RBFOX1 REGULATES MRNA TRANSLATION TO PROMOTE GERM CELL DIFFERENTIATION

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To my mom Maria Rosario-Hernandez, my dad Fernando Carreira-Rosario, my brother Rey, my sister Alayla and my grandparents.

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by

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DISSERTATION / THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

April 2016

ACKNOWLEDGEMENTS

I would like to first acknowledge my mentor Michael Buszczak for his support, encouragement and guidance throughout my graduate work. All I have accomplished would have been impossible without his help. I would like to thank current and past Buszczak lab members whose time in the lab has overlapped with mine, for their invaluable advice and companionship. Special thanks to Ömür Tastan for having trained me when I joined the lab, to Shane Scoggin for teaching most of what I know about molecular biology, Varsha Bhargava for the generation of Rbfox1 KO allele. To Nevine Shalaby, Dhananjay Chaturvedi, Victor Palacios, Varsha Bhargava and Mayu Inaba for their constant input into my project and also for their friendship, encouragement and cooperation during graduate school. I am grateful to my Thesis Committee: Drs. Nicholas Conrad, Eric Olson, Adrian Rothenfluh and Andrew Zinn for all their vital suggestions to my studies. I would like to thank Nancy Street for her precious advice at critical times in my graduate career. I must thank the GEMS community for suggestions and reagents. I also want to thank the *Drosophila* community for their generosity in sharing reagents. To Dr. Mani Ramaswami and Dr. Jens Hillebrand for their collaboration in studying Rbfox1 function in the nervous system. To Rahul Kollipara for his key bioinformatics analysis. To the Department of Molecular Biology, especially the NA8 laboratories and their members, for providing an excellent environment to do any imaginable experiment.

Thanks to all my science teachers from elementary school to college that inspired me to become a scientist. I also want to thank Berfin Azizoğlu. Having her on my side has been key to all my accomplishments over the last year. My family back at home and my friends in Dallas have provided me with an essential support system during graduate school.

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Publication No. _____

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The University of Texas Southwestern Medical Center at Dallas, 2016

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Germ cells are the only cells that can give rise to an embryo. During differentiation, female germ cells that will give rise to oocytes form a syncytium called a germline cyst. The mechanisms that regulate germline cyst development remain poorly understood. In *Drosophila,* germline stem cells (GSCs) undergo an asymmetric division, giving rise to a stem cell and a cystoblast that then divides four times to produce a 16-cell germline cyst. This 16-cell cyst will then continue differentiation until it forms a mature oocyte. *Drosophila RNA-binding Fox-1 (Rbfox1)*, also known as *Ataxin-2 Binding Protein 1 (A2BP1)*, mutant females exhibit a germ cell differentiation defect that results in germline cystic tumors. The

Rbfox genes encode several isoforms, many of which contain a highly conserved RNA recognition motif (RRM). Disruption of human RBFOX homologs have been linked with a number of different neurological disorders and cancers. Some of these isoforms localize to the nucleus while others localize to the cytoplasm. Nuclear forms have well-established roles in regulating alternative splicing. However the function of Rbfox in the cytoplasm remains unclear. Here, we demonstrate that cytoplasmic *Drosophila* Rbfox1 regulates germline cyst development. We further show that Rbfox1 represses the translation of mRNAs that contain (U)GCAUG elements within their 3' UTRs. We have identified *pumilio* (*pum*) as a critical Rbfox1 target gene. *Pum* is an RNA-binding protein essential for germline maintenance across species. During germline cyst differentiation, Rbfox1 silences *pum* mRNA translation thereby promoting germ cell development. Mis-expression of *pum* results in the formation of germline cystic tumors that resemble *Rbfox1* mutant phenotype. In addition, these cysts breakdown and dedifferentiate back to single, mitotically active cells. Together these results reveal that cytoplasmic Rbfox family members regulate the translation of specific target mRNAs to promote differentiation. In the *Drosophila* ovary, this activity provides a genetic barrier that prevents germ cells from reverting back to an earlier developmental state. These findings have thus advanced our understanding of germline development and the molecular function of Rbfox proteins, with implications in cellular differentiation and Rbfox-related disorders.

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LIST OF DEFINITIONS

A2bp1- Ataxin 2 binding protein 1

Bam- bag-of-marbles

BMP- Bone Morphogenetic Protein

Brat- Brain tumor

Bgcn- benign gonial cell neoplasm

CB- Cystoblast

dFMRP- *Drosophila* Fragile X mental retardation protein

Escort Cell- (EC)

GSC- Germline Stem Cell

Hts- hu li tai shou

IDR- Intrinsically Disorder Region

IF-Immunofluorescence

IRES- Internal Ribosomal Entry Site

mRNP- Messenger Ribonucleoprotein

NeuN- Neuronal Nuclei

Otu- Ovarian tumor

PAT- Poly(A) Tail-Length

PGC- Primordial Germ Cell

Pum- Pumilio

Puf- Pumilio and Fbf

qRT- PCR quantitative RT-PCR

RBP- RNA Binding Protein

Rbfox1- RNA binding feminizing on X 1

RBM9- RNA Binding Motif Protein 9

Rbp9- RNA binding protein 9

RRM- RNA Recognition Motif

Sxl- Sex-lethal

CHAPTER ONE
Introduction to Germline Cyst Differentiation

The ultimate stem cell

Germ cells are the only cells that can give rise to an embryo. While the sperm brings a haploid genome, the oocytes carry additional components crucial for embryogenesis and the survival of the next generation. The oocyte provides all the organelles, mRNAs and proteins necessary for the very first steps of development before zygotic transcription takes place. It also carries the mitochondrial genome that each cell will inherent in the next generation (**Fig 1A**). In addition, the oocyte contains all the factors sufficient to promote pluripotency as proven by somatic nuclear transfer cloning. Thus, understanding how eggs form is a fundamental question in developmental biology. A deeper understanding of normal oocyte development holds promise to improve our ability to generate pluripotent cells for stem cell-based therapies.

To become a mature egg, female germ cells undergo several steps of differentiation. Many of these steps are highly conserved from *Drosophila* to humans, although occurring at different stages of life (**Fig 1B**). During embryogenesis, the germ cell lineage is formed by either induction through cell-extrinsic factors or by preformation through germ plasma inheritance (Extavour and Akam, 2003). The germ cells then migrate into the developing gonad. Once the cells colonize the gonads, they undergo sex determination while expanding in number through mitosis. Germ cells will then enter several rounds of specialized synchronous mitotic divisions where cytokinesis is not completed and the cleavage furrows

remain open. In turn, germ cells form clusters of interconnected clonal cells known as germline cyst or germline nest. One or multiple cells within a cluster, depending on the species, will grow in size, go through meiosis and differentiate into an egg, while the remaining cells will eventually die. Formation of germline cyst prior to entering meiosis has been observed in all higher insects and vertebrates examined, including humans (Pepling, 2006). Thus, understanding the mechanisms by which germline cysts form is crucial to gain a better appreciation of how oocytes are formed in humans.

Germline cyst: All for one

Evolutionary pressures have favored the formation of syncytium (multinucleated cells) in several tissues, skeletal muscle being a classical example. After differentiation, individual skeletal muscles fuse to form muscle fibers. Multiple cells sharing a cytoplasm work together to form long fibers that can synchronously respond to stimuli. In the case of the female germline, it is not such a fair game. Most of the cells within a cyst eventually die. The bright side is that the survivor(s) will have the ability to give rise to an entire organism. Cells predestined to die provide nutrients, proteins, mRNA and even organelles to the developing oocyte, and thus are referred to as supporting cells. These components are transported from the supporting cells to the oocyte through intercellular bridges in a microtubule-dependent manner (Pepling et al., 1999). Before undergoing cell death, supporting cells empty all the remaining cytoplasm into the developing oocyte in a process called dumping (Buszczak and Cooley, 2000). While this process occurs throughout adulthood in *Drosophila*, mammalian germline cysts are observed during embryogenesis and

early postnatal period. Here, germ cells are tightly packed within the fetal ovary, and thus are more challenging to study. Nonetheless, recent studies from the Allan Spradling and Lei Lei labs demonstrate that organelle transfer occurs from sister cells into the developing oocyte. They further show that this process is essential for proper oocyte differentiation in mice (Lei and Spradling, 2016). Given that the last common ancestor between insects and mammals lived about 550 million years ago, it seems that formation of cyst is a robust strategy to generate oocytes.

Germline cyst formation in *Drosophila*

The availability of sophisticated genetic tools and the accessibility of the tissue makes *Drosophila* an excellent model organism to study germline cyst formation. In *Drosophila,* each female contains a pair of ovaries; each ovary is made out of 16-20 ovarioles. Each ovariole is a tube-like structure filled with sequentially developing egg chambers. Germline cysts are formed at the most anterior side of the ovariole in a structure called the germarium. Two to three germline stem cells (GSCs) reside within the germarium adjacent to the cap-cells that provide a stem cell niche (**Fig 1.2**). Activation of the canonical BMP signaling in GSCs by the niche leads to the transcriptional silencing of *bag-of-marbles* (*bam*). Upon division, GSC daughters displaced away from the stem cell niche no longer experience BMP signaling and transcribe *bam*, resulting in germline cyst commitment (Chen and McKearin, 2003). The committed cell called a cystoblast (CB) then undergoes four synchronized incomplete divisions to form a cyst composed of 16 cystocytes interconnected through cytoplasmic bridges. Once the 16-cell cyst is formed, all cystocytes exit the cell cycle and

several enter meiosis. As cyst development proceeds, meiosis gets restricted to one of the cystocytes. This cell will become the oocyte. The other 15 cells within the cyst become the supporting cells known as nurse cells in *Drosophila*. During cyst formation, cells are constantly wrapped by somatic cells called escort cells (EC) that help them move along the A-P axis. Between the end of region 2 (region 2B) and region 3, other type of somatic cells, called follicle cells, encapsulate the germline cyst as it leaves the germarium to form an egg chamber. Once the egg chamber is formed, the nurse cells enter several endoreplication cycles and provide nutrients, mRNAs, proteins and organelles to the developing oocyte throughout oogenesis. The oocyte will continue growing and progressing through the meiotic program (Pepling et al., 1999; Spradling, 1993).

Cysts within the germarium are characterized by the presence of a structure called the fusome. The fusome is a continuous membrane-rich organelle that spans through the ring canals of each cystocyte within a germline cyst. It contains molecules that are normally found in the endoplasmic reticulum (ER), and it forms a continuous membrane network with the ER in a cyst (Lin et al., 1994; Snapp et al., 2004). The membrane skeletal proteins α-Spectrin, β-spectrin and the adduccin-like protein Hu-li-tao-shao (Hts) are enriched in the fusome. The cysts formed in *hts* and *α-Spectrin* mutant ovaries display less than 16 cells and lose their synchrony (de Cuevas et al., 1996; Yue and Spradling, 1992). Mutant cysts also fail to specify the oocyte. Therefore, it has been proposed that the fusome plays an essential role during cyst formation by facilitating transport between cystocytes, first to synchronize divisions, and second to promote polarized transport towards the oocyte once the 16-cell cyst is formed.

In addition to the crucial role of fusome in cyst development, its visualization has been an invaluable tool to study cyst formation. During cyst formation, the fusome undergoes invariable morphological changes used to mark specific stages of cyst differentiation. Initially, the fusome is rounded in the GSC and CB, and it gradually branches with each cyst division. In regions 2B and 3, the fusome starts to degrade and disappears soon after the cyst exits the germarium and an egg chamber is formed. Thus, abnormalities in fusome morphology can provide clues to defects in differentiation. For instance, persistence of fusomes at later stages is a hallmark of delay in or blockage of cyst maturation. Several mutants have been isolated with variations of such a phenotype.

Germline cystic tumors

Mutations in genes necessary for proper cyst formation result in cystic tumors. In a cystic tumor, cells cannot progress through the normal differentiation program. Instead, cysts fail to exit the cell cycle and to form normal egg chambers. As a consequence, actively dividing cysts accumulate abnormally in the germarium. Occasionally, a layer of follicle cells will wrap multiple individual germline cysts to form an egg chamber that lacks nurse cells and oocytes. This type of structure is referred to as tumorous pseudo-egg chamber. Mutations that result in this type of phenotype have been mapped to just a handful of genes listed on Table 1.1, arguably reflecting how little we know about cyst differentiation.

A major event in cyst formation occurs when one of the GSC daughters leaves the stem cell niche and commits to differentiate into a germline cyst. Loss of *bam* in the germline results in tumor formation, marked by the accumulation of germ cells arrested in a pre-

cystoblast (pre-CB) like state. By contrast, ectopic expression of *bam* within GSCs results in their precocious differentiation. Therefore, Bam is both necessary and sufficient for germ cell differentiation in the *Drosophila* germline (McKearin and Ohlstein, 1995; McKearin and Spradling, 1990; Ohlstein and McKearin, 1997). *Benign gonial cell neoplasm* (*bgcn*) mutants display a similar phenotype to *Bam* mutants, where cells fail to enter the cyst differentiation program (pre-Bam Class, Table1.1) and evidence suggests that Bgcn acts together with Bam to repress translation of specific genes (Li et al., 2009; Ohlstein et al., 2000).

Once the germ cell is committed to become a cyst, Bam expression is turned off at the 8-cell stage to continue the differentiation process. Mutants like *mei-P26*, *Sex-lethal* (*Sxl)* and *fused* (*fu*) retain high levels of Bam and fail to enter this transition. In these mutant ovaries, germ cells are arrested in a stage between CB and 8-cell cyst (Bam+ Class) (Chau et al., 2009; Narbonne-Reveau et al., 2006; Neumuller et al., 2008). A distinct class of cystic tumor mutants successfully goes through the Bam On to Off transition; however, they cannot enter the 16-cell cyst stage (post-BAM Class). *Arrest* and *RNA binding protein 9* (*Rbp9*) belong to this class (Lee et al., 2000; Sugimura and Lilly, 2006). Excessive accumulation of cysts in these mutants is not as severe as the pre-Bam or Bam+ mutants, and their pseudo-egg chambers are considerably smaller. This suggests that either these cysts divide less often or that the follicle cell layer more efficiently wraps Bam negative cysts compared to Bam positive cysts.

Based on the phenotypic characterization of these cystic mutants, the genes responsible for these phenotypes are predicted to exhibit dynamic expression patterns different from each other. Careful examination of the expression patterns of these genes over

the years confirmed this hypothesis, with the exception of Bgcn, which might be needed for multiple stages (**Fig 1.3**). When the expression pattern of all the genes are put together, a highly dynamic expression profile emerges. This suggests that major molecular changes occur to drive differentiation during normal cyst development. Thus, one can speculate that genes with similarly dynamic expression patterns will play important roles in cyst development.

Rbfox1 marks a previously unrecognized step during cyst formation

A visual expression pattern screen aimed to isolate genes important for germline cyst development identified the protein trap CC00511 with a highly dynamic expression during early cyst development (Tastan et al., 2010). This protein trap mapped to the gene *Ataxin 2 binding protein 1* (*A2bp1*) also known as *RNA binding Feminizing on X 1* (*Rbfox1*). Antibodies against this protein confirmed its dynamic expression pattern. Rbfox1 is absent in GSCs, CBs are 2-cell cysts, and comes on in the 4-cell cysts, reaching a peak during the 8 to 16-cell cyst stages. Once the cyst enters region 2B, Rbfox1 levels start to decline (**Fig 1.4**). This expression pattern is unique, since it bridges the early cyst differentiation marker Bam with late differentiation markers like high levels of Nanos (Nos) (**Fig 1.5**). These observations suggest that Rbfox1 might play an essential role in cyst formation. Loss-of-function studies by previous members of our lab indeed showed that Rbfox1 is necessary for normal cyst formation (Tastan et al., 2010) (**Fig 1.6**). Rbfox1 mutant ovaries exhibit a cystic tumor consisting of cysts at stages ranging from 4- to 16-cells that do not degrade their

fusome. These tumors maintain Bam expression, which is indicative of a block in differentiation prior 8-cell cyst (**Fig 1.6**).

Rbfox1 is the only *Drosophila* homolog of an RNA-binding protein family known to play a central role in alternative splicing of specific transcripts within the nucleus. In *Drosophila* ovary, Rbfox1 is enriched in the nucleus of somatic cells, whereas it is mostly in the cytoplasm in the developing cyst (**Fig1.4**). The function of Rbfox1 during cyst development and the role of these proteins in the cytoplasm were largely uncharacterized at the time this project started. My project aimed to understand the role of cytoplasmic Rbfox1 in the context of germline cyst differentiation.

In chapter three of my thesis, I present data showing that the cytoplasmic isoform of Rbfox1 is required for proper germline differentiation where it acts as a translational repressor. This represents a novel function for the heavily studied Rbfox family of genes. Taking a step further, this study prompted us to investigate the target genes repressed by Rbfox1. In chapter four, I present data to show *pumilio* (*pum*), an essential gene for germ cell maintenance across species, is a major target of Rbfox1 during germline differentiation. This work implies that germ cells must turn off previous differentiation programs in order to progress to the next step in differentiation. Unexpectedly, we also found that overexpression of Pumilio or loss of Rbfox1 causes germ cells to dedifferentiate, suggesting that germ cells can revert back to a previous differentiation state when their normal developmental path is blocked. Thus, my research has advanced our understanding of germline development and the molecular function of Rbfox proteins, with implications in cellular differentiation and Rbfox-related disorders.

In addition to the results presented, each chapter has an introduction relevant to the specific problem under study and closes with a discussion of the presented results. In chapter five I briefly summarize the findings presented and suggest some future direction in the field.

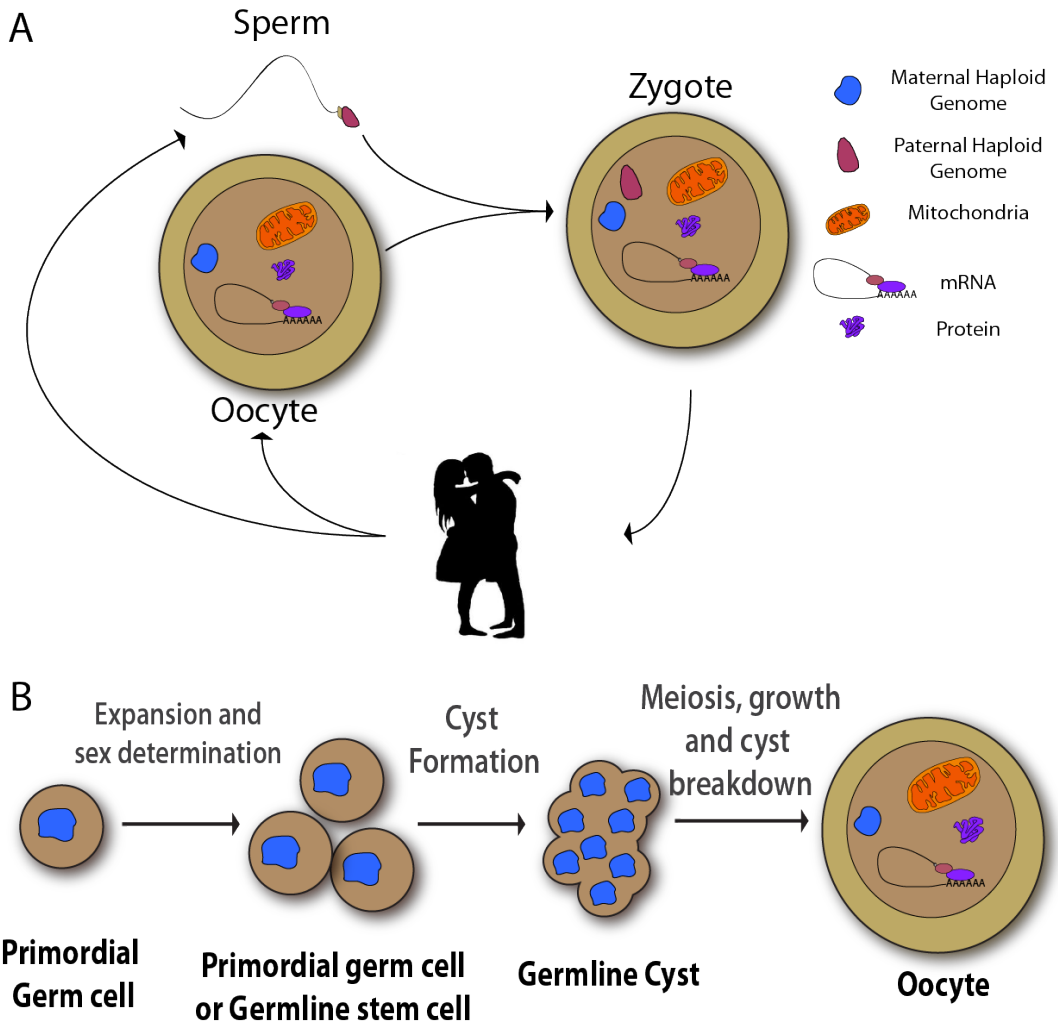


Fig 1.1 Oocyte development. (A) Upon fertilization of the oocyte by the sperm a zygote is formed. This zygote will then give rise to an entire organism. Organisms will then pass their genetic information through newly formed sperms and oocytes. Upon fertilization the egg contain mRNAs, proteins and organelles essential for embryogenesis. (B) Major differentiation steps of the female germline to develop into an mature oocyte in many multicellular organisms, including *Drosophila* and humans.

