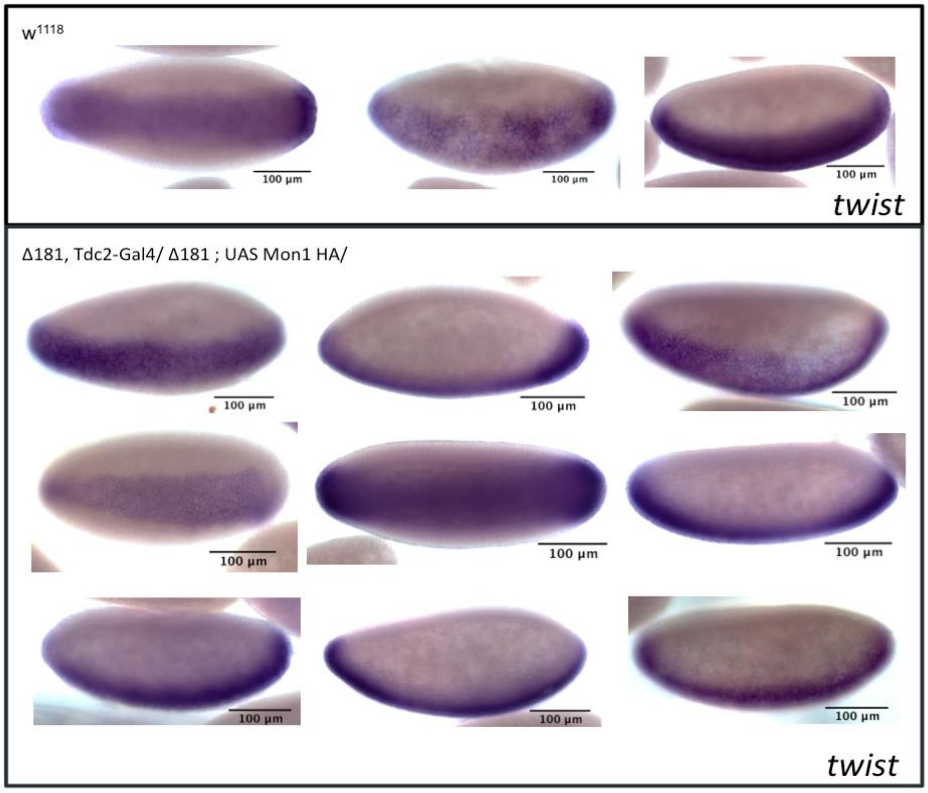

Fig 20: D-V patterning genes seem unperturbed in *Dmon1* maternal nulls. *In-situ* hybridization against *twist* in 0-3 hour *Dmon1* maternal null embryos shows a normal localisation of the mRNA at the ventral side, compared to wild type. Top panel shows 3 wild type embryos with a normal localization of *twist* mRNA in the posterior. The mRNA can be seen confined to the ventral side of the embryo. Bottom panel shows *Dmon1* maternal null embryos showing unperturbed localisation of the mRNA, compared to a wild type embryo. The staining seen in the mutants were comparable to that of wild type embryos. (Embryos imaged anterior left, dorsal up, in 10X magnification, Bright-field imaging on Apotome microscope).

The D-V patterning genes such as *sog* and *rhomboid* also showed a relatively unperturbed localisation in the mutants embryos, compared to wild type embryos. Both *sog* and *rhomboid* showed normal lateral localisation of their mRNAs in the *Dmon1* maternal null embryos.

Ovary *In-situ* show reduced localisation of *oskar* in oocytes of later stages

As mentioned before, the setting up of embryonic axes is preceded and directed by deposition of maternally derived factors in the oocyte. Since *osk* and *nos* are deposited by the nurse cells in the oocytes, *in-situ* hybridisation was done on ovaries of the Mon1 rescue flies from which the embryos were obtained for the pole plasm components.

This led us to investigate the role of Mon1 in localising oocyte polarity determination factors, such as *oskar* and *nanos*.

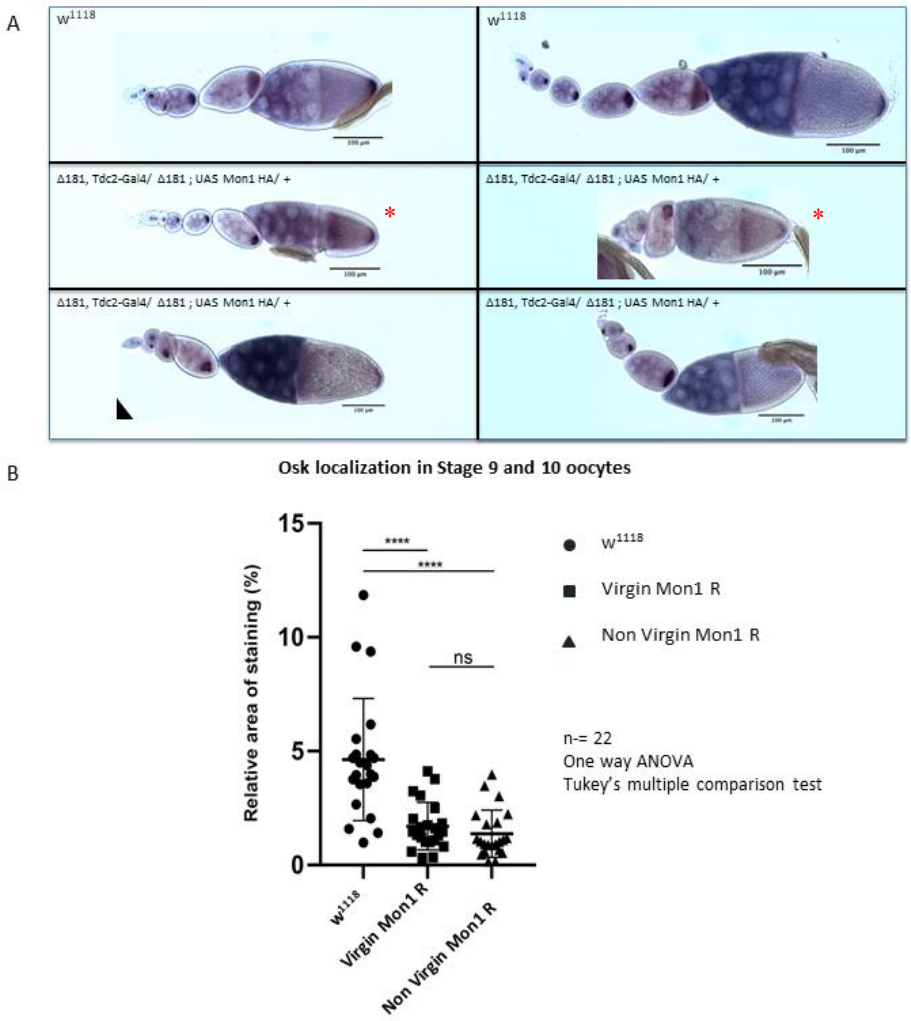

Fig 21: *Oskar* localisation in Stage 9 and 10 oocytes is perturbed in *Dmon1* rescued (Mon1 R) females. *In-situ* experiments in the ovaries of *Dmon1* rescued females show a mislocalisation of *oskar* in stage 9 and 10 oocytes. A lack of area of staining of the mRNA or a complete lack of localisation of the mRNA was observed. **(A)** Ovarioles of the *Dmon1* rescued females stained against *oskar*. Top panel shows wild type ovarioles, with stage 9 or 10 oocytes. *Oskar* can be seen localised to the posterior of the stage 9 and 10 oocytes, in a cap like localisation area. Bottom panels show ovarioles from *Dmon1* rescued females. While some of the stage 9 or 10 oocytes show posterior localisation comparable to wild type oocytes, others show very less or a lack of posterior staining (marked by asterisk in panel 6). In addition to this, a patch of anterior staining can be observed in stage 10 oocytes in the case of the mutant ovarioles, which is not observed in wild type (marked by asterisk in panel 5). Ovarioles imaged in anterior left posterior right orientation, in 10X magnification, Bright field imaging using Apotome microscope. **(B)** Quantification of the area of localisation of *oskar* in the posterior of the stage 9 and 10 oocytes of the *Dmon1* rescued females. Area of localisation was marked using FIJI/Image-J software for wild type and mutant (virgins and non- virgins) stage 9 and 10 oocytes. N=1, n=22 oocytes. The data was statistically analysed using the Graphpad-Prism software, using one way ANOVA and Tukey's multiple correlation test. There was a significant reduction in the area of

localisation of the mRNA in the mutant oocytes, in virgin and non- virgin flies. p value < 0.05 (*), <0.01 (**), <0.001 (***),ns- non significant.