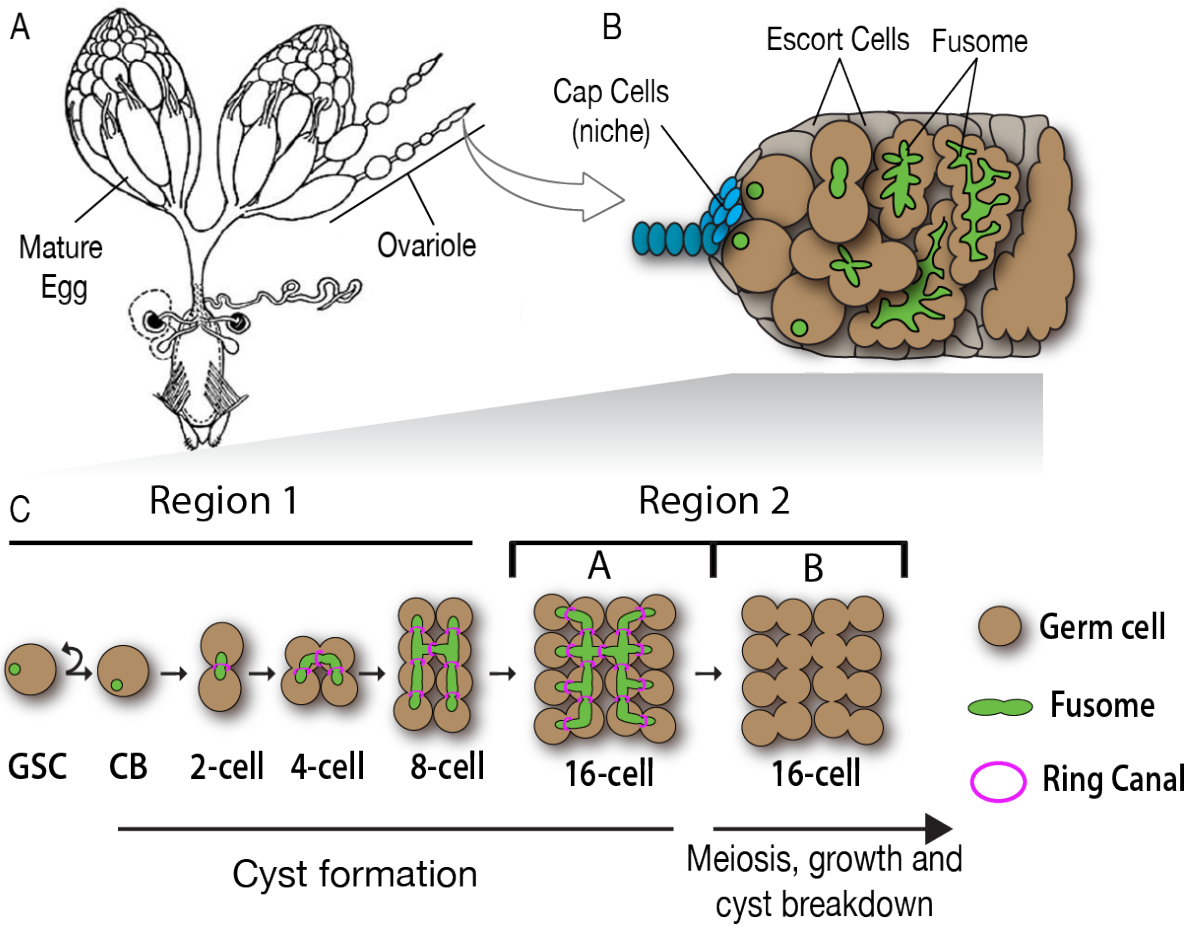


Fig 1.2 *Drosophila* oogenesis. (A) Drawing of a *Drosophila* reproductive system. (B) Schematic of a germarium. (C) Schematic of *Drosophila* germline cyst differentiation. Germline stem cells (GSC) give rise to cystoblast (CBs) that then divide four times to form 16-cell cysts. These divisions are incomplete and cells remain connected through cytoplasmic bridges called ring canals (magenta). During these divisions the fusome (green) branches (region 1 and 2A). Fusomes are specialized organelles that become increasingly more branched up until the 16-cell cyst stage, when they begin to undergo degradation (de Cuevas and Spradling, 1998). The fusome is labeled with the adducin-like protein hu li tai shou (Hts). After germ cells complete their mitotic divisions, the fusome degrades (region 2B). Persistence of fusomes typically indicates a defect in germ cell differentiation.

Gene	Molecular Function	LOF Phenotype	Class
bag-of-marbles (bam)	Indirect RNA binding; Competitor	GSC-like tumor	Pre-Bam
Benign gonial cell neoplasm (bgcn)	RNA helicase	GSC-like tumor	Pre-Bam
Sex Lethal(Sxl)	Direct RNA Binding	CB and Cystic tumor	Bam+
meiotic P26 (mei-P26)	Direct and/or indirect RNA binding, Ubiquitin Ligase	CB and Cystic tumor	Bam+
fused (fu)	Ser/The Kinase	Cystic Tumor	Bam+
RNA binding protein Fox/ A2bp1 (Rbfox1)	Direct RNA Binding	Cystic Tumor	Bam+
arrest (Bruno)	Direct RNA Binding	Cystic tumor	Post-Bam
RNA Binding protein 9/Hu-homolog (Rbp9)	Direct RNA Binding	Cystic tumor	Post-Bam
Ovarian tumor (Otu)	De-Ubiquitinating Enzyme	Unknown	Unknown

Table 1.1 Genes involved in *Drosophila* cyst development

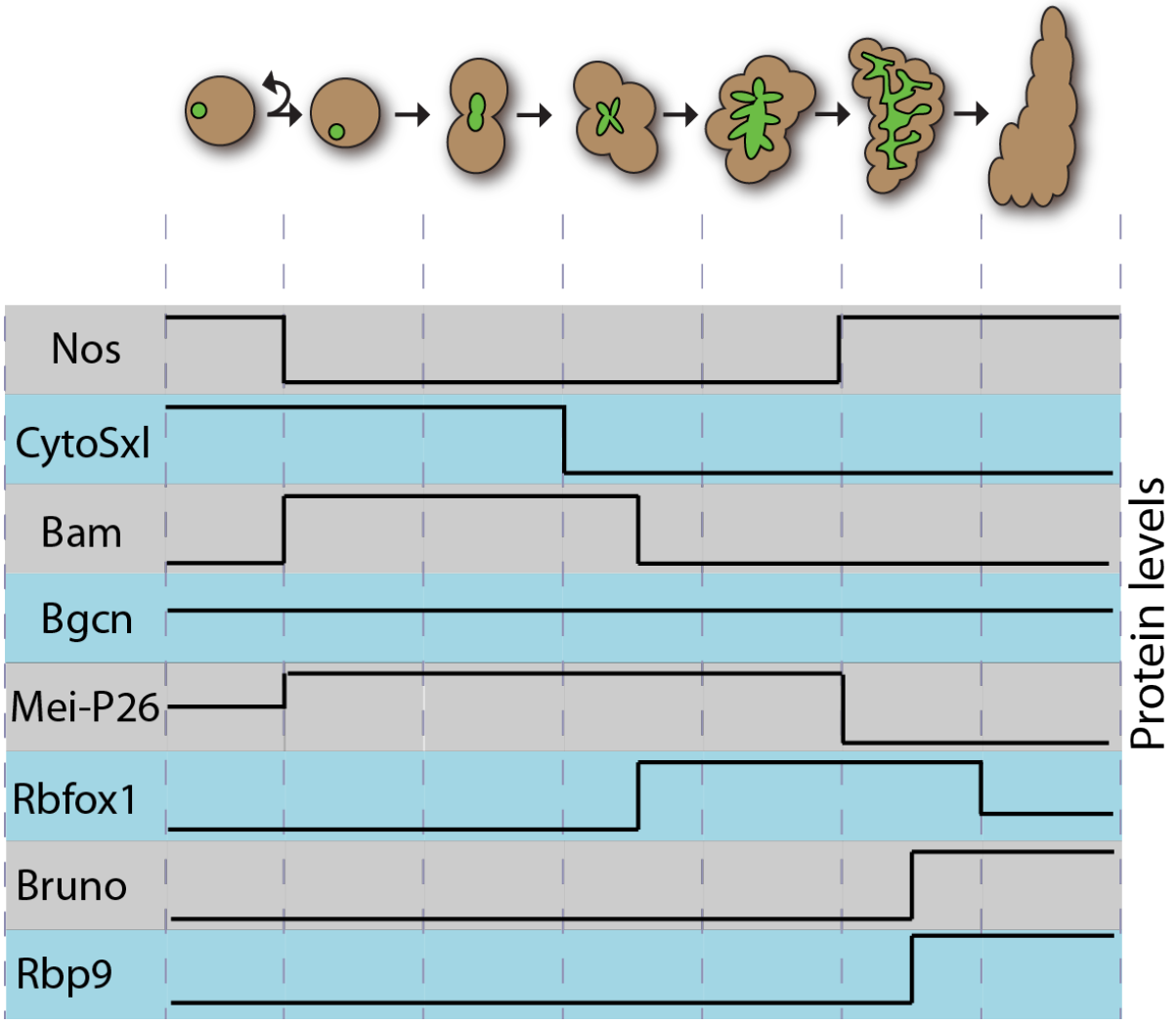


Fig 1.3 Protein expression pattern of genes involved in cyst development. This figure depicts highly dynamic changes in expression of genes involved in cyst development during cyst formation.

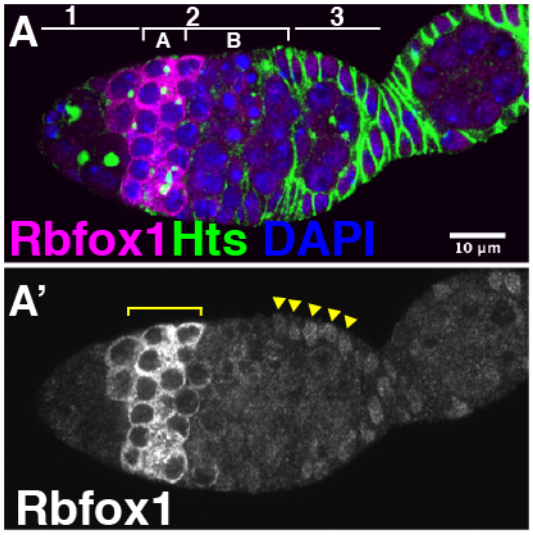


Fig. 1.4 Rbfox1 localizes to the cytoplasm within the germline. (A) A wild-type germarium stained for Rbfox1 (magenta), the fusome marker Hts (green) and DNA (blue). Regions 1, 2A, 2B and 3 are indicated. (A') Rbfox1 staining alone. The bracket marks germ cells that express high levels of cytoplasmic Rbfox1. Small arrows mark somatic follicle cells that express nuclear Rbfox1.

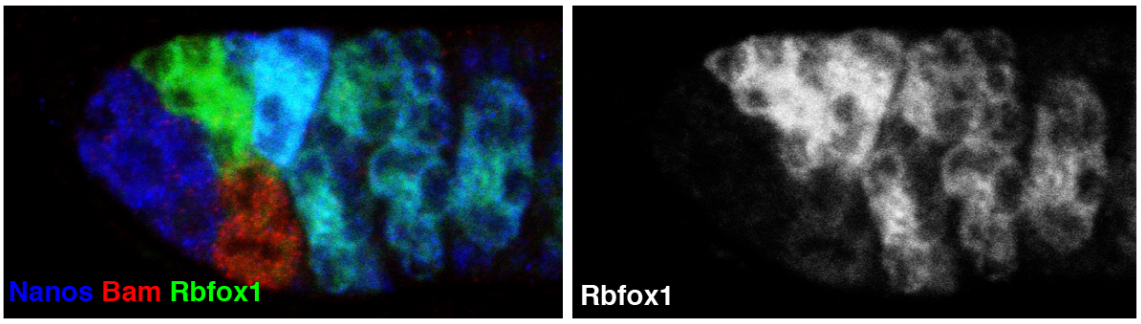


Fig 1.5 Rbfox1 expression pattern bridges early with late cyst markers. Rbfox1 expression follows the early differentiation marker Bam (Red) and precedes late differentiation markers such as high levels of Nanos (blue). This dynamic expression pattern suggests a role for Rbfox1 during cyst development. (Image taken from(Tastan et al., 2010)).

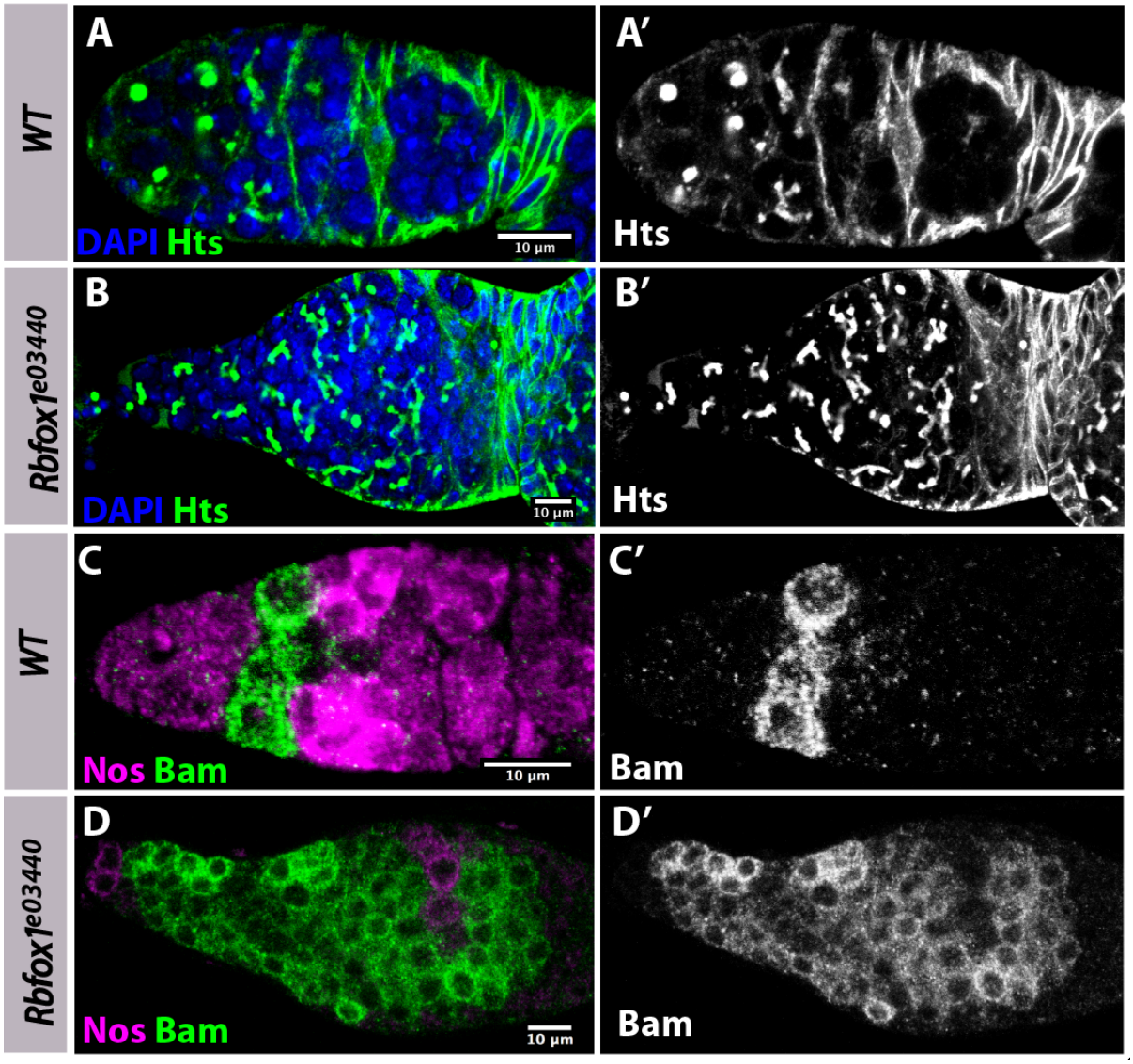


Fig 1.6 Loss of *Rbfox1* results in cystic tumors. WT (A-A') or *Rbfox1* mutant (B-B') ovaries stained for Hts (green) and DNA (blue) revealed that Rbfox1 mutants form germ cell tumors. Persistence of fusomes typically indicates a defect in germline cyst differentiation. (C-D') Immunofluorescence for Nanos (magenta), a GSC and 16-cell cyst marker, and Bam (green), a cystoblast to 4-cell cyst marker, showed that Rbfox1 mutants fail to enter the 16-cell stage and remain in a Bam-positive stage. (Image C-D modified from (Tastan et al., 2010))

CHAPTER TWO
Materials and Methods

Fly stocks

Fly stocks were maintained at 20-25°C on standard cornmeal–agar–yeast food. Vasa-gal4 was a gift from Y. Yamashita. *Df(3L)ED4457* and *pumRNAi* (BL#36676) were obtained from Bloomington Stock Center and $Rbfox1^{e03440}$ and $Rbfox1^{f02600}$ were obtained from Harvard Stock Center. UAS-pum-pum3'UTR and UAS-pum-Tub3'UTR were a gift from Elizabeth Gavis and originally described in (Menon et al., 2004). UAS-Rbfox1-RF was inserted into ZH-51D (BL#24483), UAS-Rbfox1-RN into ZH-22A (BL#24481), recombineered Rbfox1[FF>AA] and Rbfox1[WT] genomic constructs into VK00037 (BL#24872), all Rbfox sensors and pum 3'UTR reporters into ZH-51D (BL#24483) and shRNA constructs into attP6 (BL#34768) using phiC31 integrase (Rainbow Transgenics Inc.). Guide and donor plasmids to generate Rbfox1[dsRed.1] allele where injected in nos-Cas9 attP40 line by Rainbow Transgenics Inc. as well.

Molecular Biology

Cloning Rbfox1-RF and Rbfox1-RN

RNA was extracted from *hs-bam;bam*$^{\Delta 86}$ mutant ovaries and made into cDNA using SuperScript II-Strand Kit (Life Technologies), together with a reverse primer for the unique cytoplasmic exon sequence. Using this cDNA PCR was performed with CACC_Rbfox1-RF and Rbfox1-uniqueexon-R primers with KAPA HiFI under GC rich conditions (KAPA Biosystems). Amplified products were cloned into pENTR vectors following manufacture's protocol. Remaining predicted 3'end ORF was added using BstZI and AscI sites. Using LR

clonase reaction (Invitrogen) Rbfox1-RF/RN were cloned into pAHW (Drosophila Gateway Vector Collection). HA tagged versions of Rbfox1-RF/RN were cloned into KpnI and XbaI sites of pJFRC28 (Pfeiffer et al., 2012).

shRNA lines against RF/RN unique exon

shRNA oligos targeting RF/RN unique exon were annealed and inserted into EcoRI and NheI sites of Valium 22 vector (Ni et al., 2011).

[FF>AA] RRM flies

We used recombineering techniques (Carreira-Rosario et al., 2013) to insert a Zeomycin cassette into a modified version of CH321-94L16 P[acman] clone (Tastan et al., 2010). Specifically the cassette was inserted in the intronic region upstream of the exon that contains portion encoding for F158 and F160 residues. Cassette with homology arms were added to Zeiomycin cassette through PCR with Rbfox1-Zeo_F and F158A,F160A_Rbfox1-Zeo_R primers for [FF>AA] or Rbfox1-Zeo_F and F158A,F160A_Ctl_ Rbfox1-Zeo_R for [WT].

3'UTR sensors

Tub 3'UTR with nested wild type or control elements were generated by splicing by overlap extension (SOE) PCR and cloned into pCasper-Attb vector containing Venus (Li et al., 2012). Two more rounds of SOE-PCR were used to fuse a segment of the *vasa* promoter and Tub5'UTR to the reporters. Final PCR products were cloned into pCasper-Attb. To generate PumGCAUG and PumACAUA reporters wild type or mutated forms of pum-RA 3'UTR were synthesized (Integrated DNA Tech.) and clone into vasP-tub5'UTR-Venus reporter mentioned above.

Generation of *Rbfox1*$^{dsRed.1}$ allele

To generate the Rbfox1$^{dsRed.1}$ allele, guide RNAs were designed using http://tools.flycrispr.molbio.wisc.edu/targetFinder and synthesized as 5'-unphosphorylated oligonucleotides, annealed, phosphorylated and ligated into the BbsI sites of pU6-BbsI-chiRNA plasmid (Gratz et al., 2013). Homology arms were PCR amplified and cloned into pHD-dsRed-attP (Gratz et al., 2014)(Addgene). Guide RNAs and the donor vector were co-injected into nos-Cas9 attP embryos at the following concentrations: total volume of 200 µl with 250 ng/µl pHD-dsRed-attP donor vector and 20 ng/µl of each of the pU6-BbsI-chiRNA plasmids containing the guide RNAs (Rainbow Transgenics Inc.).

Immunohistochemistry

Adult ovaries from flies fed with wet yeast for 24-48 hours were dissected in 1XPBS and fixed in 4% (vol/vol) formaldehyde for 10 min. Ovaries were then washed with PBT (1X PBS, 0.5% BSA, and 0.3% Triton-X 100) three times for a total of 30min, permeabilized and block for at least 1 hour and incubated with primary antibody overnight at 4°C. Ovaries were washed and incubated in secondary then washed again and mounted in Vectashield containing DAPI (Vector Laboratories). The following primary antibodies were used: guinea pig anti-Rbfox1 (1:5000) (Tastan et al., 2010), rat anti-Pum (1:1000) (Joly et al., 2013a), rabbit anti-GFP (1:1000) (Life Technologies), rat anti-HA 3F10 (Roche) and rabbit anti-pH3(Ser10) 1:250 (Upstate). Mouse anti-Hts (1B1) (1:20), rat anti-VASA (1:20), mouse anti Sxl M18 (1:20) and mouse anti dFMRP 5B6 (1:20) (Developmental Studies Hybridoma Bank). To labeled Rbfox1 in mice tissues we used rabbit anti-Fox-1 N-14 (1:200) (Santa

Cruz). We used biotinylated Peanut Agglutinin to label ring canals (Vector Laboratories)(1:500). Fluorescence-conjugated secondary antibodies from (Jackson Laboratories) (1:300).

RNA EMSA

GST-Venus or GST-Rbfox1-RRM recombinant protein was mixed in 10mM HEPES (pH 7.5), 0.2% Tween-20, 50 mM KCL, 2 mM DTT, 1 μg/μl yeast tRNA, 0.05 μg/μl BSA and 200 U/mL of RNAse inhibitor to a final concentration of 4.5 nM. Non-labeled competitor RNA (1X equals 0.2 ng/ul) was added and incubated for 5 minutes at R/T. 1X DIG labeled RNA was added to each reaction and incubated for 25' at R/T. Samples were resolved on a 4% polyacrylamide non-denaturing TBE mini-gel at 4°C. RNA was transferred to a Hybond –N+ membrane, UV-crosslinked and processed as in the Northern Blot section.

RNA-IP

Ovaries were dissected in 1XPBS and cross-linked using 0.08% formaldehyde in 1XPBS for 10 minutes. Fixation was quenched with 2 M glycine. Ovaries placed on ice and rinsed with 1xPBS and then RIPA buffer (50 mM Tris-HCl, 200 mM NaCl, 0.4% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 200 mM NaCl). The samples were lysed in RIPA plus (RIPA, 2.5 mg/ml yeast tRNA, 1 mM PMSF, 50 U/ml Roche RNAse inhibitor) and clarified by centrifugation. For the Rbfox1 sensor mRNA, RIP lysates were mixed with pre-washed Anti-FLAG M2 affinity beads. For *pumilio* RIP lysates were mixed with either Rbfox1 polyclonal antibody or pre-serum, incubated at 4°C for two hours followed by

addition of protein A agarose beads (Sigma). Samples were then incubated O/N at 4°C under constant mixing. The beads were then washed two times with 500 µL RIPA plus supplemented with 1M Urea at room temp and incubated in reverse buffer (1 mM Tris-HCl (pH 6.8) 5 mM EDTA, 10 mM DTT, 1.0% SDS) at 70°C for 45 minutes. Proteinase K was added to a final concentration of 0.1mg/mL and incubated for 25 minutes at 37°C. RNA was isolated using Trizol and analyzed with either One-Step RT-PCR (Qiagen) or by cDNA synthesis followed by qPCR using SYBR Green Master Mix (Applied Biosystems).

Collection of ovaries enriched for Rbfox1-positive cysts

hs-bam/+;bam$^{\Delta 86}$ young flies (more than a 1.5 week old flies easily die after heat shock) were collected and store at 18ºC for 3 to 4 days with wet yeast. This minimizes any leaky expression from the *hs-bam* transgene. Flies were then heat shocked for 45 minutes twice with a 12 hour recovery time in between. 2.5 days after the first heat shocked ovaries were dissected and lysed accordingly to the specific experiment.

Northern Blot

RNA was isolated ovaries using Trizol (Life Technologies). Samples were denatured in NorthernMax formaldehyde loading dye containing Ethidium Bromide (Ambion) for 15' at 65°C and immediately placed on ice. Then samples were resolved on a denaturing agarose gel (1XMOPS, 2.2Mformaldehyde, 1.5%agarose). rRNA was imaged and then RNA was downward transferred to a Hybond-N+ following Ambion NorthernMax kit protocol. RNA was UV-crosslinked to membrane. Membrane was incubated with hybridization buffer

(Ambion NorthernMax) for 4 hours at 60°C and then DIG-labaled antisense *Venus* probe was added to a final concentration of 50ng/uL and incubated O/N. Next day the membrane was rinse once and washed 2 times for 5' with in 2XSSC, 0.1% SDS followed by two washes for 20 minutes at 68°C under constant agitation. Membrane was then washed for 2min in washing buffer (maleic acid buffer (0.1M maleic acid, O.15M NaCl , pH to 7.), 0.3% Tween 20) followed by 1hr incubation in blocking solution (1% w/v Blocking Reagent (Roche) in maleic acid buffer). Membrane was incubated O/N at 4°C in blocking solution with Anti-Digoxigenin-AP, Fab (Roche) at 1:20,000. Next day membrane was washed for a total of 45' in washing buffer, equilibrate in 0.1M Tris-HCl (pH9.5), 0.1M NaCl for 3' and developed using CDP-Star reagent (NEB).

Venus antisense probe was generated by mixing 150ng of Venus PCR product with T7 site on 3'end, 40U of T7 RNA polymerase (Roche), DIG RNA labeled mix (Roche), RNAse Inhibitor (Roche) in transcription buffer (Roche) in a 20uL reaction and incubated at 37°C for 3 hrs. RNA was purified with LiCL and EtOH.

Poly(A) Tail-Length (PAT) assays

We used Poly(A) Tail-Length Assay Kit (Affymetrix) following the instructions provided by the manufacturer.

qRT-PCR

cDNA was generated using SuperScript II-Strand Kit (Life Technologies). 5uL of diluted (dilution depended on the amount of starting RNA) cDNA was mixed with 10uL of SYBR

Green PCR Master Mix (Life Technologies), 0.2uL of 10uM forward primer and 0.2uL of 10uM reverse primer in a final volume of 20uL. Samples were run on a CFX96 Real Time System (Biorad).

Polysome fractionation

9 mL of 1.6X10^6 S2 R+ cells /ml were plated on a 100mm and the 24 hrs cells were transfected with 2.0ug of pA-HA-Rbfox1-RF using Effectene (Qiagen) following protocol provided by manufacture. 48 hrs after transfection cells were collected into a 15mL canonical tube, cyclohexamide was added to a final concentration of 100ug/mL and incubated on ice for 5 minutes. Cells were pelleted and lysed by resuspending in polysome Lysis buffer (15mM Tris-HCL pH 7.5, 15mM $MgCl_2$, 300mM NaCl, 1% TritonX-100, 100ug cyclohexamide, 1mg/mL heparin, 100U/mL RNAse inhibitor, 1 tablet per 10 mL Roche Protein Inhibitor Coktail) follow by 5 minutes incubation on ice. Nuclei was pelleted by centrifuging for 5 minutes at 4°C at 12Kg. Supernatant was applied to a 5 mL 17.5 to 50% sucrose gradient. Gradient was made as described in Masek et al. (Masek et al., 2011). Gradient was ultracentrifuge for 2hrs at 4°C in a SS55A-0067 at 100Kg. Twelve 450uL fractions were collected by pipetting and split into two parts. RNA was isolated from one part using 750uL of Trizol (Invitrogen). Purified RNA pellet was dissolved in 20uL of RNAse free water and half was used to precipitate protein. To precipitate protein from the other half, 4X volume of -20°C acetone was added to each fraction, vortex and incubated O/N at -20°C. Tubes were centrifuged at 16Kg at R/T for 5 minutes, supernatant was removed, 1 mL of -20°C 70% acetone was added, vortexed and spin for 5 minutes at 16Kg. Supernatant was

removed and pellet was air dry and resuspend in 150uL of 1X sample buffer. For ribosomal RNA analysis 3 uL of the extracted RNA from fractions were ran in a 1% agarose denaturing gel (1XMOPS, 2.3M formaldehyde) and visualized by UV. For western blotting analysis 30 uL of each protein fraction were used to run SDS-PAGE and blotted for the specific antibodies. Primary antibodies for western blots: Rabbit anti eIF3J (1:1500) and rabbit anti eIF3B (1:1500) were gifts from Matthias Hentze group, RpL11 (Pierce) (1:750), mouse anti-CycA (Developmental Studies HybridomaBank)

CHAPTER THREE
Cytoplasmic Rbfox1 promotes germline cyst development and represses translation

Introduction

RNA-binding proteins play an integral role in mRNA metabolism, splicing, transport and translation. An increasing number of studies link mutations in genes encoding RNA-binding proteins with a variety of diseases, highlighting the importance of these proteins with regard to human health (Lukong et al., 2008; Ramaswami et al., 2013). Rbfox proteins represent one such family and contain a highly conserved, centrally located RNA-recognition motif (RRM) flanked by intrinsically disordered regions (IDRs) (Auweter et al., 2006; Jin et al., 2003; Ponthier et al., 2006). Mammals have three Rbfox paralogs: *RBFOX1* (*A2BP1*), *RBFOX2* (*RBM9*) and *RBFOX3* (*NeuN*). Nuclear isoforms of these genes regulate alternative splicing by directly binding to intronic (U)GCAUG elements, resulting in the exclusion or inclusion of downstream or upstream exons respectively. In mice, disruption of *Rbfox1* in neurons leads to neuronal hyperactivity, while loss of *Rbfox2* results in cerebellum development defects (Gehman et al., 2012; Gehman et al., 2011). *Rbfox1* and *Rbfox2* have been implicated in a number of diseases including cancer, diabetes and neurological disorders such as autism, mental retardation and epilepsy (Barnby et al., 2005; Bhalla et al., 2004; Bill et al., 2013; Davis et al., 2012; Hu et al., 2013b; Mikhail et al., 2011; Sebat et al., 2007; Wen et al., 2015). In all these examples, the observed phenotypes have been ascribed to perturbations in normal mRNA splicing patterns.

Different isoforms of all three Rbfox family members localize to the cytoplasm in a variety of different tissues across species (Dredge and Jensen, 2011; Gehman et al., 2012; Hamada et al., 2013; Kiehl et al., 2001; Lee et al., 2009; Shibata et al., 2000). While the molecular functions of these isoforms remain poorly understood, both nuclear and cytoplasmic isoforms appear to act as tumor suppressors in the context of glioblastomas (Hu et al., 2013a). Loss of cytoplasmic Rbfox1 has also been associated with colorectal cancer (Sengupta et al., 2013) and abnormal cytoplasmic inclusions of Rbfox1 are often observed in SCAII (Spinocerebral Ataxia Type II) patients (Shibata et al., 2000). Recent studies have also shown that Rbfox proteins bind to many different 3'UTRs in the mammalian brain (Lee et al., 2016; Weyn-Vanhentenryck et al., 2014). These observations suggest that Rbfox proteins carry out additional functions beyond their established roles in splicing.

The *Drosophila* genome contains a single Rbfox homolog. Antibodies directed against sequences shared by all nine Rbfox1 isoforms showed that while many tissues express nuclear Rbfox1, differentiating germline cysts within the germarium exhibit a burst of cytoplasmic Rbfox1 expression (Tastan et al., 2010) (**Fig. 1.4**). Mutations in *Drosophila Rbfox1* result in germline tumor formation (**Fig1.6**) (Tastan et al., 2010). Here, we use the *Drosophila* ovary as a system to study the role of cytoplasmic Rbfox1 during germline differentiation. We show that cytoplasmic Rbfox1 is necessary for *Drosophila* germline development and regulates the translation of specific mRNAs by binding to (U)GCAUG elements contained within their 3'UTR sequences. Thus, our study reveals a splicing-independent function of Rbfox proteins, the disruption of which may contribute to *RBFOX*-linked diseases.

Results

The *Drosophila Rbfox1* locus encodes two functional cytoplasmic isoforms

Current annotations indicate that the *Drosophila Rbfox1* gene encodes at least eight different isoforms. All of the corresponding proteins contain a nuclear localization signal (NLS) in their C-termini, except for the Rbfox1-PF isoform (**Fig. 3.1A**). We cloned a cDNA corresponding to an additional transcript (*Rbfox1-RN*) that also lacks an NLS from ovarian RNA. With the exception of one exon these two isoforms share all exons. Isoform RF includes exon 15 but not 13 and vice versa for Rbfox1-RN. Although both of these exonic sequences code for IDRs, exon 15 encodes a poly-Q stretch and a Histidine stretch not present in exon 13. We transfected S2 cells with HA-tagged versions of these clones and analyzed their protein product on a SDS-PAGE. Rbfox1-PF shows a specific band around 115kDa while the Rbfox1-PN runs around 90kDa. Both of these sizes were strikingly larger than the predicted size (76.4 kDa for PF and 61.1kDa for PN) suggesting that these isoforms may be subject to post-translational modifications (**Fig. 1C**). To test this idea we expressed Rbfox1-RF in bacteria. The sizes of recombinant Rbfox1-PF from bacteria and Rbfox1-PF extracted from S2 cells were indistinguishable on a SDS-PAGE (data not shown). This suggests that the large differences between predicted and observed protein sizes are not due to post-translational modifications. Most likely, the extensive IDR including poly-Q and poly-G stretches present in Rbfox1 proteins explains their unexpected behavior on an SDS-PAGE. We then tested whether these isoforms indeed localize to the cytoplasm as predicted. We generated transgenic animals carrying HA tagged *Rbfox1-RF or Rbfox1-RN* cDNA expression constructs and found that the corresponding proteins localized to the cytoplasm as

expected (**Fig.3.1D-E**). We concluded that Rbfox1-RN and Rbfox1-RF encode for isoforms that localized to the cytoplasm.

Next we asked whether these isolated isoforms functioned within the germline. The hypomorphic mutation *Rbfox1*^{e03440} results in female sterility and a germ cell tumor phenotype, marked by the accumulation of germline cysts that fail to differentiate beyond the early stages of their development (Tastan et al., 2010) (**Fig. 3.2A**). We tested whether Rbfox1-RF and/or Rbfox1-RN can rescue this phenotype. While all egg chambers formed in *Rbfox1*^{e03440}/Df are tumorous, expression of Rbfox1-RF within the germline led a dramatic rescue with nearly all egg chambers containing 16 cells (**Fig 3.2**). When *Rbfox-RN* was expressed in this same mutant background most of the egg chambers formed contain 32 cells (**Fig 3.2C**). Although this is not normally observed in wild type, egg chambers with 32 cells form eggs that can give rise to healthy progeny and reflects a rescue of the tumorous phenotype observed in the mutant background. In conclusion, both isoforms rescued the tumorous phenotype associated with the *Rbfox1*^{e03440} allele.

We then generated two inducible shRNA lines targeting different regions of the exon sequence unique to the *Rbfox1-RF* and *Rbfox1-RN* transcripts. When expressed in the germline, these shRNA constructs caused a tumorous phenotype, mimicking the defects observed in *Rbfox1* mutants (**Fig. 3.3**). A CRISPR/Cas9 strategy was also used to delete the *Rbfox1-RF* and *Rbfox1-RN* specific exon (**Fig. 3.4**). This mutant, *Rbfox1*$^{dsRed.1}$, exhibited loss of cytoplasmic Rbfox1 expression during early germline cyst development, whereas nuclear Rbfox1 isoforms appeared largely unaffected (**Fig. 3.4C**). This allele resulted in female sterility marked by the formation of tumors comprised of undifferentiated germ cells (**Fig**

3.4). All together these experiments conclusively indicate that cytoplasmic *Rbfox1* isoforms promote early germline cyst differentiation.

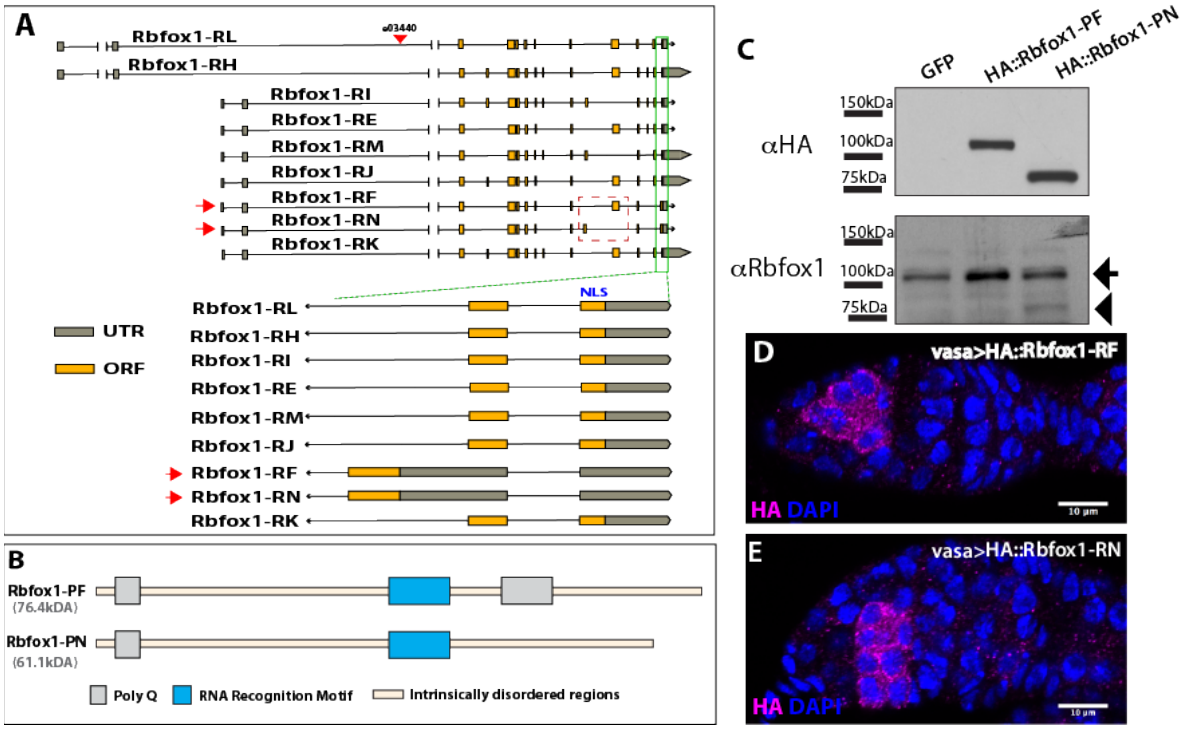


Fig 3.1. *Drosophila Rbfox1* locus encodes for two distinct cytoplasmic isoforms. (A) Schematic of the Rbfox1 locus showing that *Rbfox1-RF* and *Rbfox1-RN* both use an alternative splice site that results in exclusion of a nuclear localization signal (NLS) contained in the other annotated isoforms (B) Schematic of Rbfox1-PF and Rbfox1-PN protein products with their predicted molecular weight in parenthesis. Both contain a centrally located RNA Recognition Motif flanked by IDRs including two poly-glutamine (poly-Q) stretches on PF and one poly-Q on PN. (C) Western blot analysis probed with anti-HA (top) or anti-Rbfox1(bottom) antibodies. Samples where extracted from S2 cells transfected with the indicated constructs. Arrow indicates Rbfox1-PF and arrowhead Rbfox1-RN. (D-E) *vasa-gal4>UAS-HA::Rbfox1-RF* (*vasa-gal4>HA::Rbfox1-RF*) (D) or *vasa-gal4>UAS-HA::Rbfox1-RN* (*vasa-gal4>HA::Rbfox1-RN*) (E) germaria stained for the HA-tagged transgene (magenta) and DNA (blue).

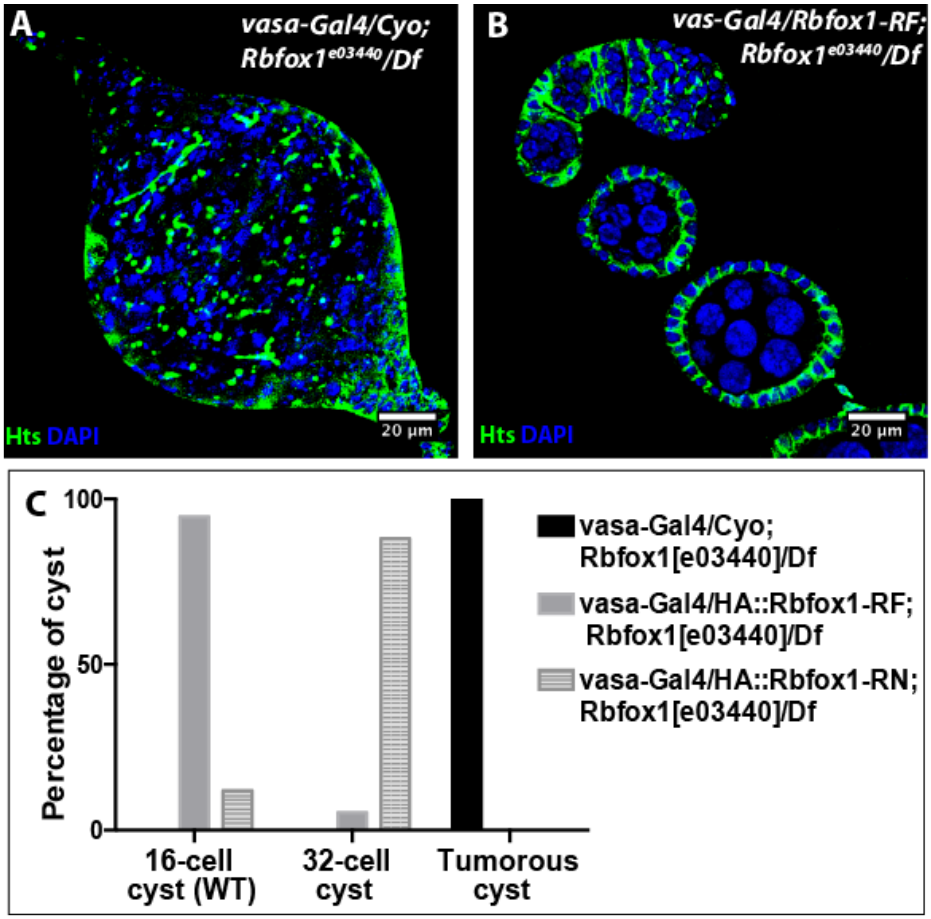


Fig 3.2. Rbfox1-RF and RN rescue *Rbfox1* tumorous phenotype. (A-B) Germaria from (A) *vasa-gal4/CyO*; *Rbfox1^{e03440}/Df* (*vasa-gal4>Rbfox1^{e03440}/Df*), (B) *vasa-gal4>UAS-HA::Rbfox1-RF*; *Rbfox1^{e03440}/Df* (*vasa-gal4>HA::Rbfox1-RF; Rbfox1^{e03440}/Df*) and females stained for Hts (green) and DNA (blue). (C) Quantification of Rbfox1 mutant phenotypes and the rescue of these defects by expression of the *Rbfox1-RF* or *Rbfox1-RN* transgene.