In-situ hybridisation in the ovaries of *Dmon1* rescued flies was conducted to identify whether the pole plasm components are mislocalised in the oocytes. The mRNAs of both *oskar* and *nanos* were targeted. Only the staining against *oskar* was successful and showed a posteriorly localised staining in the control ovaries. Upon examination, using the area of localisation as a proxy for the amount of *oskar* mRNA localised in the posterior, it was observed that the area of localisation of the mRNA was reduced, compared to that of w^{1118}. Moreover, there was a patch of increased staining in the anterior of the stage 10 oocytes of the mutants, as compared to that of w^{1118}. It seemed like the transport and localisation of *oskar* mRNA seemed to get affected in the absence of *Dmon1*. The phenotype was consistently seen in oocytes of virgin and non-virgin flies, ruling out any effect caused by mating.

In-situ for Nanos did not show any posterior localisation in the wild type control and the maternal nulls and so will require further standardisation of the staining procedures. Additionally, the later stage oocytes did not show any uptake of the probe, which would require to standardise the procedure further for such oocytes.

Dmon1 **rescued (Mon1 R) ovaries do not show a significant change in total Rab levels**

To analyse whether Rab levels in the ovaries of *Dmon1* rescue flies, Western blotting was done for the dissected ovaries of these flies. Virgin flies of w^{1118} controls and *Dmon1* rescue were collected and fed with media garnished with yeast for 2 days before dissection. Dissection was done in 1X PBS and the ovaries were subsequently transferred to RIPA buffer containing PIC. The tissue was crushed with a pestle and processed for SDS-PAGE.

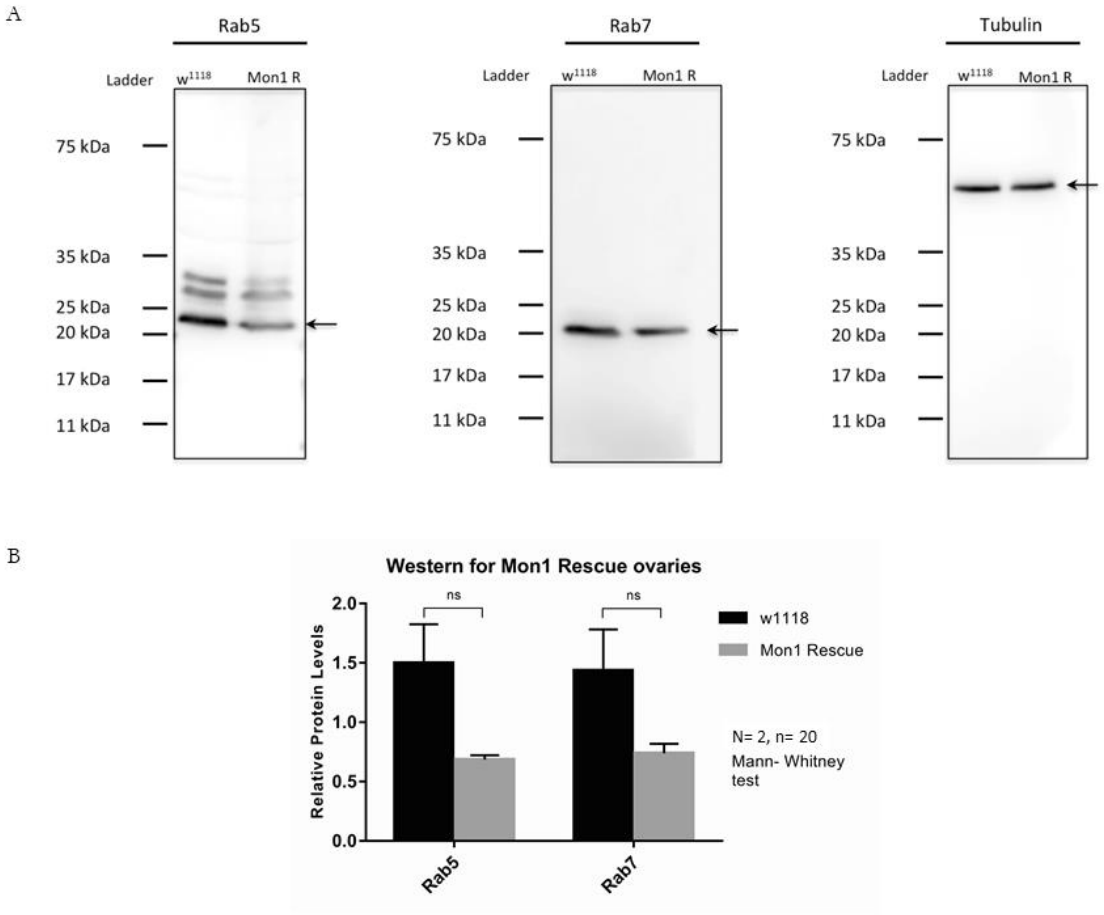

Fig 22: Rab5 and Rab7 levels in *Dmon1* rescued oocytes are comparable to the wild type

Total protein levels of Rab5 and Rab7 in the ovaries of *Dmon1* rescued females were seen to be comparable to that of wild type. **(A)** Western blotting against Rab5 and Rab7 total protein levels in the ovaries of the *Dmon1* rescued females. N=2, n= 20 ovaries. The blots show a slight reduction in the staining intensities of Rab5 and Rab7 in case of the mutant ovaries as compared to the wild type. Tubulin was used as a loading control for the purpose of quantification of the bands of the blots. Mon1R: *Dmon1* rescued flies. **(B)** Quantification of the staining intensities of the western blotting for Rabs. Statistical analysis for total Rab5 and Rab7 protein levels in the ovaries of the *Dmon1* rescued females was done using the Graphpad-Prism software. n= 20 ovaries, N=2. Mann-Whitney test was done on the data. The total protein levels in the mutant ovaries show no significant reduction as compared to the wild type, shown by the p values. p value < 0.05 (*), <0.01 (**), <0.001 (***), ns- non significant.

Although the blots showed a slight reduction of the total protein levels, statistical analysis for the intensities of the bands for the 2 experiments conducted showed no significant reduction of the protein levels for both Rabs in these ovaries compared to the wild type.

Dmon1 maternal null embryos do not show significant differences in Rab levels

To assess whether Rab levels were disrupted in the *Dmon1* maternal null embryos, a Western blot analysis for the same was conducted. 0-2.5 hour embryos were collected

and processed so as to ensure obtaining embryos in the window of the activity of maternal effect genes, before zygotic gene activation.

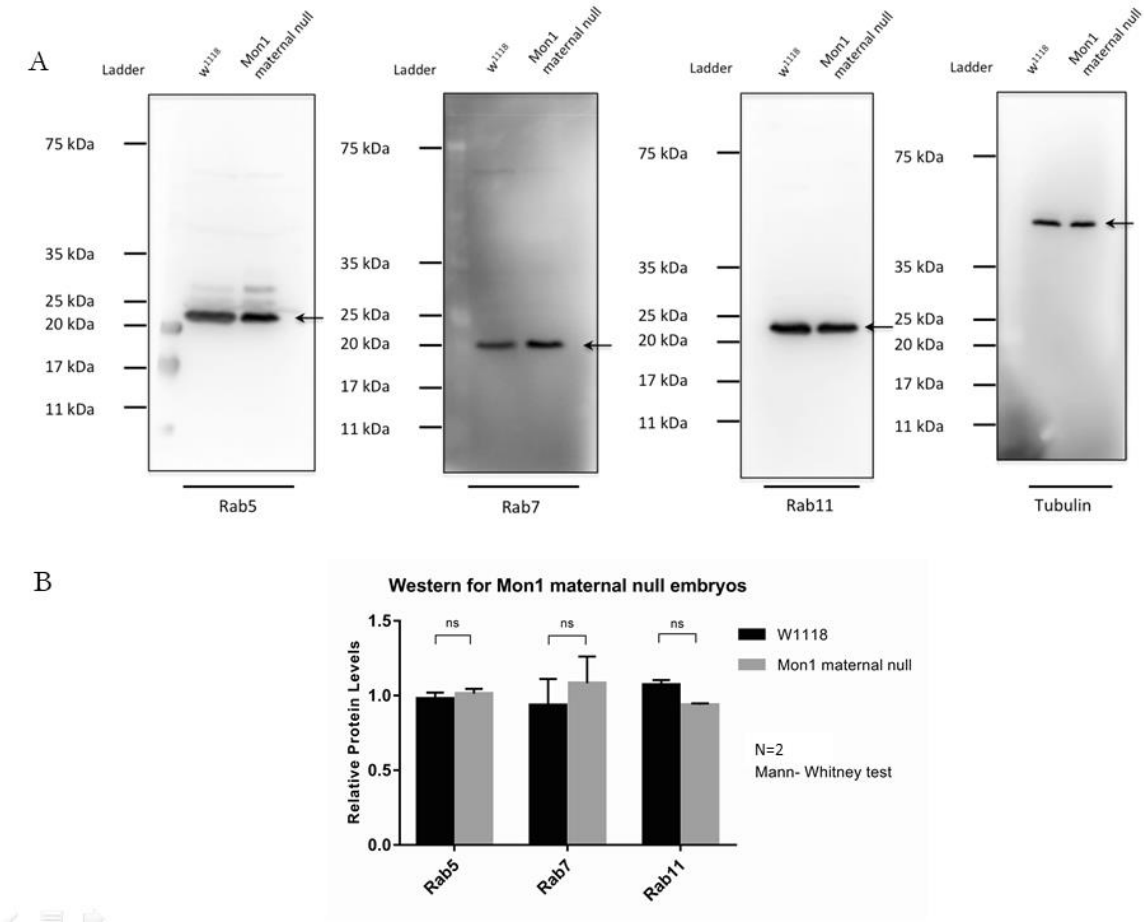

Fig 23: Rab5, Rab7 and Rab11 levels in *Dmon1* maternal nulls embryos are comparable to the wild type. Total protein levels of Rab5, Rab7 and Rab11 in the *Dmon1* maternal null embryos were seen to be comparable to that of wild type embryos. **(A)** Western blotting against Rab5, Rab7 and Rab11 total protein levels in *Dmon1* maternal null embryos. N=2. Loading on SDS- PAGE was done after conducting protein estimation of the lysate, which was prepared after multiple 0-2.5 hour egg collection from a cage for an experiment. The intensities of Rab5, Rab7 and Rab11 bands in case of the mutant embryos seem comparable to the wild type, although Rab7 shows a slight increase in intensity in the above figure, as compared to the wild type. Tubulin loading was done in order to use as a loading control for quantification of the band intensities of the blots. **(B)** Quantification of the staining intensities of the western blotting for Rabs. Statistical analysis for total Rab5, Rab7 and Rab11 protein levels of the *Dmon1* maternal null embryos was done using the Graphpad-Prism software. N=2. Mann-Whitney test was done on the data. The total protein levels in the mutants show no significant reduction as compared to the wild type, shown by the p values. p value < 0.05 (*), <0.01 (**), <0.001 (***), ns- non significant.

Statistical analysis of the western blots shows that the Rabs 5, 7 and 11 levels showed no significant change in these mutant embryos as compared to wild type, despite any slight variation seen on the blots.

Localisation of Rabs are affected in the oocytes of the *Dmon1* rescue flies

To see whether the localisation of Rabs 5, 7 and 11 is affected in the oocytes of the *Dmon1* rescue flies, antibody staining was conducted against Rabs 5, 7, 11. It was observed that there was a loss of staining inside the periphery of the stage 10 oocytes observed in the mutants for Rab7 and 11, but not for Rab5. All the eight stage 10 oocytes imaged showed this phenotype of a loss of staining for the Rabs 7 and 11.

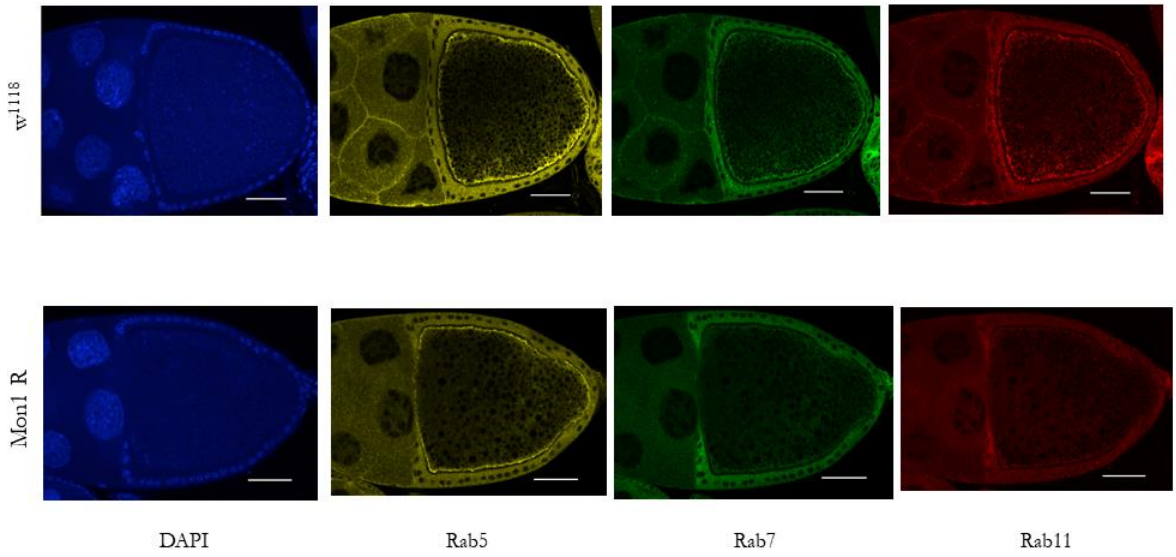

Fig 24: Rab 7 and Rab 11 shows perturbation in their localization in *Dmon1* rescued oocytes. A perturbation in the localization of the Rabs 7 and 11 were observed in stage 9 and 10 oocytes upon immunostaining of the ovaries of the *Dmon1* rescued females. Top panel shows wild type stage 10 oocytes stained against nuclei (DAPI), Rab5, Rab7 and Rab11. Bottom panel shows stage 10 *Dmon1* rescued oocytes stained against nuclei (DAPI), Rab5, Rab7 and Rab11. In case of Rab7 and 11, there is a lack of staining observed at the periphery of the oocyte, below the follicle cells. Rab 5 staining seems to be unperturbed in the mutants as compared to wild type. N=8, N=1. It seemed that the localization of the Rabs to the periphery of the oocyte seemed to be disrupted upon the absence of *Dmon1*. Images were taken in 40X magnification, confocal imaging using Leica SP8 microscope. Oocytes are positioned in an anterior left, posterior right orientation.

CHAPTER IV

Discussion

Dmon1 as a maternal effect gene

Although the roles of *Dmon1* in the context of oogenesis, in the context of the nervous system and the physiology of the organism, have been studied previously, its roles in early embryonic development are less explored. This study provides information on the role played by *Dmon1* as a maternal effect gene. From the preliminary lethality assay conducted, along with the cuticle preparation, it was identified that *Dmon1* has a maternal effect in early embryonic development. The finding that a loss of maternally deposited *Dmon1* gene product causes a high rate of early embryonic lethality indicates that the gene is required in the early stages of development. The cuticles of these maternal null embryos showed a range of patterning defects, identifying the role of *Dmon1* in orchestrating early embryonic development and patterning. The observation that this phenotype cannot be rescued by providing a paternal copy of the gene, in the genome of the organism, confirms the hypothesis that the phenotypes observed are due to a maternal lack of function mutation for the gene, and that it is a maternal effect gene. Lack of maternal transcripts or other gene products in the oocyte or in the embryo shows a range of phenotypes, each of which can be characterized separately.