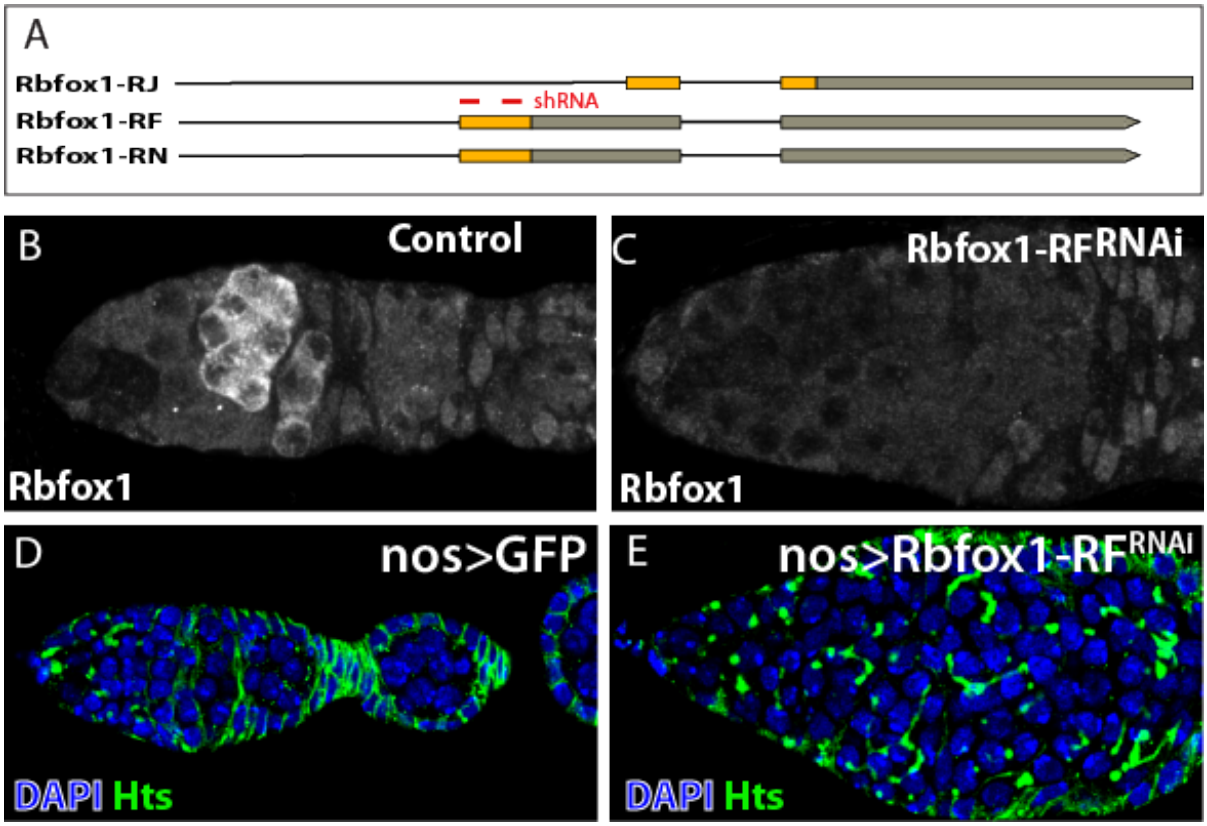
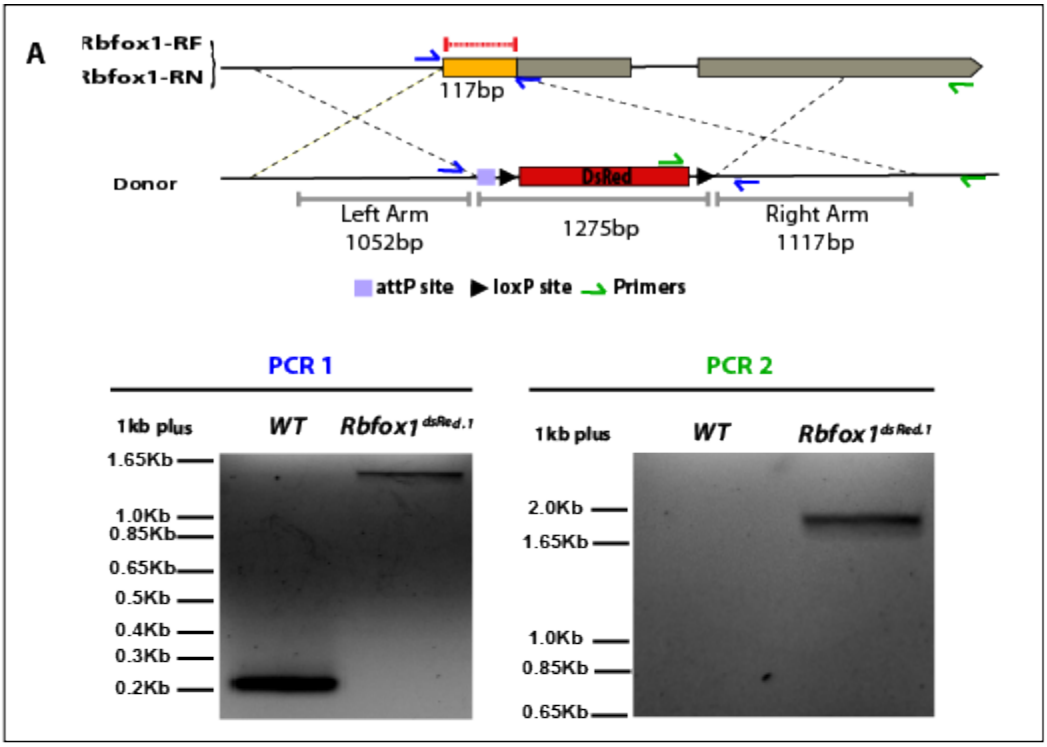


Fig 3.3. shRNA targeting RF/RN unique exon leads to cystic tumors. (A) Schematic of 3' end genomic region of *Rbfox1-RF* and *Rbfox1-RN*. Red bars depict RNAi targeted regions design to affect RF and RN expression but not other isoforms. (B-C) *nos-gal4>UAS-GFP* (Control) and *nos-gal4>UAS-Rbfox1-RF*^{RNAi} (*Rbfox1-RF*^{RNAi}) germaria stained for Rbfox1 to validate Rbfox1 knockdown. (D-E) *nos-gal4>UAS-GFP* (Control) and *nos-gal4>UAS Rbfox1-RF*^{RNAi} (*Rbfox1-RF*^{RNAi}) germaria stained Hts (green) and DNA (blue).



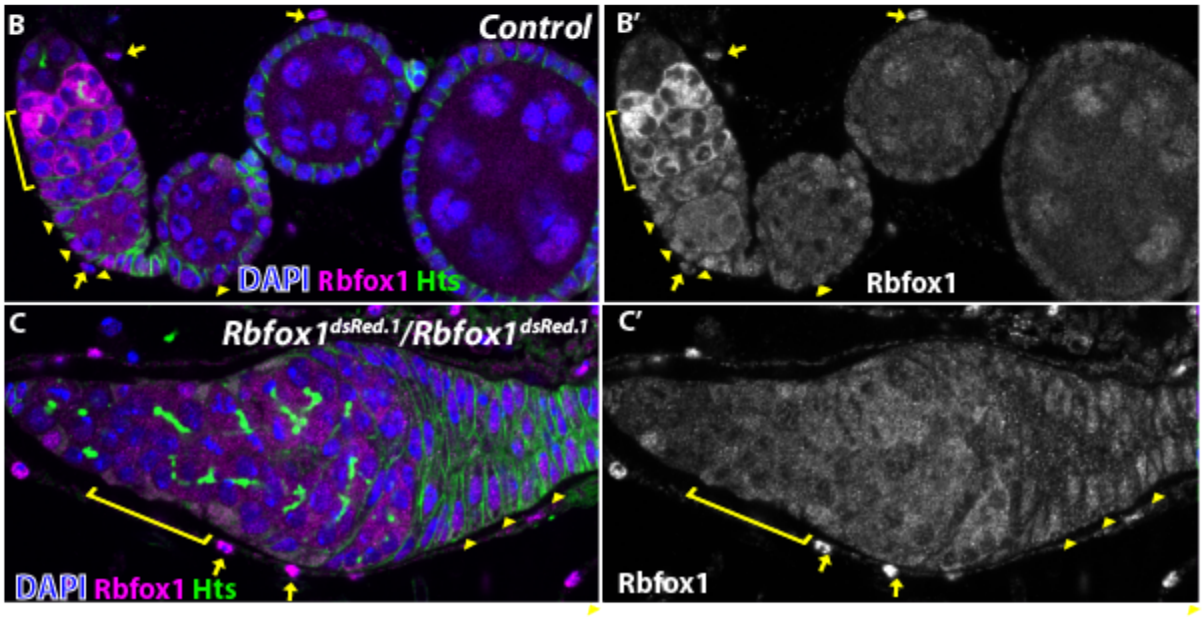


Fig 3.4. CRISPR/Cas9 mediated unique exon replacement causes germline tumors. (A) PCR verification that the *Rbfox1*$^{dsRed.1}$ allele harbors a *3xP3-RFP* cassette in place of sequence specific for cytoplasmic isoforms of Rbfox1. (B-C) Control (B-B') or *Rbfox1*$^{dsRed.1}$ homozygous (C-C') germaria stain for DAPI, Rbfox1. B' and C' Rbfox1 alone.

Cytoplasmic Rbfox1 acts as a translational repressor

Drosophila Rbfox1 contains a highly conserved RNA Recognition Motif (RRM), which shares over 90% amino acid identity with its human homolog (**Fig 3.5**). To test whether the function of Rbfox1 during early cyst development depends on its ability to bind to RNA, we generated a genomic rescuing construct in which F158 and F160 were replaced by alanines (Rbfox1[FF>AA]). These are highly conserved amino acids within the RRM crucial for the ability of Rbfox1 protein to bind to its RNA targets (Auweter et al., 2006). A wild type genomic construct rescued the Rbfox1 tumorous phenotype, while the *Rbfox1[FF>AA]* construct did not (**Fig 3.5**). These results indicate that cytoplasmic Rbfox1 must bind to RNA to carry out its function within the germline.

We noticed that a pool of WT cytoplasmic Rbfox1 exhibits a granular distribution while *Rbfox1[FF>AA]* protein shows a diffuse distribution throughout the cytoplasm (**Fig 3.6**). Many RNA-binding proteins form messenger ribonucleoprotein (mRNP) aggregates visible under light microcopy. These include stress granules, processing bodies and germ cell granules (Anderson and Kedersha, 2009). In response to different kinds of stress, such as nutrient deprivation or heat shock, these granules typically increase in size. To characterize whether the Rbfox1 granules we observed behave in a similar manner we performed co-localization experiments with *Drosophila* Fragile X mental retardation protein (dFMRP), a protein known to localize to stress granules, using heat shocked fly ovaries. Upon heat shock, Rbfox1 granules became more prominent and partially colocalized with the dFMRP particles (**Fig 3.7**). IDRs within RNA-binding proteins are thought to be necessary and sufficient to form RNA granules. This conclusion is largely based on overexpression analysis *in vitro*.

Our experiments suggest that RNA-binding contributes the formation of large mRNPs under physiological conditions. A recent paper that carefully titrated protein concentration to mimic physiological conditions showed that indeed RNA-binding promotes RNA granule formation (Molliex et al., 2015).

Localization of Rbfox1 to stress granules led us to investigate whether Rbfox1 regulates translation. Many RNA-binding proteins form mRNPs that localized to cytoplasmic RNA granules where they regulate translation through direct interaction with cis-acting elements within 3'UTRs of target genes. We first examined whether Rbfox1 binds to the same (U)GCAUG RNA element as its mammalian homolog through RNA-EMSA. Recombinant *Drosophila* Rbfox1 RRM associates with *in vitro* transcribed RNA that contains (U)GCAUG elements. By contrast, the *Drosophila* Rbfox1 RRM domain did not bind to RNA containing (U)GCAUA elements (**Fig 3.8**). To directly test the hypothesis that cytoplasmic Rbfox1 regulates translation of specific mRNAs through a 3'UTR dependent mechanism, we engineered two different sensors; a 3XRbfox1 sensor and a mutant sensor (**Fig 3.9**). These two sensors have the exact same promoter and they both have the GFP variant Venus as a reporter gene. Downstream of Venus they contain the *α-tubulin* 3'UTR with either 3XUGCAUG elements in the 3XRbfox1 sensor or 3XUGCAUA elements in the mutant sensor (**Fig 3.9A**). These reporters lacked introns, avoiding any complication that might result from the regulation of splicing. We then performed RNA-immunoprecipitation (RNA-IP) from cross-linked tissue extracts to verify that cytoplasmic Rbfox1 proteins associated with the 3XRbfox1 sensor mRNA *in vivo* (**Fig 3.9B**). We then looked at the protein expression of these sensors in the ovary. While the mutant sensor showed fairly

uniform expression throughout the ovary, the Rbfox1-sensor showed a down-regulation exclusively in Rbfox1-positive cells (**Fig 3.10 A-B'**). To confirm that Rbfox1 causes this down-regulation we looked at the Rbfox1 sensor in an Rbfox1 mutant background. Here, Rbfox1 sensor expression levels never decreased **(Fig 3.10C**). Next we asked how many Rbfox1 binding sites were needed to observe the Rbfox1 repressive effect. The 1X Rbfox1 sensor was expressed throughout the germarium (**Fig 3.11B**), while the 2X Rbfox1 and 3X Rbfox1 sensors exhibited decreased expression in Rbfox1 expressing germ cells (**Fig. 3.11C-D**). The down-regulation observed in the 3X Rbfox1 sensor was more pronounced than the 2X Rbfox1 sensor. Then we asked whether Rbfox1 induces mRNA instability or somehow sequesters translational machinery from target transcripts. We performed quantitative RT-PCR (qRT-PCR) and Poly(A) Tail-Length (PAT) assays using the 3XRbfox1 and mutant sensors as read-outs. These experiments showed that the presence of (U)GCAUG sequences does not induce obvious changes in reporter transcript stability or poly-A tail length (**Fig 3.12**). Overall, our results show that cytoplasmic Rbfox1 blocks gene expression through 3'UTRs at the level of translation.

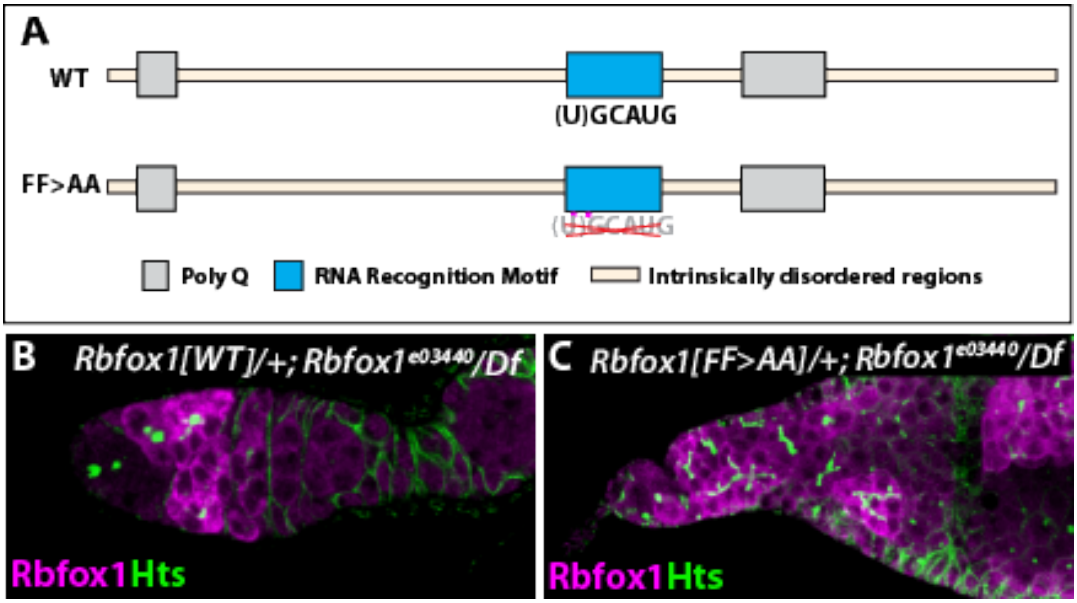


Fig 3.5 Rbfox1 requires RNA-binding for its function within the germline. (A) Schematic of Rbfox1 protein product. On top, full length (WT) Rbfox1 contains a highly conserved RNA recognition motif (RRM) (blue box), which has affinity for (U)GCAUG elements. In the FF>AA mutant F158 and F160 were mutated to alanines (depicted by magenta asterisks) to prevent RNA-binding. (B) A genomic construct containing WT Rbfox1 rescues the *Rbfox1*^{e03440} cystic tumor phenotype. (C) A genomic construct that harbors Rbfox1 [FF>AA] fails to rescue the *Rbfox1*^{e03440} phenotype.

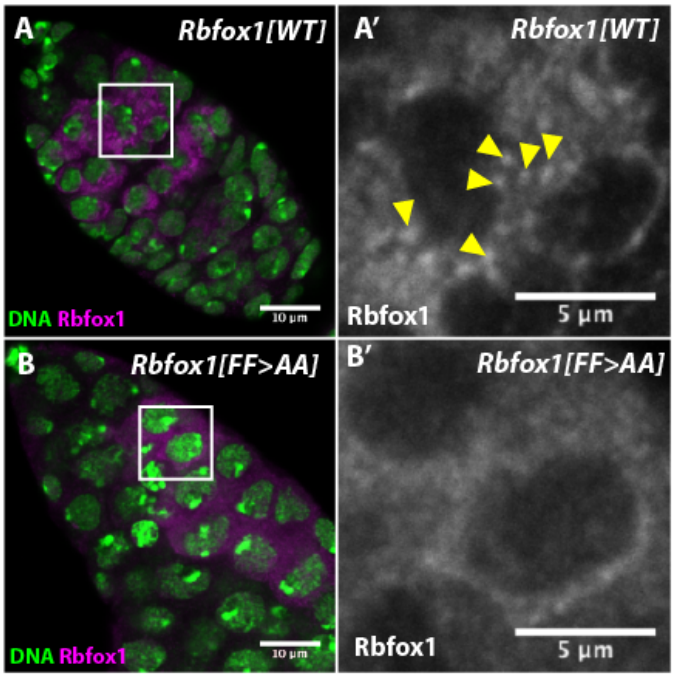


Fig 3.6 Rbfox1 granules required RNA-binding for their formation. Animals carrying a WT (A) or a Rbfox1[FF>AA] (B) genomic construct in an *Rbfox1*^{e03440} mutant background stained for DNA and Rbfox1. (A' and B') Inset of indicated region showing Rbfox1 signal alone. Arrowheads point to large RNA granules. This granules are absent in Rbfox1[FF>>AA].

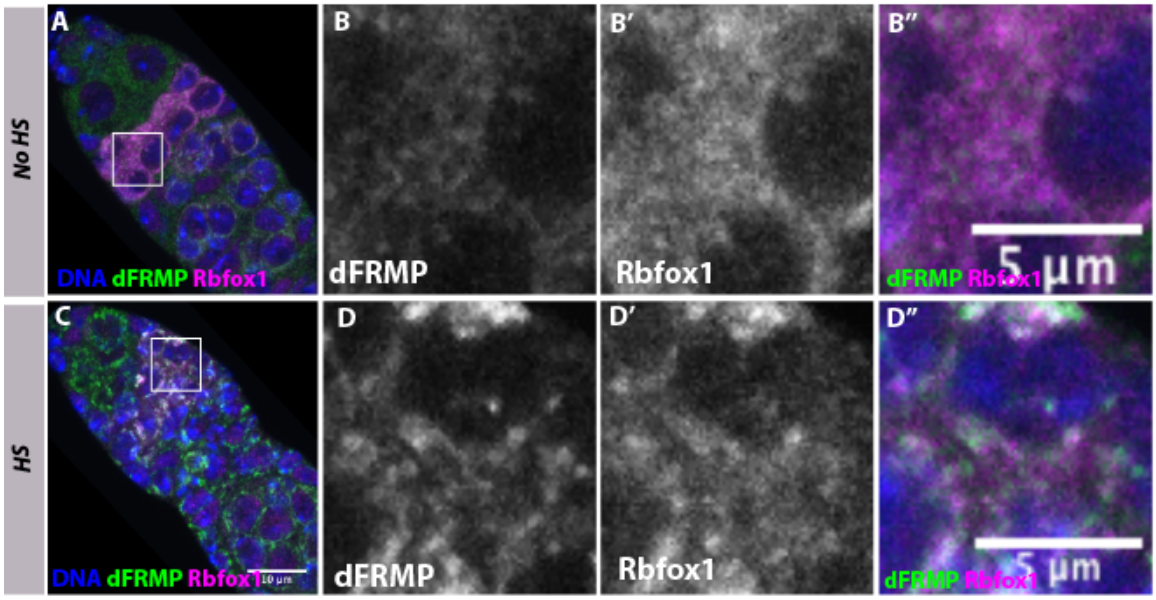


Fig 3.7 Pool of Rbfox1 localizes to cytoplasmic granules. Dissected ovaries from not heat shocked (A-B'') or heat shocked (C-D'') flies stained for dFMRP (green) and Rbfox1 (magenta). Upon heat shock Rbfox1 cytoplasmic granules become more prominent (D). These granules partially localizes with the stress granule marker dFMRP (D'').

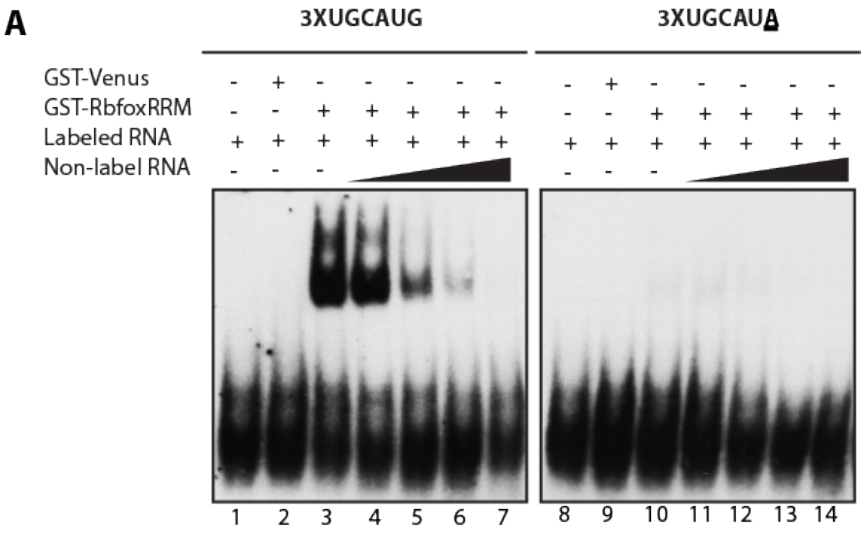


Fig 3.8 Drosophila Rbfox1 binds to (U)GCAUG elements *in vitro.* Recombinant Rbfox1-RRM (lanes 3-7 and 10-14) or Venus (lane 2 and 9) fused to GST were mixed in a binding reaction with DIG labeled ssRNA fallowed by analysis on a electrophoresis native gel. Addition of GST-Rbfox1RRM to the binding reaction results in a shift on the RNA mobility that is outcompeted when non-label RNA is added (lanes 3-7) when UGCAUG RNA was used. This shift is not observed with the 3XUGCAUa RNA (lanes 8-14). The first lane of each gel represents free RNA. Non-labeled RNA was added at 1, 10, 20 and 100 times the amount of labeled RNA (lanes 4-7 and 11-14).