Dmon1 maternal nulls show a distinct nuclear phenotype

In the antibody staining experiments conducted, a phenotype which stood out in the early *Dmon1* maternal null embryos was a perturbation in the distribution and division of the nuclei of the embryos in the periphery of the embryos. The embryos showed an irregular and patchy distribution of nuclei on their surfaces, as compared to a wild type embryo. In dividing nuclei, the distribution of the genetic material was also observed to be asymmetric. This could point into a hypothesis where endocytic mechanisms perturbed in *Dmon1* mutants lead to an improper regulation of nuclear division and distribution throughout the embryo. This would require further experiments for its characterisation.

Mislocalisation of patterning genes underlie Antero-posterior and Dorso-ventral (A-P and D-V) patterning defects

As the cuticles of these *Dmon1* maternal null embryos show a variety of phenotypes, such as loss of head structures, perturbation of denticle bands, loss of tail and other terminal structures, in varying intensities, it shows that both antero-posterior and dorso-ventral patterning is perturbed. A-P patterning perturbation can be identified from the loss of head and tail structures, and the perturbation in the denticle bands, along with phenotypes resembling *twist* or *dpp* mutants, serve as a readout for D-V patterning defects.

Looking at perturbations in early patterning genes provides us with useful insights on the developmental defects undergoing in the organisms. This approach follows from the fact that the body axis and polarity of the embryos is determined by the localisation of different morphogens in the oocytes. These morphogens are maternally deposited factors, which can be mRNA or protein of the particular gene. The antero-posterior axis of the oocyte is specified by the *bicoid* mRNA localisation at the anterior end of the oocyte and by the localisation of *oskar* mRNA at the posterior end of the oocyte. *Oskar* mRNA acts as a pole-plasm determinant, and pole-plasm components direct the development of germline in the embryo, along with dictating patterning of the abdominal region of the organism (EPHRUSSI AND LEHMANN 1992; GONZALEZ-REYES *et al.* 1997). The dorso-ventral axis of the oocyte is first determined by the localisation of *gurken* mRNA at the dorsal-anterior position, above the nucleus of the oocyte (GONZALEZ-REYES *et al.* 1997). This, later on, activates signalling via the EGFR receptors, to direct the region to acquire a dorsal fate. After a cascade of signalling events, starting from the follicle cells, to accumulate and localise dorsal protein in the ventral side of the oocyte leads to the determination of the ventral side and the D-V axis of the oocyte (GONZALEZ-REYES *et al.* 1997). These initial gradients that set the polarity of the oocyte set up the body axis of the embryos, and orchestrate patterning.

The *in-situ* experiments conducted to look at patterning genes show a pole-plasm phenotype in these embryos. A mislocalisation of the pole-plasm localised mRNAs of *oskar* and *nanos* was observed. Instead of being localised at the posterior terminus of the embryo, these pole-plasm determinants were observed to be displaced from their expected positions, to the side of the embryos. In some of them, such a localisation was missing, too. This phenotype was not observed in the wild type embryos, suggesting a

possible cause for the patterning defects observed in these organisms. Although we had expected to see perturbation in the D-V patterning genes, such as *twist*, *snail*, *rhomboid* and *sog*, owing to the fact that the cuticles showed phenotypes reminiscent of D-V patterning defects, the mRNA localisation of these patterning genes showed no significant perturbation in these early embryos.

As mentioned before, setting up of the embryonic axes is preceded and directed by deposition of maternally derived factors in the oocyte that dictates oocyte polarity. The localisation of these transcripts, such as that of b*icoid*, *oskar*, and *gurken*, depends on mechanisms within the ovaries during oogenesis, which leads to specification, development and polarity determination of the oocyte.

Following oocyte determination, where one of the sixteen cells that arises after a series of incomplete cell divisions in the germline is specified to become the prospective oocyte, the remaining 15 cells endocycle, where replication of the genome occur and nuclei grow in size, without cytoplasmic divisions, and function as nurse cells which deposit gene products or transcripts into the oocyte. This process of deposition of gene products is mediated by transport along microtubules. Different genes such as *bicaudal-D*, *orb* and *egalitarian* determine oocyte specification and microtubule organisation into this specified cell (GONZALEZ-REYES *et al.* 1997). This led us to investigate the role of *Dmon1* in localising oocyte polarity determination factors, such as *oskar*.

The *in-situ* staining on ovaries revealed a lack of posterior localisation of *oskar* in the stage 9 and 10 oocytes of the *Dmon1* rescued females. There was a decrease in the area of localisation observed in most of them, as well as no localisation of the mRNA in a few. There was anteriorly localised mRNA staining observed in these oocytes which could possibly be a result of a lack of proper transport of the *osk* mRNA in these egg chambers. This point to the hypothesis that a lack of a functional *Dmon1* allele in the female can lead to a lack of proper localisation or a perturbation in the localisation of maternally deposited *oskar* or other pole plasm components.

Endocytic mechanisms that regulate pole-plasm anchoring might be perturbed in *Dmon1* mutants

Endocytic mechanisms have been previously implied in development, as well as oocyte polarity. A previous study on the *oskar* mRNA localisation and translation in oocytes links pole-plasm anchoring to the functioning of Rab11 (DOLLAR *et al.* 2002).

The study indicates that there exists an asymmetry in the localisation of membrane trafficking components in the *Drosophila* oocyte. In stage 8-10 oocytes, a localisation of Rab11 in the posterior pole is present. While the study suggests a reinforcement of this localisation by *osk*, it also suggests the requirement of Rab11 in the localisation and translation of the *osk* mRNA. This process is dependent on the organisation of microtubules by Rab11, where it is required for the microtubule plus end organisation towards the posterior of the oocyte. Oskar has been implied in the proper anchoring of the *osk* mRNA at the posterior pole, previously (RONGO *et al.* 1995), and the study suggests that Rab11 is involved in the proper localisation of translation factors required for *osk* mRNA, apart from the anchoring function.

Another study conducted has also previously identified the role of Rab11 in *oskar* localisation (JANKOVICS *et al.* 2001). It suggests that a disruption in the polarity of the microtubules in the oocyte that transport *osk* mRNA is brought about in Rab11 mutants, which leads to *osk* mRNA localising ectopically in the oocytes. The study shows the role endocytic membranes play in cytoskeletal organisation and mRNA localisation in oocytes.

In the study conducted by HARISH *et al.*, 2019, it was observed that a loss of *Dmon1* led to an increase in the dendritic arborisation that involved Rab11. It was hypothesized that this could be due to altered endocytic flux towards the Rab11 dependent recycling endosomal pathway. Linking the study on the influence of Rab11 in *osk* localisation, and the regulation of RE vesicular flux by *Dmon1*, one can look at the involvement of *Dmon1* in the localisation and anchoring of pole-plasm components like *osk*.

The subsequent steps to investigate this regulatory mechanism revealed that total Rab5, Rab7 and Rab11 protein levels were unperturbed in the *Dmon1* maternal null embryos. A similar result of unperturbed protein levels of Rab5 and Rab7 in the ovaries of the *Dmon1* rescued females was also observed. Immunostaining of ovaries of the *Dmon1* rescued flies against Rab 5, 7 and 11 revealed a loss or reduction of Rab 7 and 11 compartments from the periphery of the stage 10 oocytes. These results indicate that *Dmon1* is possibly involved in the localisation of Rab compartments in the oocyte, and not in their translational regulation.

Taking insights from previous work on *Dmon1* and *osk* localisation, further experiments can be conducted in order to link how *Dmon1* might regulate Rab 11 mediated microtubule organisation in oocytes, which is the primary mechanism put forth

by previous studies to explain the regulation of *oskar* localisation by Rab 11. This can lead us to find a possible mechanism involving the regulation of pole-plasm component localisation and anchoring that is regulated by *Dmon1* mediated endocytic flux. Investigating further into this will give us insights on how endocytic mechanisms involving *Dmon1* regulate morphogen gradients and early embryonic patterning.

CHAPTER V

Materials and Methods

Fly stocks

Sl. no.	Genotype	Short name	BDSC name/ Source
1	*+/+; ⊿181, Tdc2-GAL4/CyO-AGFP; +/+*	Neuronal driver line balanced with Mon1 null.	DEIVASIGAMANI *et al.* 2015
2	*+/+; +/+; ⊿181/CyO-AGFP; UAS-Mon1-HA/TM3 Ser*	Mon1 overexpression line balanced with Mon1 null.	DEIVASIGAMANI *et al.* 2015
3	*+/+; UAS CD4-GFP/ CyO; +/+*	Membrane GFP line	
4	*+/+*	w^{1118}	25705
5	*+/+; MatGAL4/MatGAL4; MatGAL4/MatGAL4*	Maternally expressed GAL4; alpha-tubulin promoter	RATNAPARKHI *et al.* 2006
6	*+/+; If/CyO; MKRS/Ser*	Balancer line	BDSC
7	*otu- GAL4::VP16/+; nos-GAL4/+; nos-GAL4::VP16/+*	Maternal Triple Driver line	31777

Table 1: Fly lines used in the experiments conducted

The fly lines were maintained on 6% standard agar cornmeal-yeast media, at 25°C. Crosses were set up in the above-mentioned conditions in bottles or vials of the media.

Genetic crosses for Obtaining *Dmon1* rescued flies

Crosses were set up between the Δ181, 9313/Cyo females and Δ181/CyO; UAS-Mon1-HA/TM3 Ser males to obtain progenies that are Δ181 homozygous, with Dmon1 overexpression in the OPN neurons.

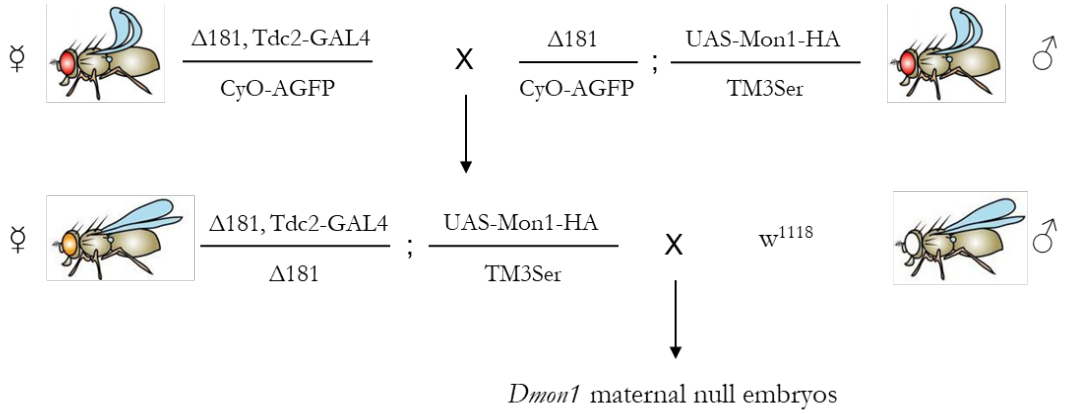

Fig 25: Schematic representation of the crosses set up to obtain *Dmon1* rescued females, used to obtain *Dmon1* maternal null embryos. Δ181, Tdc2-GAL4/CyO-AGFP virgin females were collected and kept at 18 ^{0}C before setting up a cross with Δ181/CyO-AGFP; UAS-Mon1-HA/TM3 Ser males. The crosses were set in 6 % fly media bottles, and kept at 25 ^{0}C. The straight winged, orange eyed, virgin female progenies, which are of the *Dmon1* rescued phenotype, Δ181, Tdc2-GAL4/ Δ181; UAS-Mon1-HA/ + genotype, were collected to obtain *Dmon1* maternal null embryos, after setting up a cross with w^{1118} males.

Characterisation of the Δ181 lines

Genomic PCR was done in order to confirm that the Δ181 deletion was present in the parent fly lines of the *Dmon1* rescue flies, of the following genotypes: Δ181, Tdc2-GAL4/CyO-GFP and Δ181/CyO-GFP; UAS-Mon1-HA/TM3 Ser. Primers used for this purpose were obtained from Deivasigamani et.al. publication (DEIVASIGAMANI *et al.* 2015). The forward primer used: 11926_ 2F, 5'-AGCACGACAGTCTGTGGCAGG-3'; Reverse primer used: Ex31660_2R, 5'-AACCGACAGATACACGAGCATT-3'

3rd instar larvae of the Δ181 homozygous genotype were selected from the vials by screening for an absence of Actin-GFP expression, using fluorescence channels of the stereoscope. Straight winged, light orange-eyed flies were also selected, which showed the phenotype of Δ181 homozygotes. w^{1118} flies were used as a positive control for obtaining the full gene product. The flies and larvae collected were crushed and processed for DNA extraction referring to the single fly DNA prep protocol by Georg Dietzl in Barry Dickson's Lab, IMP Vienna 12/2002. PCR was done following standard protocol: PCR mix of 20 µL, containing 1X Taq buffer, 1.8mM $MgCl_2$, 150 µM dNTPs, 0.5 mM of both forward and reverse primers, 1 µL of template DNA, 0.2 µL of Taq DNA polymerase, and the final volume made up using 12 µL of nuclease free water. PCR mix reagents obtained from Thermofisher. Initial denaturation step was done for 5

mins, and for 30 secs in the subsequent cycles, at a temperature of 95 ^{0}C. Annealing step of the primers was done below the primer melting temperature, at 56 ^{0}C for 30 secs, and extension was done at 72 ^{0}C for a period of 2 mins 10 secs. The final extension cycle was for 10 mins, and the machine was set to hold at 4 ^{0}C at the end of 30 cycles.

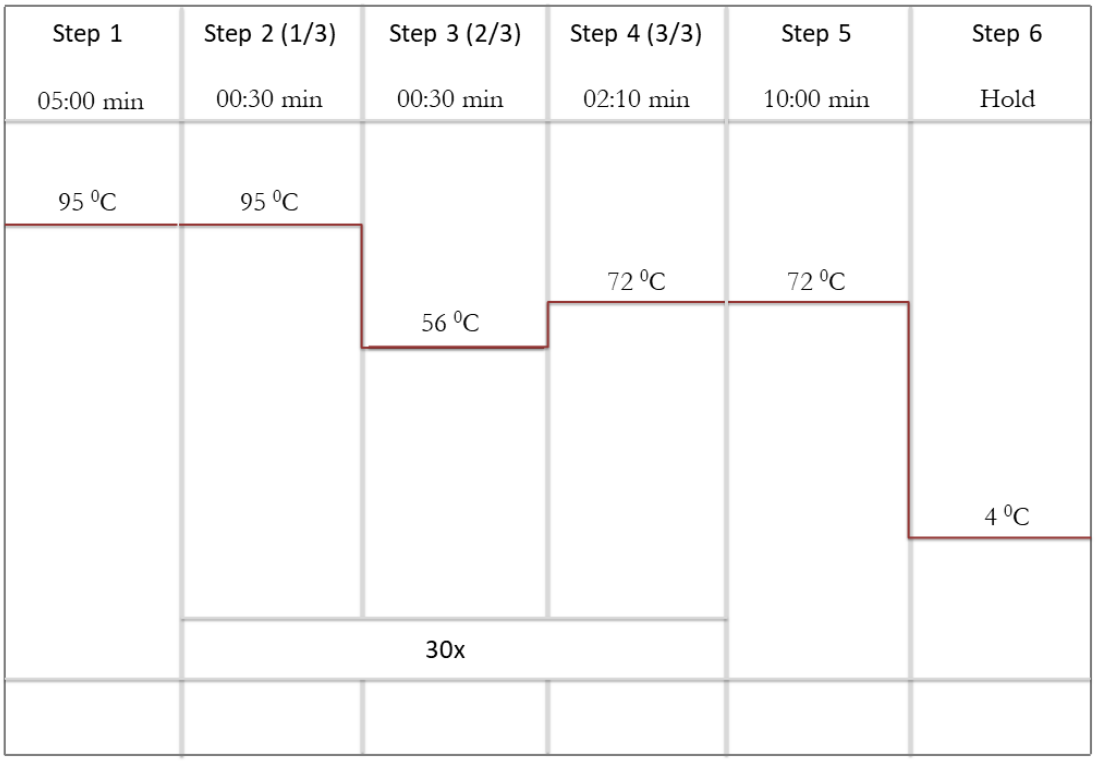

Fig 26: Schematic of PCR done for genotyping of *Dmon1* fly lines. PCR was done for identifying *Dmon1* Δ181 deletion in the fly lines using primers specific to *Dmon1*. 30 cycles of denaturing, annealing and extension were performed. Denaturing temperature: 95 ^{0}C, initial one for 5 mins, and the subsequent ones for 30s; annealing temperature: 56 ^{0}C, for 30 secs; extension temperature: 72 ^{0}C, for 2 mins 10 secs, and an extended period of 10mins at the end of 30 cycles. Hold temperature: 4 ^{0}C.