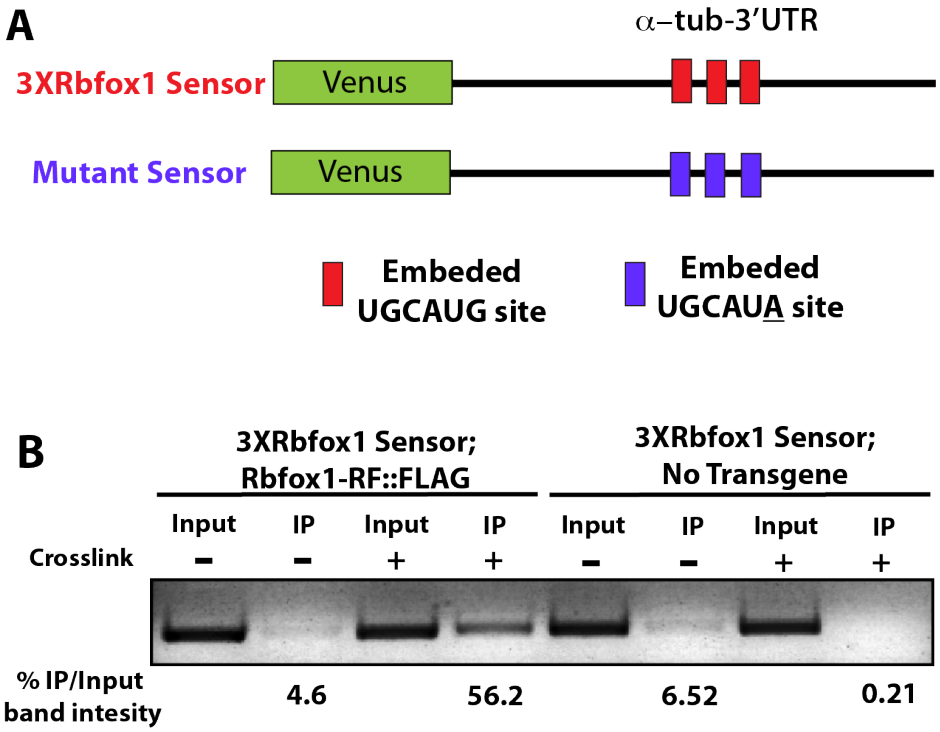


Fig 3.9 Sensors to study Rbfox1 function *in vivo*. (A) Schematic of Mutant and Rbfox1 sensors. Sensors contain same elements use in the RNA EMSA (Fig. 3.8) inserted within the alpha-tub 3'UTR (B) Rbfox1-PF physically interacts with Rbfox1 sensor mRNA. FLAG immunoprecipitation was performed on ovaries expressing both a Rbfox1-RF::HF transgene and the Rbfox1 sensor (lines1-4) or just the Rbfox1 sensor (lines 5-8) followed by qRT-PCR using primers against Venus mRNA. To control for non-physiologically relevant protein-RNA interactions tissues were fixed with 0.08% formaldehyde (FA) (lines 3,4, 7 and 8) and IPs were extensively washed with 1M urea before RNA isolation. A clear enrichment of the sensor mRNA was observed on IP from ovaries expressing the Rbfox1-RF::HF transgene that were fixed (line 4).

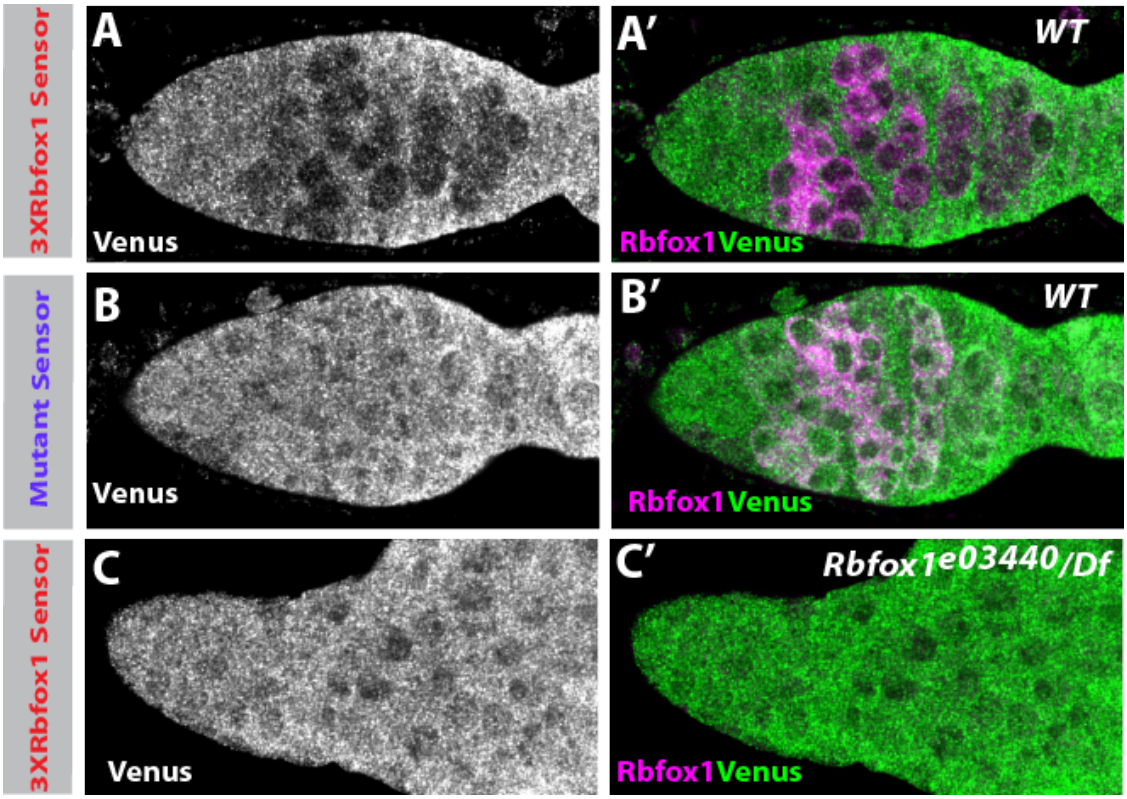


Fig 3.10 Rbfox1 represses Rbfox1 sensor but not mutant sensor. WT (A-B) or Rbfox1 mutant (C) ovaries expressing the 3XRbfox1 sensor (A, C) or mutant sensor (B) were stained for anti-Rbfox1(magenta) and anti-GFP (green). Protein expression of the Rbfox1 sensor but not mutant sensor is downregulated in Rbfox1-positive cells (compare A with B). This down-regulation is no longer observed in Rbfox1 mutants (C).

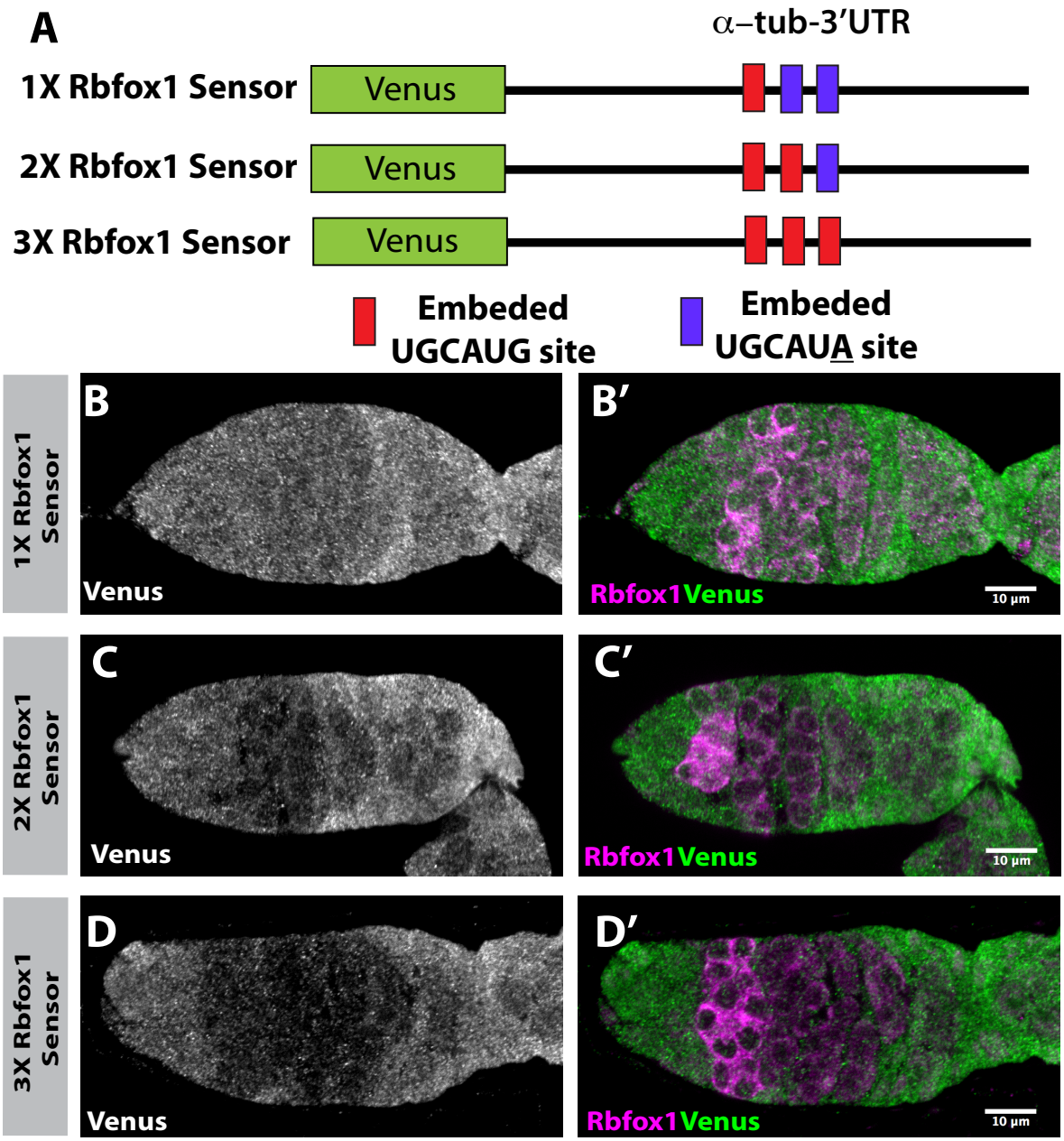


Fig 3.11 Amount of Rbfox1 binding sites in 3'UTR positively correlate with the extent of repression. (A) Schematic of 1X UGCAUG (1X Rbfox1 sensor), 2X UGCAUG (2X Rbfox1 sensor) and 3X UGCAUG (3X Rbfox1 sensor) sensors. (B-D') Ovaries expressing the corresponding sensors stained for Venus (green) and Rbfox1 (magenta).

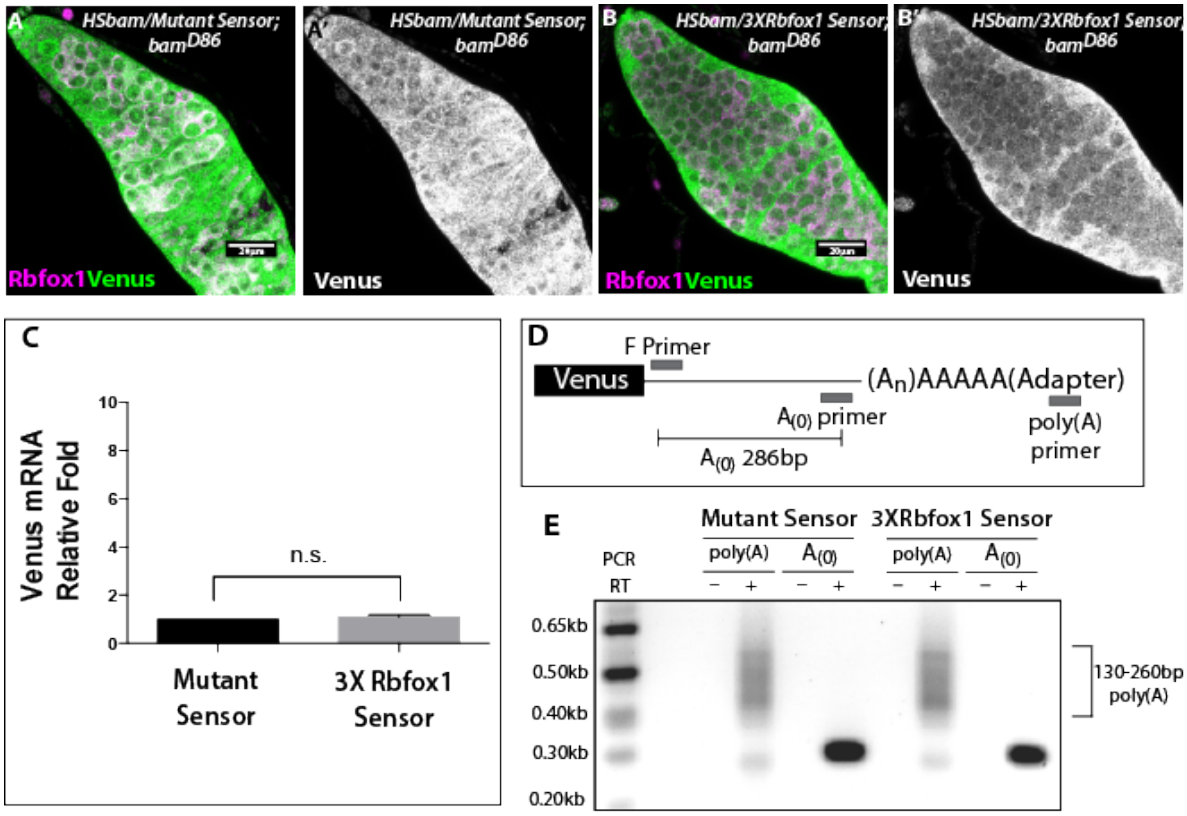


Fig 3.12 Rbfox1 do not affect mRNA stability. (A-B') Germaria from flies carrying the 3XRbfox1 sensor (A) or mutant sensor (B) in a *bam*$^{\Delta 86}$ mutant background with a heat shock-inducible *bam* (*hs-bam*) transgene stained for Rbfox1 (magenta) and the Venus (green). 2.5 days after Bam induction by heat shock, germ cells undergo synchronized differentiation and express high levels of Rbfox1. This system provides material enriched for cysts expressing Rbfox1. (C) qRT-PCR analysis of RNA extracted from ovaries containing mutant or 3X Rbfox sensor using primers against Venus. Venus mRNA levels were normalized to *α-Tubulin* mRNA. No significant difference in the mRNA levels between sensors was observed. (D) Method used to detect poly(A) tail lengths. Addition of a G/I adapter preceded an RT followed by PCR with either an A(0) reverse primer or a poly(A) reverse primer (PAT assay). (E) PAT assay with RNA extracted from ovaries carrying the mutant or the 3X Rbfox1 sensor. Sensors showed a similar Poly-A tail length that ranges from 130bp to 260bp.

Potential Rbfox1 mechanism of action

The qRT-PCR and PAT assays results led us to hypothesize that Rbfox1 represses translation instead of inducing mRNA degradation. Many RNA-binding proteins prevent eIF4F formation on the 5′-7-methylguanosine cap (5'-cap) to prevent translation (Hinnebusch and Lorsch, 2012). The vast majority of eukaryotic mRNAs recruit eIF4F to the 5'-cap for proper translation. To test whether Rbfox1 acts at this step we asked whether Rbfox1 could repress translation of a transcript that bypasses eIF4F-5'-cap interaction for translation. We generated a 3XRbfox1 sensor containing the *Drosophila hsp70a* 5'UTR upstream of the Venus reporter. *Hsp70a* 5'UTR contains an internal ribosomal entry site (IRES) that allows 5'-cap independent translation (Hernandez et al., 2004). Protein levels of *α-tub* 5'UTR reporter but not of *hsp70a* reporter dropped after heat shock (**Fig. 3.13**). Since cap-dependent translation is blocked upon heat shock we concluded that the hsp70a 5'UTR reporter is translated in a 5'cap-independent manner (Lamphear and Panniers, 1991). *Hsp70a*-5'UTR-3XRbfox1 sensor protein levels were lower than *hsp70a*-5'UTR-mutant sensor in Rbfox1-positive cells (**Fig 3.14**). This result supports a model were Rbfox1 acts downstream of eIF4F recruitment to the 5'-cap. Supporting this idea we observed that Rbfox1 co-fractionates with eIF3 members on a sucrose gradient rather than with previous fractions like other proteins known to prevent eIF4F assembly (**Fig3.15**) (Villaescusa et al., 2009). Once eIF4F gets recruited, the eIF3 complex together with other proteins mediate several steps of translational initiation: recruitment of 40S ribosomal subunit, ribosomal scanning and 60S ribosomal subunit joining to form monosomes. After a monosome is formed, the eIF3 complex is released from the transcript follow by elongation and polysome formation. Co-fractionation

with eIF3 members led us to hypothesize that Rbfox1 prevents a step upstream of polysome formation. We tested this idea by looking at the behavior of mutant and 3XRbfox1 sensors mRNA on a sucrose gradient. Although we did not observe an obvious enrichment on any particular fractions, we detected a reduction of Rbfox1 sensor mRNA amount in polysomal fractions compared to mutant sensor mRNA in the same fractions (**Fig. 3.16**). Collectively these data support a model where Rbfox1 prevents translation downstream of eIF4F recruitment and upstream of translational elongation.

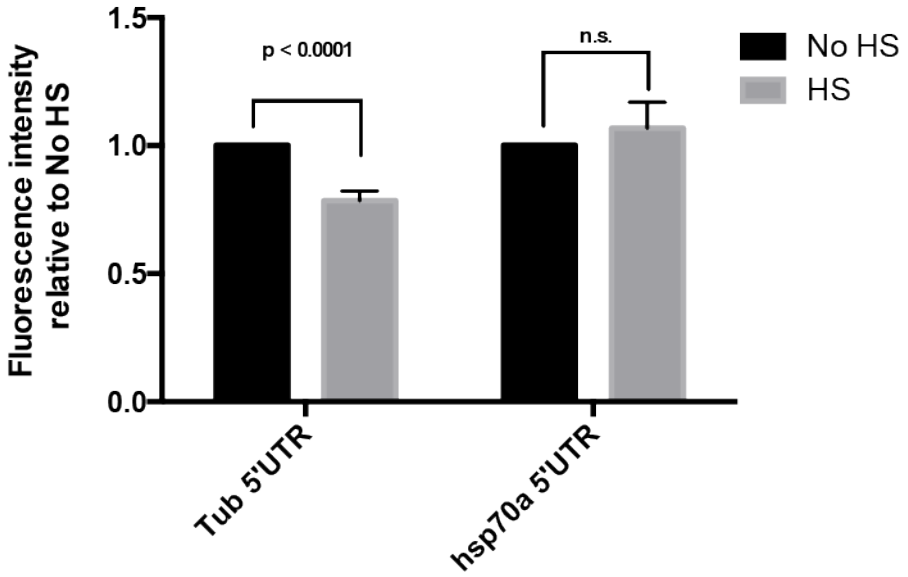


Fig 3.13 hsp70 IRES reporters validation. (A) Protein levels of Venus reporters were measured through fluorescence intensity quantification of immunefluorescently labeled Venus. Upon heat shock, protein levels of of *α-tub* 5'UTR reporter significantly diminished, presumably due to shut down of 5'-cap-dependent translation. However *hsp70a* 5'UTR reporter expression levels remain unchanged.