The amplified DNA fragments were visualised under UV light, imaged using Gel-Doc, after agarose gel electrophoresis in a 2% gel, to separate and visualise an 850 bp deletion product specific for the Δ181 deletion.

Characterisation of the Δ181, Tdc2-GAL4/CyO-AGFP line to confirm a functional Tdc2-GAL4 construct

To identify whether the Tdc2-GAL4 construct was functional, a cross was set up between the Δ181, Tdc2-GAL4/ CyO-AGFP females and UAS CD4-GFP/ CyO male flies. Progeny larvae were screened for the lack of GFP, of which half of them will be of

the desired genotype. Alternatively, the flies with straight wings can also be screened and stained against GFP. The brain and ventral nerve cord of the 3rd instar progeny larvae were immunostained against GFP to check for GFP expression in the OPN neurons.

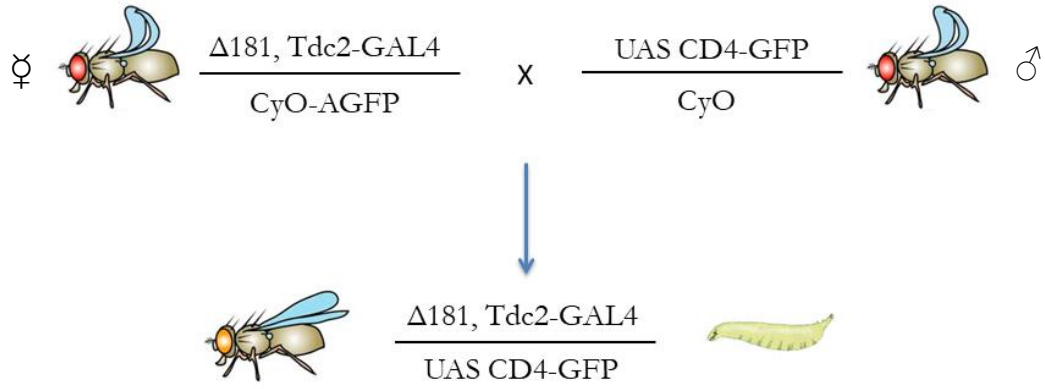

Fig 27: Schematic representation of the cross conducted to test the GAL4 fly line. Δ181, Tdc2-GAL4/CyO-AGFP virgin females were collected and kept at 18 ^{0}C before setting up a cross with UAS CD4-GFP/ CyO males. The progeny larvae were screen for the absence of GFP, and the brain and the ventral nerve cord were immunostained against GFP to look for the GFP expressing OPN neurons.

Larval Brain and ventral nerve cord staining

Larvae were dissected in 1X PBS and fixed using 4% PFA in 1X PBST (0.3 % Triton X in 1X PBS). Immunostaining was done after permeabilization and rehydration by giving multiple washes using 1X PBST. Blocking of the tissue was done in 2% BSA in 1x PBST, for an hour at room temperature. This was followed by incubation with primary antibody against GFP (raised in chicken), at a dilution of 1:500 in the blocking solution at 4 ^{0}C overnight. After giving PBST washes, secondary antibody (1:1000 dilution, anti-chicken secondary) incubation was done at 4°C overnight. This was followed by PBST washes, with DAPI staining at a dilution of 1:1000 in an intermediate wash step. The samples were mounted on glass slides using anti-fade mounting media (Composition: 0.5% N- propyl gallate, 20mM Tris (pH 8) in 90% glycerol) and imaged using an epifluorescence microscope (Zeiss Apotome) under 10X magnification.

Embryo collections

Medium-sized cages were set up for around 150 flies per cage. Virgin flies of the desired genotype were collected from the crosses and mated with males of the desired genotype. Crosses were set up 2 days prior to setting up the cage on 6% fly media. The flies were transferred into the cage after 2 days and acclimated for a day. The flies were

fed with yeast paste in the cage on plates containing 3% sugar, 2.5% agar medium. The cages and crosses were kept at 25°C.
Collections for varying durations depending upon the experiments were made after which the embryos were processed for cuticle preparations, immunostaining, *in-situ* hybridisations and western blots. Fixed and processed embryos were stored in methanol or ethanol at minus 10°C for long term storage, depending upon the processing undergone.

Lethality assays

Embryo collections from previously set up cages are done for a fixed duration, so as to collect sufficient numbers (~300-500) of embryos from a single cage. 200 of these embryos are transferred to another sugar-agar plate and arranged in a grid for the convenience of counting. The embryos are left for 24 hours at 25°C for hatching and the number of hatched/unhatched embryos is counted in both the control and the genotype of interest. The embryos are checked after the next 48 hours to look for slow-developing embryos that might have hatched later. The experiment is repeated thrice and the numbers are plotted and statistical tests are done to assess significance of lethality rates.

Cuticle preparations

Embryo collections were done overnight for control and mutant flies, and the collected embryos were aged on the sugar-agar plate for 22-24 hours, allowing them to deposit cuticles. The embryos were collected in a sieve, washed with tap water and dechorionated using 100% bleach for 90 secs. The embryos were later transferred to scintillations vials, and washed with 100 % methanol. Later, equal volumes of methanol and heptane were added to it. They were subsequently devitellinised by methanol-heptane devitellinisation, by vigorous shaking of the vial. The devitellinised embryos fall into the denser methanol layer, and the heptane later with the remaining non devitellinised embryos was removed. Tissue lysis for these embryos was performed overnight by 80% lactic acid digestion at 55°C on glass slides with cover slips, kept on slide warmers. The cuticles were observed and imaged using stereo microscope dark-field imaging on the Zeiss Apotome microscope at the IISER- Pune microscopy facility.

Immunostaining of *Drosophila* Embryos

Immunostaining was done on early 0-3 hour *Drosophila* embryos. The collected embryos were washed using tap water and dechorionated using 100% bleach. They are subsequently fixed using 4% PFA (Paraformaldehyde) in 1X PBS (Phosphate Buffered saline, prepared following CSH protocols: 137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 1.8mN KH_2PO_4), for 20 mins on shaking. The PFA layer on the bottom is removed and the embryos are washed in 100 % methanol. Then equal volumes of chilled methanol and heptane are added to it, and methanol-heptane devitellinisation is performed by vigorous shaking of the vial. Following this step, heptane layer is removed, and the embryos are washed with methanol. The embryos can be stored in methanol for long term storage at minus 10°C.

The processed embryos were permeabilized and re-hydrated by giving washes in 0.3% Triton X in 1X PBS (1X PBST) buffer. They were blocked for antibody staining using 2% Bovine serum albumin (BSA) in 1X PBST for an hour at room temperature. The sample is incubated with the primary antibody overnight at 4°C at the required dilution in blocking solution. After giving 1X PBST washes after primary incubation, the sample is incubated with secondary antibody at 1:1000 dilution at 4°C, for 4-6 hours or overnight. Samples undergo PBST washes after secondary antibody incubation with DAPI staining for the nuclei at an intermediate wash step, at a dilution of 1:1000. Mounting is done using anti-fade mounting media containing 0.5% N- propyl gallate, 20mM Tris (pH 8) in 90% glycerol. Alternatively, VECTASHIELD mounting media can also be used. Imaging is done on the confocal microscope Leica SP8 under 20X Magnification through appropriate fluorescent channels.

Immunostaining of Ovaries

Ovaries of the desired genotype are dissected in 1X PBS and ovarioles were separated while keeping the bunch connected. They are transferred immediately to 0.3% Triton X in 1X PBS buffer on ice, to prevent tissue damage. They are fixed using 4% PFA in 1X PBST for 20 mins, and can be stored overnight at 4 ^{0}C. The tissue is later re-hydrated and permeabilised for staining by giving 1X PBST (0.3% triton in 1X PBS) washes. This is followed by blocking in 2% BSA in 1X PBST solution. Primary antibody incubation is done overnight at the desired dilution in blocking solution at 4°C. The tissue is later washed in 1X PBST and secondary antibody incubation at a dilution of 1:1000 is done, for 4-6 hours or overnight at 4°C. 1X PBST washes are given after

secondary incubation, with DAPI staining at a dilution of 1:1000 in an intermediate washing step. The ovaries are mounted using the anti-fade mounting media or VECTASHIELD on glass slides and imaged using Leica SP8 confocal microscope at 40X magnification.

In-situ hybridization of embryos

In-situ hybridisation protocol from the Levine lab was followed. 0-3 hour old embryos were collected in egg collection cages. Embryos fixed using the antibody staining procedure are also compatible for *in-situ* hybridisation. Embryos collected are collected in a sieve and washed in 0.1 % Triton X, 0.75 % NaCl solution. They are dechorionated for 90 seconds using 100 % bleach, and fixed using the fixing solution (1XPBS, 0.5M EGTA, 5% PFA in dH2O). They are subsequently devitellinised using methanol-heptane devitellinisation. The embryos are washed in Methanol, and later in Ethanol. They can be stored at -10 ^{0}C in ethanol. The embryos are washed in equal volumes of ethanol and xylene and are soaked in Xylene for a period of 1.5 hours. They are rinsed in 1:1 solution of xylene and ethanol, and later rinsed sequentially in ethanol, methanol and 1:1 solution of methanol and PBT- Formaldehyde (5% formaldehyde in 1X PBT, PBT: 0.1 % Tween 20 in 1X PBS). This is followed by a second fixation step, using PBT- Formaldehyde, for 25 mins, followed by PBT washes. Subsequently, the embryos are subject to a Proteinase-K treatment (4 µg/ml of non-predigested proteinase-K in PBT), followed by dilution of the proteinase K using 2 mg/ml Glycine in PBT, to stop the reaction. Emrbyos are rinsed in PBT, followed by another round of fixation in PBT- Formaldehyde for 25 mins. Washes are given in 1:1 solutions of PBT and Hybridisation solution (50 % formamide, 5× SSC, 0.1 % Tween 20, 100 µg/ml denatured DNA (from herring sperm), 50 µg/ml heparin). This is followed by incubation in the hybridisation solution for an hour at 55 ℃. Probes are prepared in the hubridisation solution, , with 0.5 µL of the dig U RNA probe in 50 µL of the solution. The probe is heathed at 80 ^{0}C for 3 to 5 mins, and immediately kept on ice. The embryos are incubated for at least 18 hours at 55℃ in the hybridisation solution containing the DIG-labelled riboprobe. The embryos are subject to washing steps, first using the hybridisation solution at 55 ^{0}C for an hour, and subsequently giving four 30 min washes for 2 hours. This is followed by a wash in 1:1 solution of PBT/ hybridisation solution, and only PBT washes at room temperature. This is followed by overnight incubation of the samples with the anti-DIG antibody, at 4 ℃, at a dilution of 1:2000 in PBT. The

probes are visualised using the alkaline phosphatase substrate tablets (NBT-BCIP) dissolved in the staining buffer (100mM NaCl, 50mM $MgCl_2$, 100mM Tris, pH 9.5 and 0.1% Tween), 1 tablet for 10 ml staining solution, in staining wells. The colour reaction can be stopped by adding chilled PBS to the reaction mixture. Samples can be mounted on glass slides using 80% glycerol mounting media. Imaging of the samples was done in the bright field setting of the Zeiss Apotome microscope.

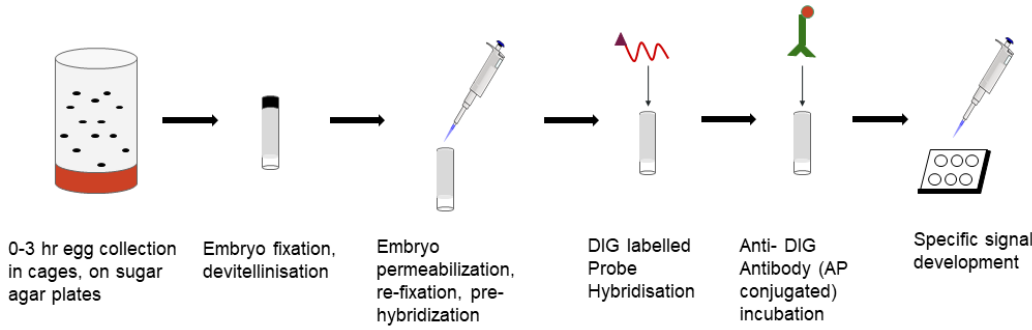

Fig 28: Schematic of *In-situ* hybridisation of embryos. 0-3 hour embryo collections are done in egg collection cages. They are fixed, devitellinised, and later is subject to multiple rounds of permeabilisation and refixation, and undergoes prehybridisation. The emrbyos are incubated with DIG labelled RNA probes, and later incubated with anti-DIG antibody, conjugated with alkaline phosphatase (AP). Signal development is done by adding the staining reagent containing NBT-BCIP tablets.

In-situ hybridization of ovaries

A modified protocol of *in-situ* hybridisation, referring to SHPIZ *et al.* 2014 and the Levine Lab embryo *in-situ* protocols, was followed for ovaries of the desired genotype. Flies were fed with media garnished with yeast 2 days prior to dissection. Ovaries were dissected in 1X PBT (1X PBS with 0.1% Tween-20) and ovarioles were separated. They were kept on 1X PBT on ice before processing for staining (maximum 2 hours). The tissue samples were fixed in 3.7% PFA in 1X PBT and washed in PBT post-fixation. They can be stored overnight at 4 ^{0}C in PBS. The ovaries were transferred to 0.6% Triton in 1X PBS, for a 10 min incubation, followed by incubation in hybridisation solution (50 % formamide, 5× SSC, 0.1 % Tween 20, 100 µg/ml denatured DNA (from herring sperm), 50 µg/ml heparin) at 42 ℃ for 1 hour. This step is followed by incubation of the tissue with denatured DIG-labelled riboprobe at 42℃ overnight. Prior to this, as mentioned for embryo *in-situ*, probes are heated in the hybridisation solution at 80 ^{0}C for 3-5 mins and kept on ice immediately. Washes are given are given the next day using the hybridisation solution at 42 ^{0}C, and later, washes with decreasing

concentrations of Formamide solutions in PBT (30 %, 20 %, 10 % Formamide), followed by PBT washes. Anti-DIG antibody incubation is done overnight at 4 ℃, at a dilution of 1:2000 in PBT. The probe is visualised using the alkaline phosphatase substrate tablets (NBT-BCIP) dissolved in the staining buffer (100mM NaCl, 50mM MgCl2, 100mM Tris, pH 9.5 and 0.1% Tween) in staining wells, 1 tablet in 10 ml of staining solution. The colour reaction can be stopped by adding chilled PBS to the reaction mixture. Samples can be mounted in 80% glycerol mounting media. Imaging of the samples was done in the bright field setting of the Zeiss Apotome microscope.

Western Blot for embryos

0-2.5 hour embryos were collected from the egg collection cages. These were washed and dechorionated using 100 % bleach, and subsequently snap-frozen and kept at -80℃, after removing all water content by blotting with a tissue from the vial, for crushing and protein extraction in the future. Once sufficient amounts of embryos were collected, the embryos were crushed in RIPA (Radio-Immunoprecipitation assay; composition: 150mM NaCl, 1% Nonidet P-40, 0.5% Sodium deoxycholate (DOC), 0.1% SDS, 25mM Tris, pH 7.4, ddH_2O) buffer containing PIC (Protease inhibitor cocktail) using a pestle. The lysate was kept on ice for 15 mins, followed by centrifugation at 4℃ at 22,000G (maximum speed) for 30 mins. The supernatant was aspirated and the protein concentration of the lysate was estimated using the BCA protein assay, mentioned below. The samples were diluted according to the colorimetric readings obtained and were boiled with SDS (Sodium Dodecyl sulphate)-Bromophenol blue sample buffer (composition: 50mM Tris, pH 6.8, 6% glycerol, 2% SDS, 0.004 Bromophenol blue, 1% beta-mercaptoethanol) at 95℃ for 10 mins, and stored at 4℃ prior to running an SDS-PAGE.