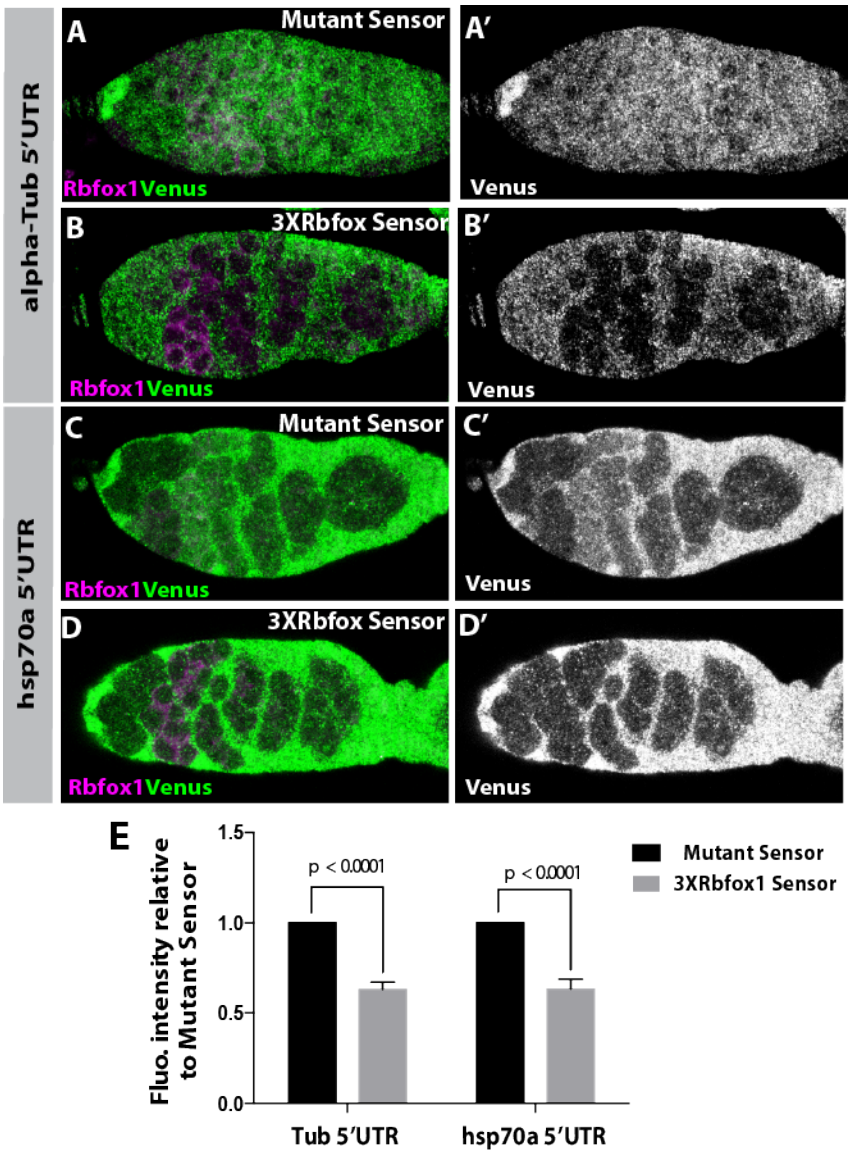


Fig 3.14 Rbfox1 represses translation in a cap-independent manner. (A-D') Ovaries expressing the *α-tub* 5'UTR Mutant sensor (A-A'), *α-tub* 5'UTR 3XRbfox1 sensor (B-B'), hsp70a 5'UTR Mutant senor (C-C') or hsp70a 5'UTR 3XRbfox1 sensor (D-D') were stained for anti-Rbfox1 (magenta) and anti-GFP (green). 3XRbfox1 sensors with either *α-tub* 5'UTR or *hsp70a* 5'UTR showed lower protein levels compare to its Mutant counterpart sensor (B' vs A' and D' vs C'). Settings between *α-tub* 5'UTR and *hsp70a* 5'UTR reporters but not between Mutant and 3XRbfox1 sensors were different because expression levels of hsp70a reporters are lower *α-tub* reporters. This presumably due to lower cap-independent translation compare to cap-dependent translation in the germline. (E) Fluorescence intensity of anti-GFP signal in cells expressing high levels of Rbfox1 was quantified for all sensors and plotted relative its respective Mutant sensor. Bars represent the average of 14 measurements and error bars are Standard of error of the mean (SEM). The hsp70a 5'UTR 3XRbfox1 sensor showed a significant reduction in intensity as compare to hsp70a 5'UTR mutant sensor. This difference is virtually identical to the one observed in the cap-dependent *α-tub* 5'UTR reporters.

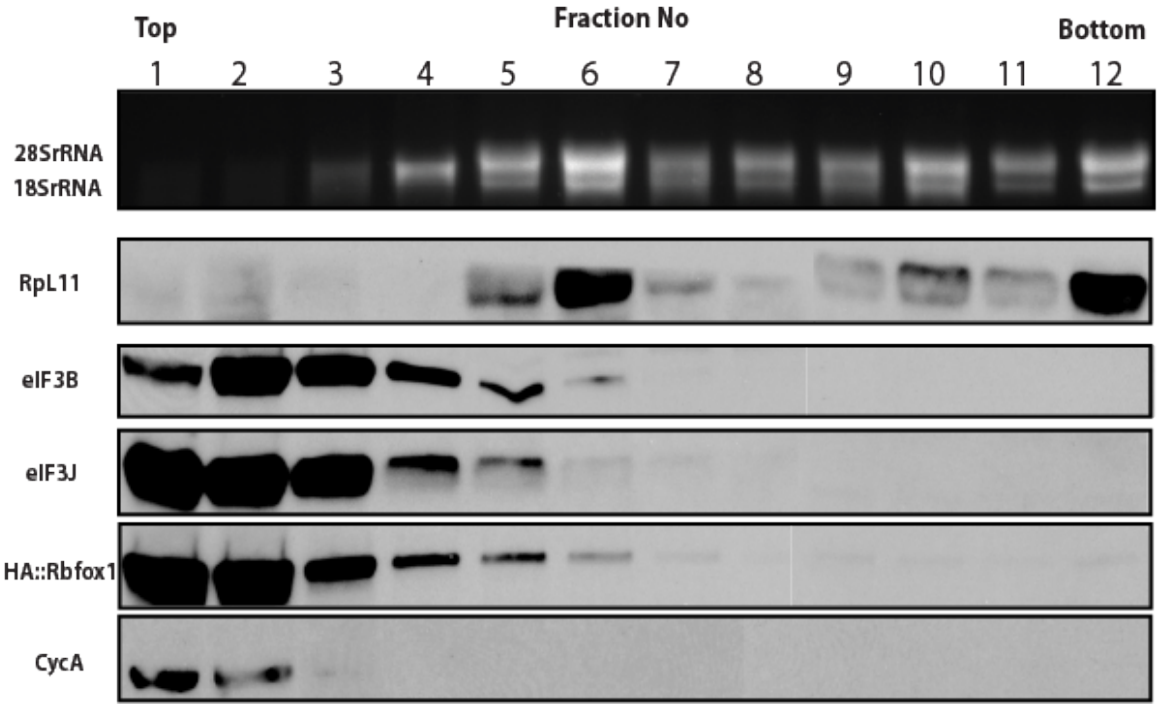


Fig 3.15 Rbfox1 co-fractionates with pre-polysomal fractions. Lysate from S2 cells transfected with HA tagged Rbfox1-RF was applied to a sucrose gradient and subjected to ultracentrifugation. Isolated RNA and protein from 12 different fractions were run on formaldehyde denaturing gel or SDS-PAGE respectively. rRNA was visualized with ethidium bromide and western blotting was perform with antibodies for RpL11, eIF3B, eIF3J, HA and CycA. Fraction 6 represents monosome as noted by high levels of both rRNA and RpL11. Fractions following this will represent polysomal fractions (fractions 7-12). Presence of small ribosomal subunit rRNA and eIF3 components indicates that fractions 3 to 5 represent 40S and 43S. Rbfox1 but not the negative control CycA co-fractionates with small ribosomal subunit and eIF3 members.

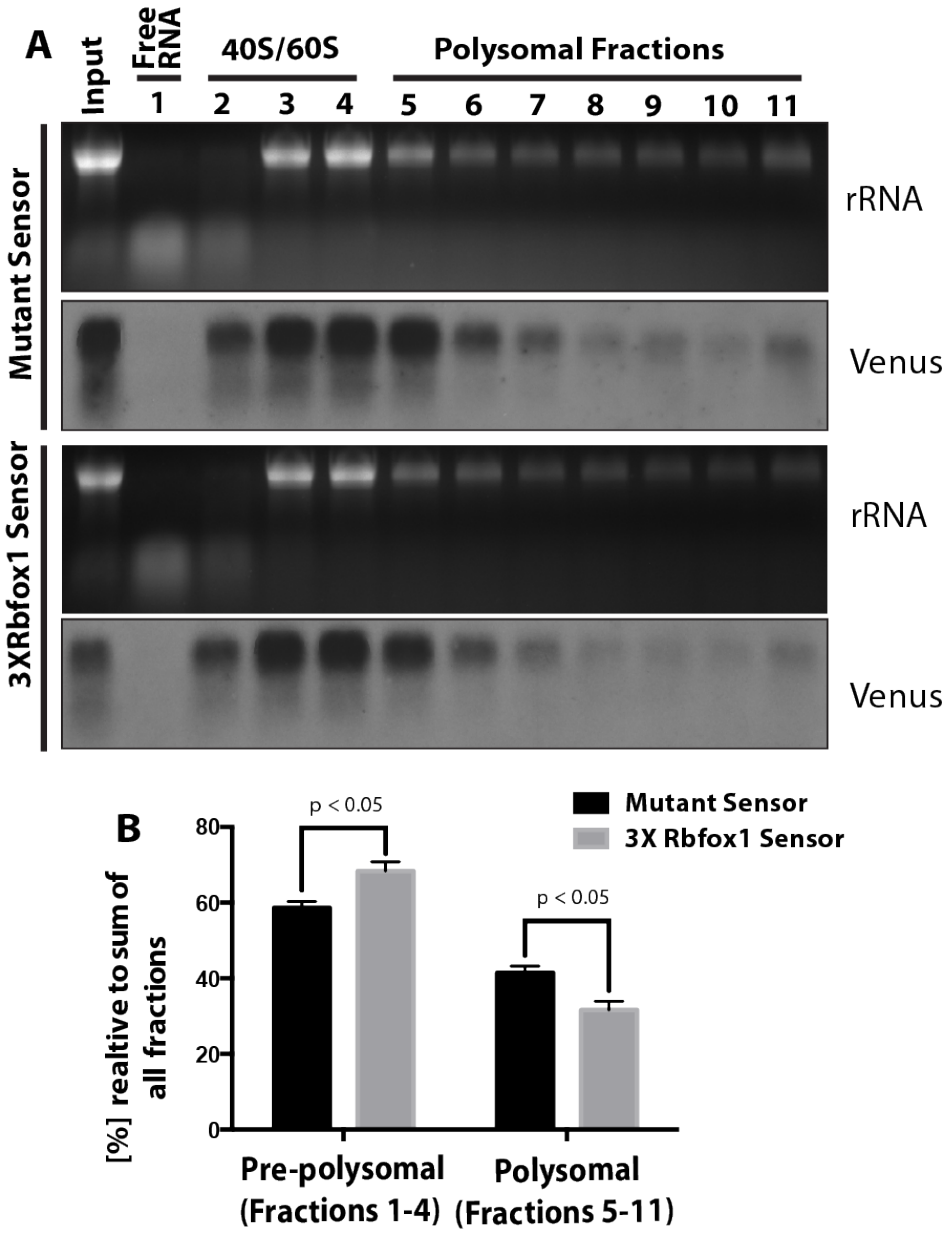


Fig 3.16 Mild enrichment of 3XRbfox1 sensor in pre-polysomal fractions. Lysates from ovaries enriched for Rbfox1 positive cysts expressing either mutant sensor (top two panels) or 3XRbfox1 sensor (bottom two panels) were fractionated on a sucrose gradient. RNA was isolated from each fraction and used for northern blot analysis using an anti sense Venus probe. Peak on rRNA levels on fraction 4 represent the monosomal fraction. The drop in rRNA on fraction 5 represents the first polysomal fraction. Amount of Venus mRNA on pre-polysomal fractions (fractions 1-4) or polysomal fractions (fractions 5-11) was quantified using band intensity. These amounts were then divided by the total of Venus mRNA counts throughout all fractions. Rbfox1 sensor is significantly lower in pre-polysomal fractions and lower on polysomal fractions as compare to mutant sensor.

Discussion

Role of cytoplasmic Rbfox in differentiation

Rbfox family members from different species localize to either the nucleus or the cytoplasm. While isoforms that localize to the nucleus play a clear role in regulating alternative splicing (Gehman et al., 2011; Hamada et al., 2013; Lee et al., 2009), the function of cytoplasmic isoforms has remained less clear. Here, we present evidence that the *Drosophila* Rbfox1 locus encodes for at least two cytoplasmic isoforms, including one previously unannotated isoform that we named *Rbfox1-RN*. Both of these isoforms are necessary for proper germline cyst development (**Fig 3.18**). These two isoforms seem partially redundant since expression of either one rescues the $Rbfox1^{403440}$ mutant phenotype. However, the Rbfox1-RF isoform rescue is more pronounced than Rbfox1-RN. This difference is not due to differences in expression levels (**Fig 3.1**). Previous work from the lab showed that different *Rbfox1* alleles disrupt expression of different isoforms (Tastan et al., 2010). A potential explanation for the difference in the degree of rescue between Rbfox1-RF and RN is that in $Rbfox1^{403440}$ mutants Rbfox1-RF levels are more affected than Rbfox1-RN. This will make $Rbfox1^{403440}$ mutants more sensitive to Rbfox1-RF expression.

Through a couple of approaches we also showed that loss of cytoplasmic Rbfox1 prevents differentiation and leads to cystic tumors within the germline. In vertebrate model organisms, Rbfox genes play crucial roles in the differentiation of a variety of cell types in development and adulthood (Bill et al., 2013; Fogel et al., 2012; Gallagher et al., 2011; Gehman et al., 2012; Gehman et al., 2011; Hu et al., 2013b). Although some of these studies suggested that Rbfox proteins might have splicing–independent functions in the cytoplasm,

the observed phenotypes have mostly been attributed solely to mis-splicing events caused by the loss of nuclear Rbfox. However, more recent studies have provided strong evidence that cytoplasmic forms of Rbfox1 play important roles in neuronal differentiation in the context of normal development and disease (Hamada et al., 2015; Hu et al., 2013b). Together with our study this argues that cytoplasmic Rbfox1 functions in the differentiation of a variety of cell types across species. In agreement with this idea, preliminary data shows that in different mammalian tissues Rbfox1 is low or absent in progenitor cells and expressed in differentiated cells (**Fig3.19-3.21**).

Novel molecular function for Rbfox1

Further experiments showed that cytoplasmic Rbfox1 regulates gene expression through a 3'UTR-dependent mechanism. *In vitro* and *in vivo* experiments presented here indicate that *Drosophila* Rbfox1 physically associates with RNAs that contain (U)GCAUG elements, similar to mammalian Rbfox proteins. Our data show that the presence of (U)GCAUG sites within mRNA 3'UTRs results in a reduction of protein expression without affecting mRNA stability. In addition, we observed that an increasing number of GCAUG sites within 3'UTRs appeared to have an additive effect on target gene expression in the context of germ cells. The presence of one site had little or no effect, while the presence of two or three sites resulted in a clear repression of protein expression in Rbfox1 expressing cells. We also show some preliminary data suggesting that Rbfox1 prevents a protein translational step between initiation and elongation in a cap-independent manner. Together, these observations suggest that *Drosophila* Rbfox1 acts to repress the translation of specific target mRNAs. This data,

however, does not rule out the possibility that Rbfox1 might act as a promoter of translation in other contexts. For example, other cis-elements present within the same 3'UTR might recruit trans-factors that block or modify Rbfox1 function. Also, different cell types or even different sub-cellular environments might contain specific factors that modulate Rbfox1 activity.

Recent studies using RNA-CLIP approaches have shown that mammalian Rbfox1, Rbfox2 and Rbfox3 all physically interact with GCAUG sites or other similar elements within 3'UTR sequences (Weyn-Vanhentenryck et al., 2014). Together with the work presented here, these studies suggest that the ability of cytoplasmic Rbfox family members to regulate protein expression has been conserved across species. The discovery of this new function has significant implications on our understanding of how Rbfox family members regulate normal development, as well as the disorders linked with disruption of Rbfox genes such as epilepsy, autism and cancer.

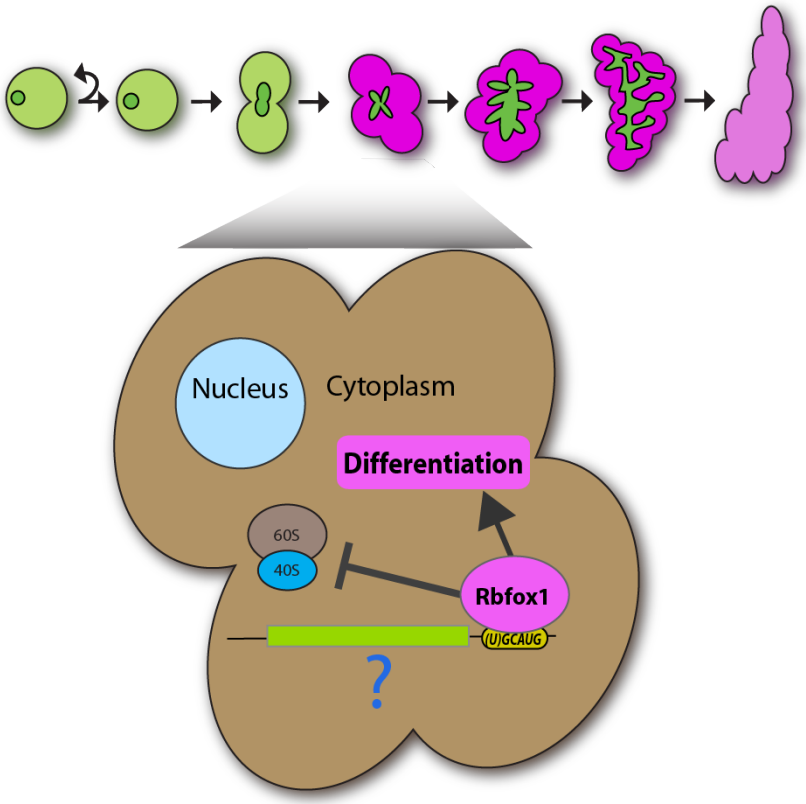


Figure 3.18 Model of Rbfox1 function during germline differentiation Rbfox1 is not present in GSC, CB and 2-cell cyst. At the 4-cell stage expression of cytoplasmic Rbfox is turned on and promotes differentiation. Here, Rbfox1 binds to UGCAUG elements within 3'UTRs to repress translation. A physiologically relevant target gene will be discussed in Chapter IV. Although data presented suggest that Rbfox1 prevents translation in a cap-independent manner, the exact mechanism by which Rbfox1 operates remains as an open question.

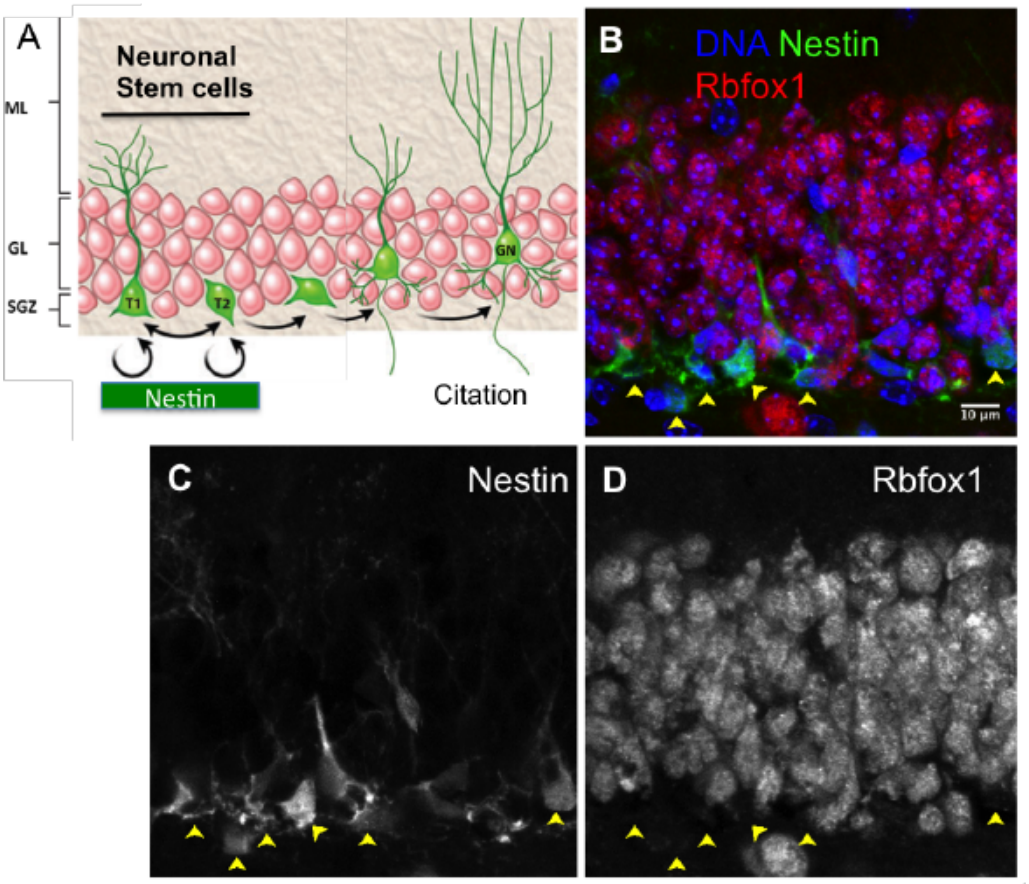


Fig 3.19 Expression of Rbfox1 in mouse neurogenic zone (A) Schematic of hippocampal subgranular zone (SGZ). Pink cells represent the granular layer where the soma of differentiated cells reside. A cell undergoing differentiation is in green. T1 stem cells give rise T2 progenitor cell through an asymmetric self-renewing division. T2 cells also undergo an asymmetric division that will give rise to a cell that enters the differentiation program to become a granular neuron (GN). T1 and T2 cells are the only cells expressing detectable levels of Nestin within this process. (B) Confocal image of SGZ slide immunostained for Rbfox1 (red) and Nestin (green). (C) Nestin staining alone. (D) Rbfox1 alone. Differentiated cells but not T1 or T2 cells (arrowheads) display Rbfox1 expression in both the nucleus and the cytoplasm.