SDS-PAGE was done on an acrylamide gel of desired concentration (12% acrylamide gel for Rab protein western blotting). Stacking gel voltage- 90V, Resolving gel voltage- 120V. After separating the proteins on the SDS-PAGE, the protein bands were transferred into a PVDF membrane at 90V for 1 hour, at 4 ^{0}C in transfer buffer (CSH Protocol). The membrane was later blocked using a 5% milk solution in 0.1 % Tween 20, 1X TBS (1X TBST) solution. Primary antibody was incubated overnight in the blocking solution at 4℃. After giving TBST washes, HRP (Horseradish peroxidase) conjugated secondary antibody incubation was done for an hour at room temperature. The blots were developed using the chemiluminescence developing reagent containing

Luminol and Sodium peroxide, mixed in 1:1 ratio, added prior to imaging, and imaged using the LAS-4000 gel doc imaging system.

BCA protein estimation assay for embryos

Bicinchoninic Acid protein estimation assay relies on the ability of the protein molecules to reduce Cu^{2+} ions to Cu^{+} ions in an alkaline solution. Decreasing concentrations of Bovine serum albumin solutions are made in RIPA buffer (2mg/ml, 1.5 mg/ml, 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml) and 25 microliter of each is added to wells of a 96 well plate, with one well containing only the RIPA buffer as blank/ control. After the lysate for western blotting is prepared, dilutions of 1/20 and 1/40 of both controls and mutants are made and added to the wells. The plate is kept for incubation at 37^{0}C for 30 mins, and colorimetric readings are taken using a Varioskan Lux reader. A standard curve is plotted for absorption versus the concentration of the BSA control. The concentration of the lysates is calculated using the equation obtained from this curve, set to y-intercept = 0. The concentration of the lysates is adjusted to be equal by adding an appropriate amount of RIPA buffer to dilute the required sample. The diluted samples are processed and western blotting is done following the standard protocol.

Western Blot for ovaries

For ovaries of comparable sizes between 2 genotypes, a fixed number of ovaries (15) were dissected in 1X PBS, and transferred to 1X PBS on ice to avoid tissue damage. Lysate preparation was done in a similar way as that for the embryos, using the RIPA buffer containing PIC. Standard SDS-PAGE followed by transfer to membranes for antibody staining was done. The blots were developed using chemiluminescence resulting from Luminol-HRP reaction as mentioned previously in the embryo western blotting protocol, and imaged using the LAS-4000 gel doc imaging system.

Statistical analyses of Data

An appropriate statistical test was done for data analysis for the experiments done using the Graphpad- Prism software.

CHAPTER VI

Bibliography

Bastock, R., and D. St Johnston, 2008 Drosophila oogenesis. Curr Biol 18: R1082-1087.

Cabrera, M., S. Engelbrecht-Vandre and C. Ungermann, 2014 Function of the Mon1-Ccz1 complex on endosomes. Small GTPases 5**:** 1-3.

Cabrera M, Ungermann C. 2010 Guiding endosomal maturation. Cell 141:404–406.

Deivasigamani, S., A. Basargekar, K. Shweta, P. Sonavane, G. S. Ratnaparkhi *et al.*, 2015 A Presynaptic Regulatory System Acts Transsynaptically via Mon1 to Regulate Glutamate Receptor Levels in Drosophila. Genetics 201**:** 651-664.

Dhiman, N., K. Shweta, S. Tendulkar, G. Deshpande, G. S. Ratnaparkhi *et al.*, 2019 Drosophila Mon1 constitutes a novel node in the brain-gonad axis that is essential for female germline maturation. Development 146.

Doherty, G. J., and H. T. McMahon, 2009 Mechanisms of endocytosis. Annu Rev Biochem 78**:** 857-902.

Entchev, E. V., A. Schwabedissen and M. Gonzalez-Gaitan, 2000 Gradient formation of the TGF-beta homolog Dpp. Cell 103**:** 981-991.

Fischer, J. A., S. H. Eun and B. T. Doolan, 2006 Endocytosis, endosome trafficking, and the regulation of Drosophila development. Annu Rev Cell Dev Biol 22**:** 181-206.

Harish, R. K., S. Tendulkar, S. Deivasigamani, A. Ratnaparkhi and G. S. Ratnaparkhi, 2019 Monensin Sensitive 1 Regulates Dendritic Arborization in Drosophila by Modulating Endocytic Flux. Front Cell Dev Biol 7**:** 145.

Hegedus, K., S. Takats, A. Boda, A. Jipa, P. Nagy *et al.*, 2016 The Ccz1-Mon1-Rab7 module and Rab5 control distinct steps of autophagy. Mol Biol Cell 27**:** 3132-3142.

Jankovics, F., Sinka, R., Erdelyi, M. 2001 An Interaction type of Genetic Screen Reveals a Role of the Rab11 Gene in *oskar* mRNA Localization in the Developing *Drosophila melanogaster* Oocyte. Genetics 158: 1177-1188

Kiontke, S., L. Langemeyer, A. Kuhlee, S. Schuback, S. Raunser *et al.*, 2017 Architecture and mechanism of the late endosomal Rab7-like Ypt7 guanine nucleotide exchange factor complex Mon1-Ccz1. Nat Commun 8**:** 14034.

LaFever L, Drummond-Barbosa D. 2005 Direct control of germline stem cell division and cyst growth by neural insulin in Drosophila. Science. 309:1071–1073.

Le Roy C, Wrana JL. 2005 Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. Nat Rev Mol Cell Biol. 6:112–126.

Mizushima, N., B. Levine, A. M. Cuervo and D. J. Klionsky, 2008 Autophagy fights disease through cellular self-digestion. Nature 451**:** 1069-1075.

Nordmann M, Cabrera M, Perz A, *et al.*, 2010 The Mon1-Ccz1 complex is the GEF of the late endosomal Rab7 homolog Ypt7. Curr Biol 20:1654–1659.

Poteryaev, D., S. Datta, K. Ackema, M. Zerial and A. Spang, 2010 Identification of the switch in early-to-late endosome transition. Cell 141**:** 497-508.

Ratnaparkhi GS, Jia S, Courey AJ. 2006 Uncoupling dorsal-mediated activation from dorsal-mediated repression in the Drosophila embryo. Development. 133:4409–4414.

Shpiz, S., Lavrov, S., Kalmykova, A. 2014 Combined RNA/DNA Fluorescence In Situ Hybridization on whole- Mount *Drosophila* Ovaries. PIWI_ Interactcing RNAs: Methods and Protocols, Methods in Molecular Biology, vol. 1093

Stainier, Y. R. D. 2002 A glimpse into the molecular entrails of endoderm formation. Genes and Development 16: 893-907

Tadros, W., and H. D. Lipshitz, 2009 The maternal-to-zygotic transition: a play in two acts. Development 136**:** 3033-3042.

Wandinger-Ness, A., and M. Zerial, 2014 Rab proteins and the compartmentalization of the endosomal system. Cold Spring Harb Perspect Biol 6**:** a022616.

Wang, C. W., P. E. Stromhaug, E. J. Kauffman, L. S. Weisman and D. J. Klionsky, 2003 Yeast homotypic vacuole fusion requires the Ccz1-Mon1 complex during the tethering/docking stage. J Cell Biol 163**:** 973-985.

Wang, C. W., P. E. Stromhaug, J. Shima and D. J. Klionsky, 2002 The Ccz1-Mon1 protein complex is required for the late step of multiple vacuole delivery pathways. J Biol Chem 277**:** 47917-47927.

Wolf, J. B., and M. J. Wade, 2009 What are maternal effects (and what are they not)? Philos Trans R Soc Lond B Biol Sci 364**:** 1107-1115.

Yousefian, J., T. Troost, F. Grawe, T. Sasamura, M. Fortini *et al.*, 2013 Dmon1 controls recruitment of Rab7 to maturing endosomes in Drosophila. J Cell Sci 126**:** 1583-1594.

Zerial M, McBride H. 2001 Rab proteins as membrane organizers Nat Rev Mol Cell Biol. 2:107–117.