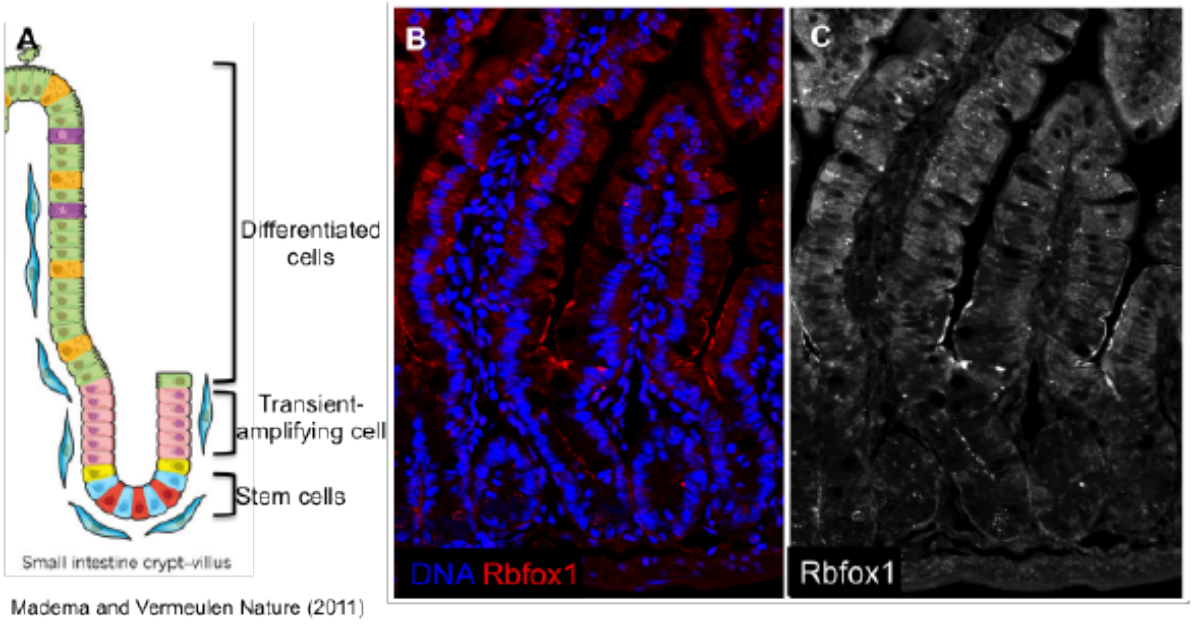


Fig 3.20 Expression of Rbfox1 in the intestinal crypt. (A) Schematic of a crypt-villus that forms epithelial lining of the small intestine. Stem cells reside at the bottom of each crypt where they divide to give rise to transient amplifying cells. Transient amplifying cells are displaced upward as they differentiate. Once cells reach the villus cells exhibit sings of terminal differentiation. (B) Mouse intestinal tissue section immunostain for Rbfox1 (red) counterstain with DAPI to label DNA (blue). Cells within the crypt express non or low levels of Rbfox1 compare to the cells in the villi which represent the terminally differentiated cells.

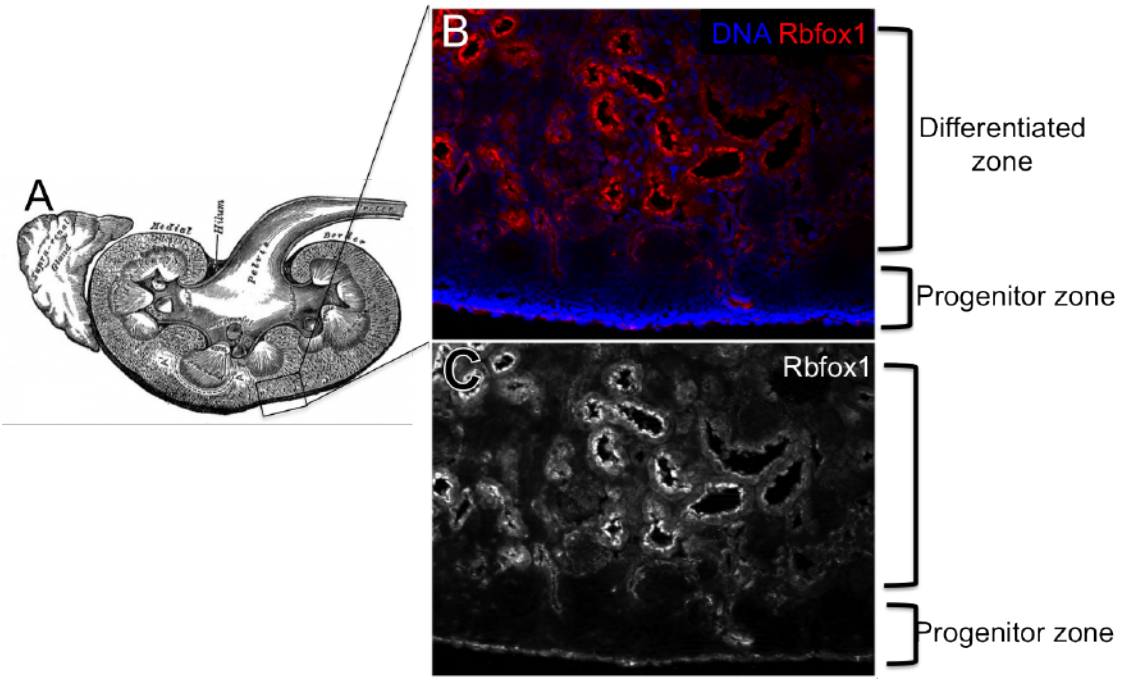


Fig 3.21 Expression of Rbfox1 in a newborn mouse kidney. (A) Drawing of a kidney to orient image on panel B. (B) Image of a P1 kidney section. Newborn mice kidney contains a pool of progenitor cells. These cells reside in a progenitor zone region close to the cortex. Differentiated cells are found further away from this region. (C) Rbfox1 is enriched in differentiated cells as compare to the progenitor cells.

CHAPTER FOUR
Repression of Pumilio protein expression by Rbfox1 promotes germ cell differentiation

Introduction

Different cell types in our body carry out distinct specialized functions. Skin keratinocytes form a protective barrier from the environment. Neurons transmit electrical signals from one cell to another. Eggs transfer genetic material to the next generation. These and all cells in our body acquire specific functions during development and throughout adulthood via a process called cellular differentiation. How this occurs is a fundamental question that needs to be answered to understand how multicellular organisms develop and function.

The Waddington epigenetic landscape is a widely used model to explain cellular differentiation. In this model, a pluripotent cell sits on the top of a hill. Down this hill, there are many paths that the cell can go through. The number of paths to choose from represents the potency and the differentiation state of the cell. As the cell goes downhill, further differentiation occurs while the potency of the cell gets restricted. Once the cell approaches the bottom of the hill, there is just one path. The cell continues this path downhill and when it reaches the lowest part of the hill final differentiation is achieved. Different genes get activated during this process. These genes commonly encode for transcription factors that bind to specific gene promoter regions to activate their transcription. Activation of these genes commits cells to specific paths. When not committed, cells turn off these transcription factors to prevent undesired differentiation. While this model applies to most studied lineages, the germline seems to follow different rules.

No transcription factor, that promotes germ cell differentiation following specification, has been identified. Instead, germ cells exploit genetic regulation at the post-transcriptional level to differentiate (Gunter and McLaughlin, 2011; Okano et al., 2005; Slaidina and Lehmann, 2014a). In *Drosophila,* the vast majority of mutants with cyst differentiation defects map to RNA-binding proteins (**Table 1.1**). For instance, the Bam-Bgcn complex and Sxl are thought to bind specific mRNAs to promote CB cyst formation (Li et al., 2009). Once the 16-cell cyst is formed, Bruno represses the expression of mitotic cyclins through their 3'UTR (Sugimura and Lilly, 2006). These studies and others have suggested that the function of many major factors involved in germline differentiation is to repress the previous genetic program at the translational level, which in turn promotes cyst differentiation. While data presented in these studies certainly support this notion, none of these studies have reported whether ectopic expression of the putative targeted gene leads to a block of differentiation at the predicted stage.

Pumilio (Pum) is an RNA-binding protein required for GSC maintenance. This protein is the *Drosophila* homolog of the Puf (PUM and FBF (fem-3 binding factor from C. elegans) family present in all eukaryotes. These proteins contain an RNA-binding domain located at the C-terminus composed of eight Puf repeats. Through this domain, Pum binds to 3'UTRs and promotes de-adenylation and/or interferes with translation to repress protein expression (Lai and King, 2013; Quenault et al., 2011; Wickens et al., 2002). Pum proteins are essential for germline maintenance across species (Miller and Olivas, 2011; Slaidina and Lehmann, 2014a; Tsuda et al., 2003; Wickens et al., 2002). *Drosophila pum* mutant ovaries exhibit a pleiotropic phenotype in the germline, including loss of GSCs. Several reports

suggest that Pum forms a complex with Nos to repress the differentiation genes Mei-P26 and Brain tumor (Brat) in GSCs, and thereby prevents premature differentiation (Harris et al., 2011; Joly et al., 2013b). In addition, Pum, together with Brat, represses expression of dMyc and Mad in the CB (Harris et al., 2011). Many of these studies and others have also observed that Pumilio is expressed early on and is turned off during cyst differentiation. How this expression of Pum is regulated and whether this down-regulation is functionally relevant remains as an open question.

In this chapter, we provide strong evidence that Rbfox1 promotes cyst differentiation, at least in part by repressing Pum protein expression through its 3'UTR. Knockdown of Pum in an *Rbfox*1 mutant background suppresses the tumorous phenotype. Moreover, ectopic expression of Pumilio results in cystic tumors that resemble the Rbfox1 mutant phenotype. We also found that cysts dedifferentiate in ovaries lacking Rbfox1 or ectopically expressing Rbfox1. All together, our results provide compelling evidence for the hypothesis that during germ cell cyst development, cells must turn off components of the previous program to progress through differentiation. When this process fails, germ cells tend to revert back to a previous state.

Results

Cytoplasmic Rbfox1 represses Pumilio expression in germ cells

In order to identify endogenous cytoplasmic Rbfox1 target genes within the germline, we compared the conservation of core GCAUG sites, with or without the variable 5' (U) residue, within mRNA 3'UTRs across different *Drosophila* and insect species. This analysis revealed

that the short 1.2 kb *pumilio* 3'UTR, which exhibits enriched ovarian expression (flybase.org), contains four GCAUG sites. Two of these are absolutely conserved across 12 and 13 species respectively (**Fig. 4.1**). *pumilio* stood out as a potentially significant Rbfox1 target mRNA: Pumilio regulates germline stem cell (GSC) maintenance by repressing the translation of specific mRNAs, and loss of *pum* results in precocious germ cell differentiation (Forbes and Lehmann, 1998; Slaidina and Lehmann, 2014a; Slaidina and Lehmann, 2014b). Next we studied the expression pattern of Pum within the germarium. We observed that a Pum protein trap (*CC00479)* is dynamically expressed during early cyst development with high levels early on followed by low to undetectable levels (**Fig 4.2A**). We also used an antibody raised against Pum and observed that, within the germline, Pum is equally expressed in GSC, CB and 2-cell cysts followed by a gradual decrease that reaches its lower level at 8-cell stage (**Fig 4.2B, D**). This pum down-regulation coincides with the up-regulation of cytoplasmic Rbfox1 (**Fig 4.2B**). Loss of cytoplasmic Rbfox1 results in increased Pum protein expression within the germline (**Fig. 4.2 C, E**). Next we compared the expression levels of *pum* mRNA and protein in ovaries carrying a synchronized population of germ cells in the presence or absence of Rbfox1. To perform this experiment we crossed Rbfox1 mutations into a *hs-bam; bam*$^{\Delta 86}$ homozygous mutant background. Loss of *bam* prevents germ cell differentiation, leading to the formation of large tumors that contain germ cells arrested in a pre-cystoblast like state. Heat-shock induction of the *bam* rescuing transgene causes all the germ cells within these tumors to undergo synchronous differentiation. By waiting a set period of time, we can isolate ovaries highly enriched for germ cells at a specific stage of development. Using this genetic background allows us to

avoid complications associated with assaying a mixed population of germ cells at different stages of differentiation. qRT-PCR analysis of samples derived using this system revealed that *pum* mRNA levels increased in the absence of Rbfox1 (**Fig. 4.3A**). However, the levels of Pum protein increased to a much greater degree in the absence of Rbfox1 (**Fig. 4.3B**), suggesting that while Rbfox1 likely promotes *pum* mRNA destabilization, it also represses *pum* mRNA translation.

To test the functional significance of increased Pum expression in *Rbfox1* mutant ovaries, we examined whether RNAi knockdown of *pum* modified the phenotype caused by RNAi knockdown of cytoplasmic *Rbfox1*. These experiments revealed that loss of *pum* strongly suppressed the germ cell tumor phenotype of $Rbfox1^{RNAi}$ ovaries, resulting in the formation of egg chambers containing nurse cells with polyploid nuclei (**Fig 4.4**). Control experiments showed that this suppression was not due to changes in Rbfox1 expression levels (**Fig. 4.5**). Through RNA IP experiments we detected a weak but consistent interaction of Rbfox1 with *pum* mRNA (**Fig. 4.6).** These data suggest that *pum* represents a functionally significant *in vivo* direct target of cytoplasmic Rbfox1-dependent gene regulation.

To determine whether the decrease in Pum expression in Rbfox1 expressing cells depends on the GCAUG elements within the *pum* 3'UTR, we constructed two reporter constructs: a wild-type *pum* 3'UTR reporter and a mutant *pum* 3'UTR reporter in which all four of the GCAUG elements were changed to ACAUA (**Fig 4.7 and Fig 4.8**). The wild-type *pum* 3'UTR reporter exhibited the same decreased expression in Rbfox1 expressing cells as Pum protein, suggesting that Pum expression is controlled, at least in part, in a 3'UTR-dependent manner (**Fig 4.7**). By contrast, the mutant reporter was expressed throughout the

early germline, and actually displayed a marked increase in many Rbfox1 expressing cysts (**Fig. 4.8**). We crossed the Pum GCAUG reporter into a Rbfox1 mutant and no longer observed the marked down-regulation. Altogether, these data strongly suggest that Pum protein expression is regulated through its 3'UTR by cytoplasmic Rbfox1.

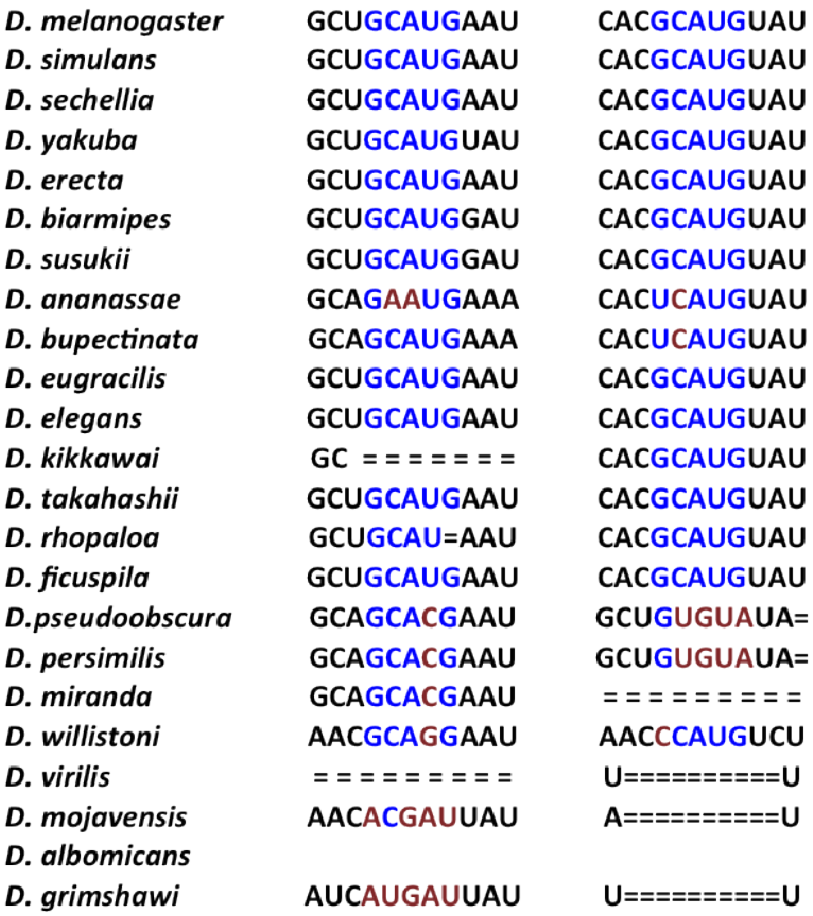


Fig 4.1 *Pum* 3'UTR contain highly conserved Rbfox1 binding sites. Schematic showing sequence conservation between GCAUG sites contained within the 3'UTR of *pum* genes in different *Drosophila* species.

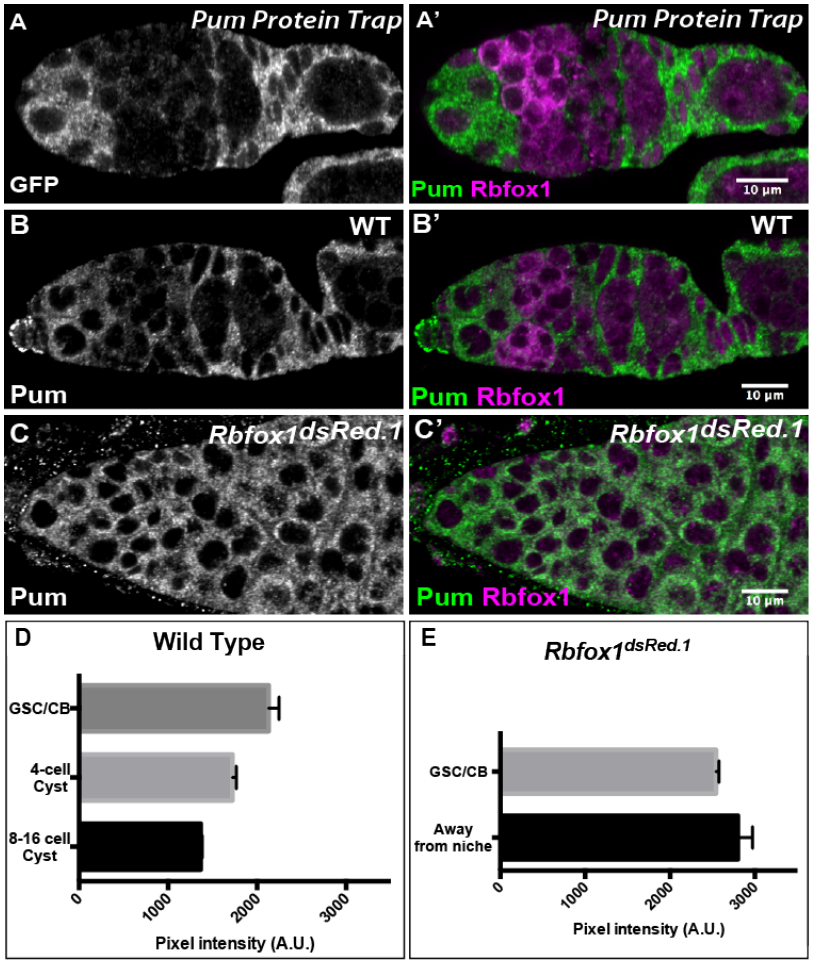
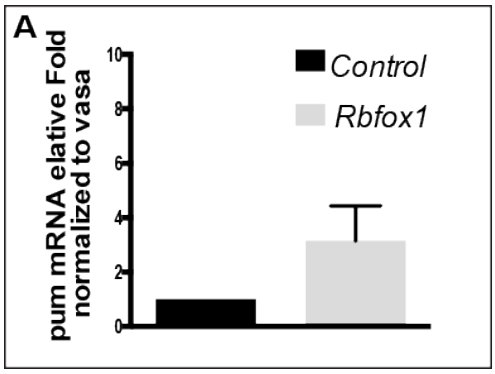


Fig 4.2 Expression of Pum in the germarium is regulated in an Rbfox1 dependent manner. (A-A') Pumilio protein trap (*CC00479*) flies stained for GFP (green) and Rbox1 (magenta). (B-C) WT (B-B') and *Rbfox1*$^{dsRed.1}$ (C-C') mutant germaria stained for Pum (green) and Rbfox1 (magneta). (D-E) Fluorescence intensity of Pum protein signal in wild type (D) and *Rbox1*$^{dsRed.1}$ (E) measured at the indicated regions.



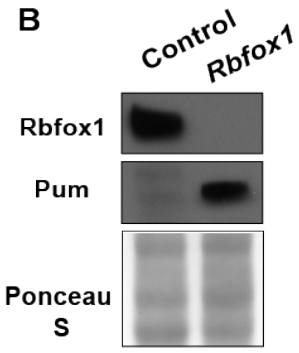


Fig 4.3 *Pum* mRNA and protein levels in *Rbfox1* mutant ovaries. (A) Quantification of *pum* mRNA levels relative to *vasa* within populations of control and *Rbfox1* mutant germ cells undergoing synchronous differentiation (p-value=0.0458). (B) Western blot showing Rbfox1 and Pum protein expression levels within populations of control and *Rbfox1* mutant germ cells as in A.

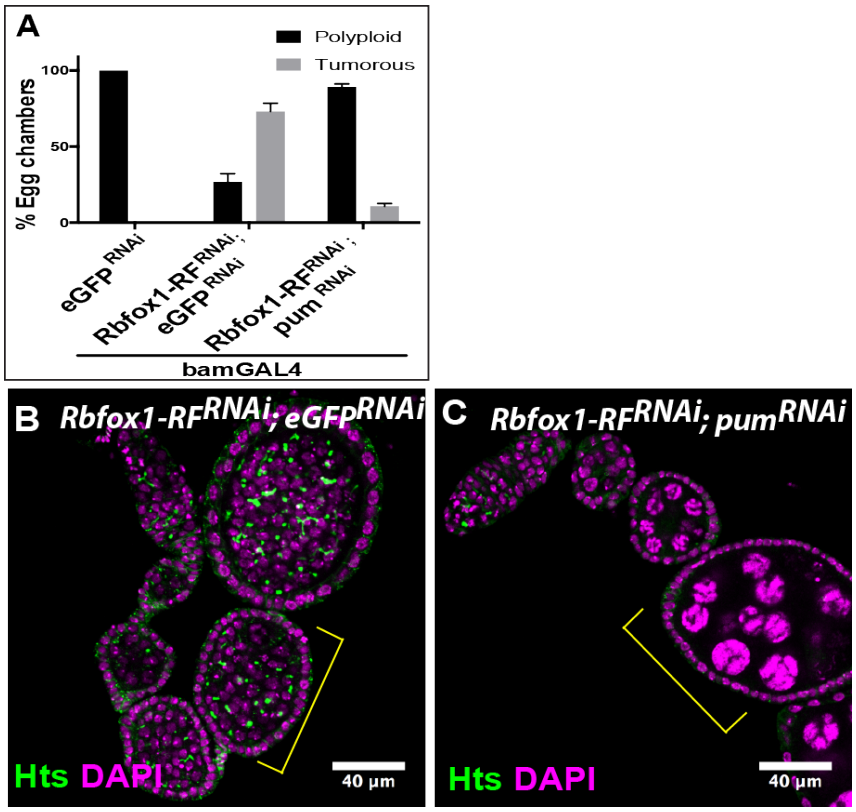


Fig 4.4 Down-regulation of Pum in Rbfox1 shRNA background promotes germline differentiation. (A) Quantification of the percentage of egg chambers that contain polyploid nurse cells or cystic tumors in the indicated RNAi backgrounds (n= >100 egg chambers). (B) *bam-gal4; UAS-Rbfox1-RF*^{RNAi}; *UAS-eGFP*^{RNAi} (*Rbfox1-RF*^{RNAi}; *eGFP*^{RNAi}) and (C) *bam-gal4; UAS-Rbfox1-RF*^{RNAi}; *UAS-pum*^{RNAi} (*Rbfox1-RF*^{RNAi}; *pum*^{RNAi}) ovarioles stained for Hts (green) and DNA (magenta).

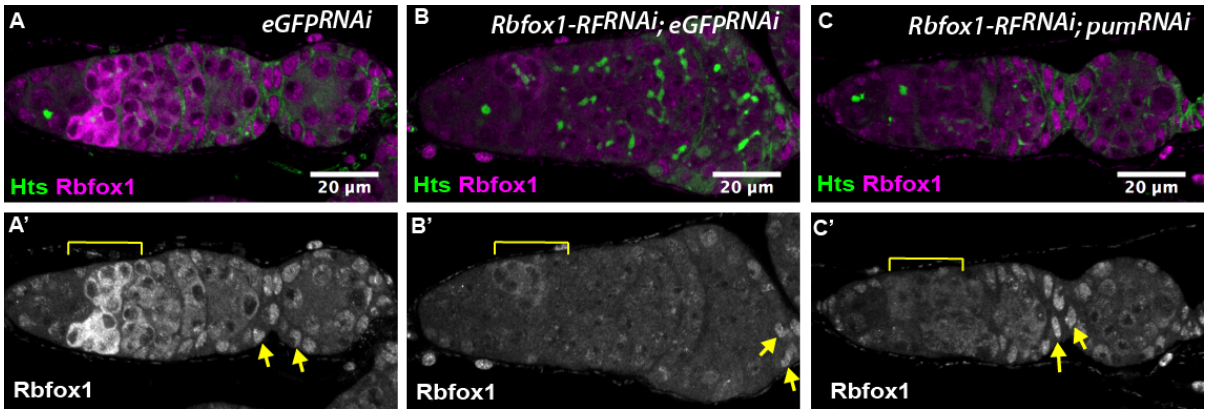


Fig 4.5 Rbfox1 levels remain low in Rbfox1-pum double knockdown. (A) *bam-gal4* driving *eGFP*^{RNAi} stained for Rbfox1 results in wild type levels of Rbfox1 within the germarium (magenta in A and white in A'). Same staining was performed with flies expressing *Rbfox1-RF*^{RNAi} (B-B') or *Rbfox1-RF*^{RNAi} and *pum*^{RNAi} (C-C'). Bracket marks region in which germline cysts usually exhibit high cytoplasmic Rbfox1 levels within the germline while arrowheads point to nuclear Rbfox1 signal in the nucleus of somatic cells.

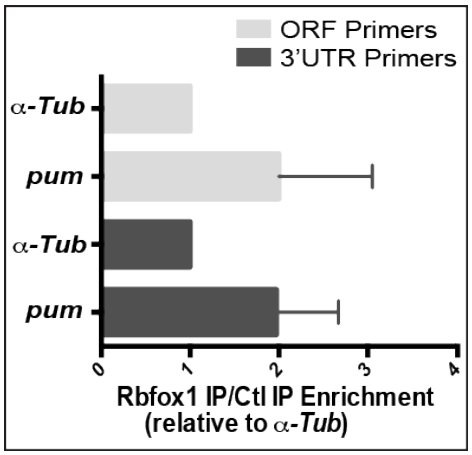


Fig 4.6 Rbfox1 interaction with *pum* mRNA. Rbfox1 IP or control IP (pre-serum) followed by qRT-PCR shows a modest enrichment for *pum* mRNA in the Rbfox1 IP. *pum* mRNA levels are shown relative to *α-Tub* mRNA. Two different sets of primers were used to amplify *pum* cDNA in the qRT-PCR; one targeting ORF and another one targeting the 3'UTR.

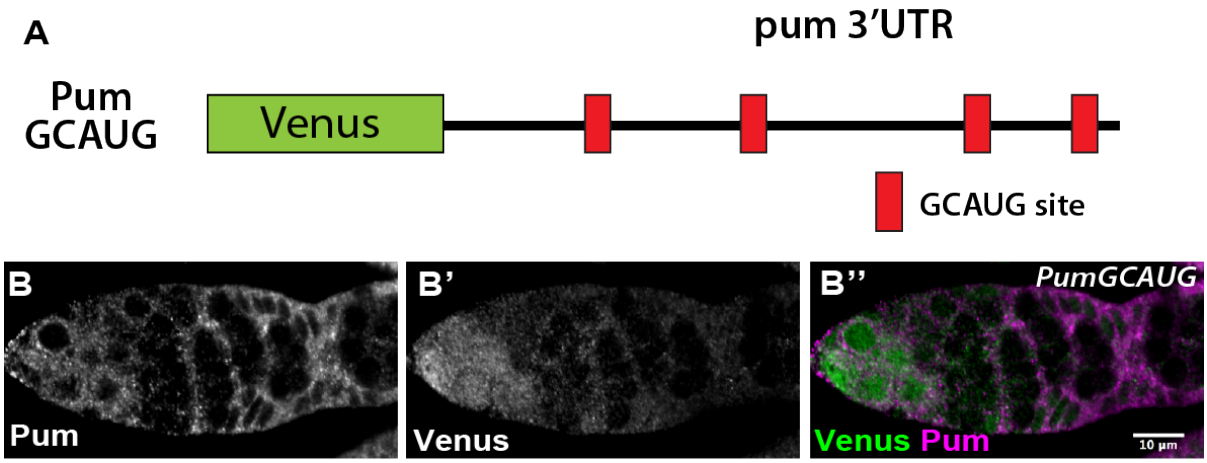


Fig 4.7 *Pum* 3'UTR reporter expression correlates with endogenous Pum expression. (A) Schematic of *pum* 3'UTR reporter. (B) Ovaries carrying the Venus-pum 3'UTR reporter (PumGCAUG) co-stained with antibodies against Venus (B' and green in B'') and Pum (B and magenta in B'').

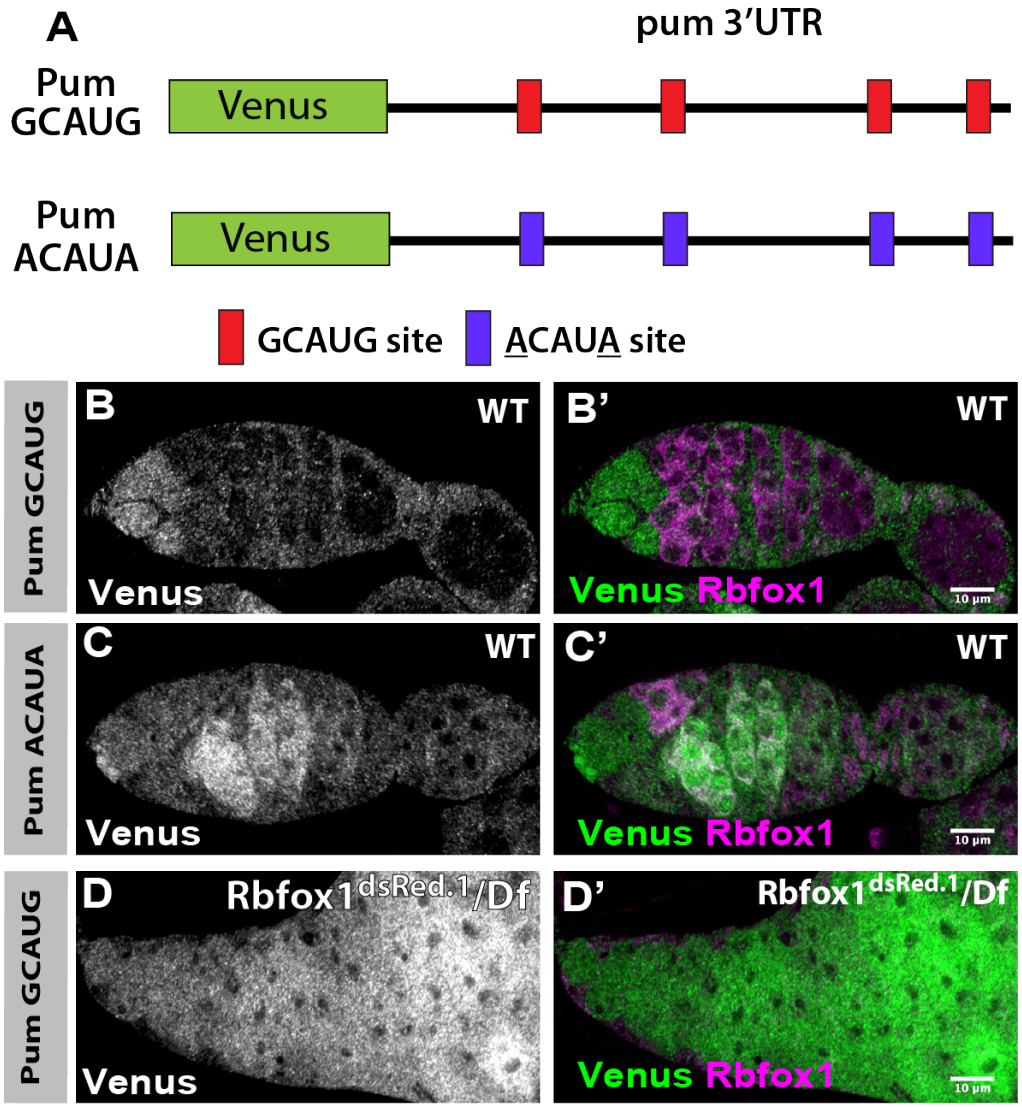


Fig 4.8 *Pum* 3'UTR responds to Rbfox1 protein levels. (A) Schematic of *pum* 3'UTR reporter. (B-C) Expression of the Pum GCAUG (B-B') and Pum ACAUA mutant *pumilio* 3'UTR reporters (C-C') (green) relative to Rbfox1 (magenta) expression in wild type backgrounds. (D-D') PumGCAUG reporter in a Rbfox1 mutant background.

Ectopic expression of Pumilio blocks germ cell differentiation

We next tested whether low or high levels of ectopic *pum* expression disrupted normal germ cell differentiation using two transgenes containing full-length *pum* coding sequence and either endogenous *pum* 3'UTR (*pum-pum*) or *α-tubulin* 3'UTR (*pum-tub*) (Menon et al.,

2004) (**Fig. 4.9**). We drove expression of these transgenes with vasa-Gal4 and examined their mRNA and protein expression levels through qRT-PCR analysis and IF respectively. We observed that, even though *pum-pum* mRNA levels were higher than *pum-tub, pum-tub* transgene led to higher protein levels than *pum-pum* (**Fig 4.9).** Germline expression of the *pum-pum* transgene resulted in a mild phenotype, whereas expression of the *pum-tub* transgene completely blocked germline cyst differentiation, resulting in a tumorous phenotype that strongly resembled the *Rbfox1* mutant phenotype (**Fig. 4.10**). These results demonstrate that mis-expression of Pum disrupts normal germ cell differentiation.

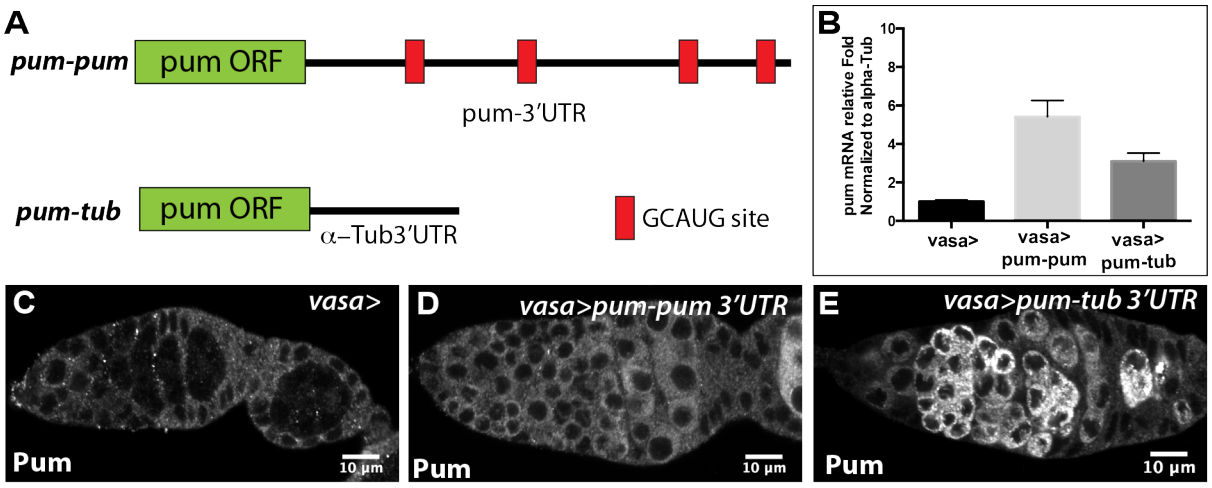


Fig 4.9 Expression of *pum-pum* and *pum-tub* transgenes in the germline. (A) Schematic of the UAS-full length *pum* transgenes that carry endogenous *pum* 3'UTR (*pum-pum*) or *α-Tubulin* 3'UTR (*pum-tub*). (B) Quantification of *pum* mRNA levels. (C-D) *vasa-gal4* (*vasa>*)(Control) (C), *vasa-gal4;UAS-pum-pum 3'UTR* (*vasa>pum-pum 3'UTR*) (D) or *vasa-gal4;UAS-pum-tub 3'UTR* (*vasa> pum-tub 3'UTR*) (E) germaria stained for Pum.

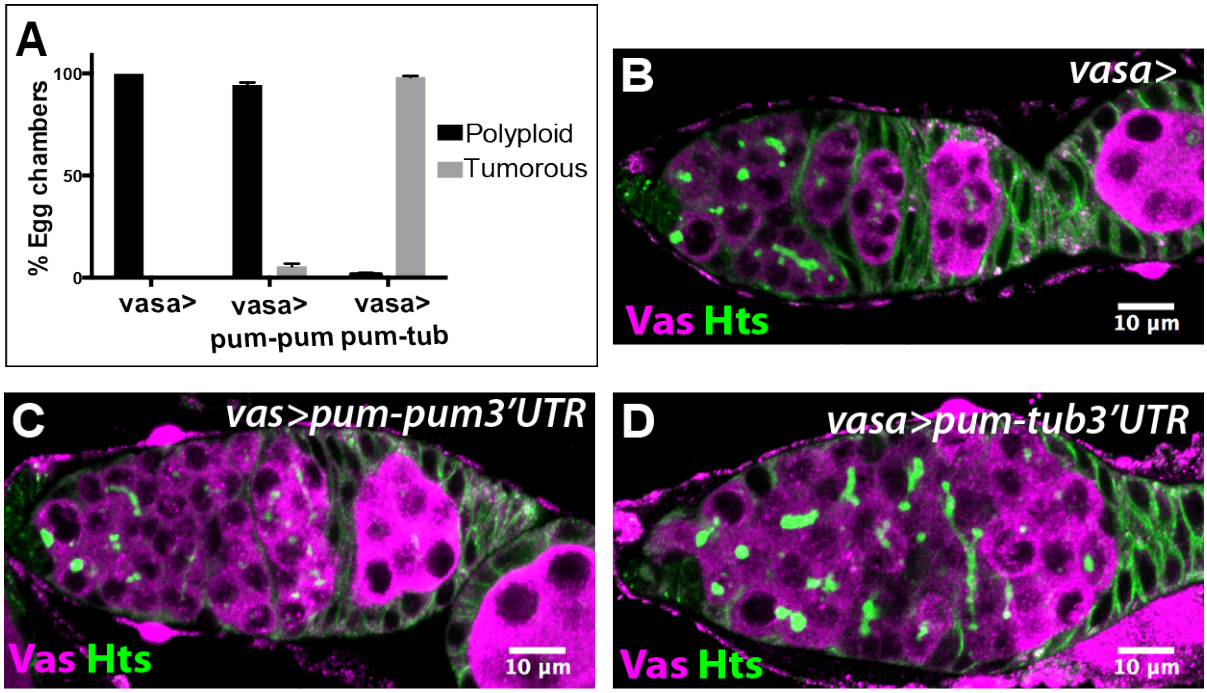


Fig 4.10 Expanded expression of Pum blocks germ cell differentiation. (A) Quantification of the percent of egg chambers with polyploid nurse cells or germ cell cystic tumors upon expression of the *pum-pum* or *pum-tub* transgenes driven by germline specific *vasa-gal4* driver (n= >100 egg chambers). (B-D) *vasa>* (control) (B), *vasa>pum-pum 3'UTR* (C) and *vasa>pum-tub 3'UTR* (D) germaria stained for Hts (green) and Vasa (Vas) (magenta).

Mis-expression of Pumilio and loss of Rbfox1 leads to dedifferentiation

Interestingly, many of the posteriorly positioned germ cells ectopically expressing Pum exhibited signs of dedifferentiation, a process previously described in both the *Drosophila* ovary and testis (Brawley and Matunis, 2004; Kai and Spradling, 2004) (**Fig. 4.11-4.12**). For example, germ cells expressing the *pum-tub 3'UTR* transgene re-acquired high levels of cytoplasmic Sxl, which typically marks GSCs, cystoblasts and 2-cell cysts (**Fig. 4.11**). These cysts also appeared to break down into single cells, as marked by changes in fusome and ring canal morphology (**Fig. 4.12**). These single dedifferentiated germ cells remained mitotically active, as reflected by phosphor-histone H3 staining, and the incidence of cell death appeared

similar in control germaria and those overexpressing *pum* (**Fig. 4.14; Fig. 4.15**). Re-examination of *Rbfox1*$^{dsRed.1}$ mutant ovaries revealed that loss of cytoplasmic Rbfox1 also resulted in breakdown and dedifferentiation of multicellular cysts (**Fig. 4.12; Fig. 4.13**). These results suggest a model whereby Rbfox1 promotes differentiation, in part, by providing a genetic barrier that prevents the inappropriate reversion of germline cysts back to an earlier developmental state (**Fig. 4.16**).

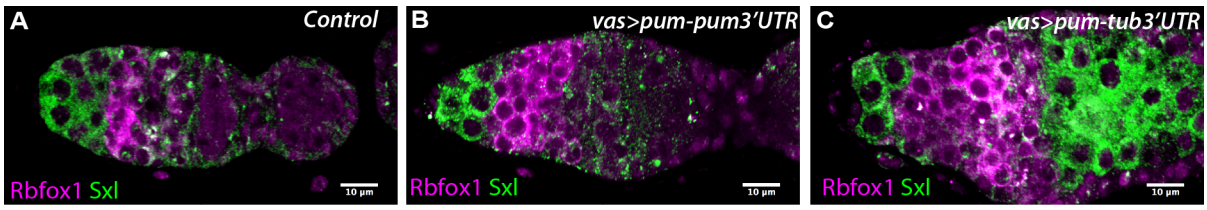


Fig 4.11 Pum miss expression leads to ectopic expression of GSC-CB marker Sxl. *vasa>* (control) (A), *vasa>pum-pum 3'UTR* (B) and *vasa>pum-tub 3'UTR* (C) germaria stained for Sxl (green) and Rbfox1 (magenta).

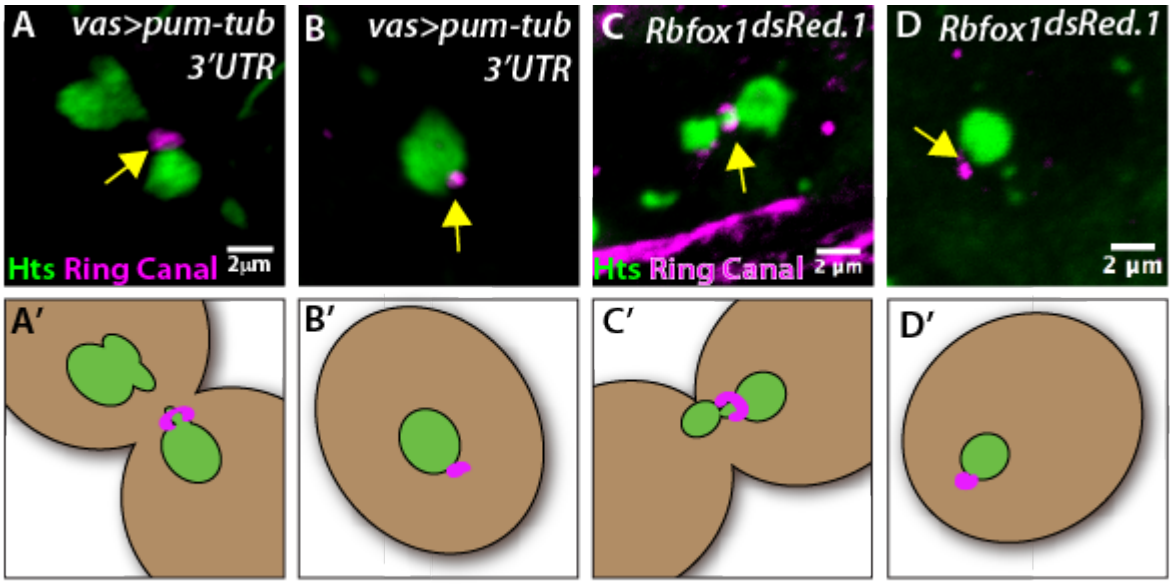


Fig 4.12 Ectopic expression Pum and loss of Rbfox1 leads to dedifferentiation. *vasa-gal4>pum-tub 3'UTR* (A-B) or *Rbfox1*$^{dsRed.1}$ (C-D) ovaries stained for Hts (green) and galactosyl (β-1,3) N-

acetylgalactosamine (magenta) (which we observe is normally enriched in ring canals) with the biotinylated lectin peanut agglutinin. Arrows point to either ring canals undergoing degradation, characteristic of cysts undergoing dedifferentiation (A-A' and C-C') or to remnants of ring canals after cyst have completed dedifferentiation to individual cells (B-B' and D-D').

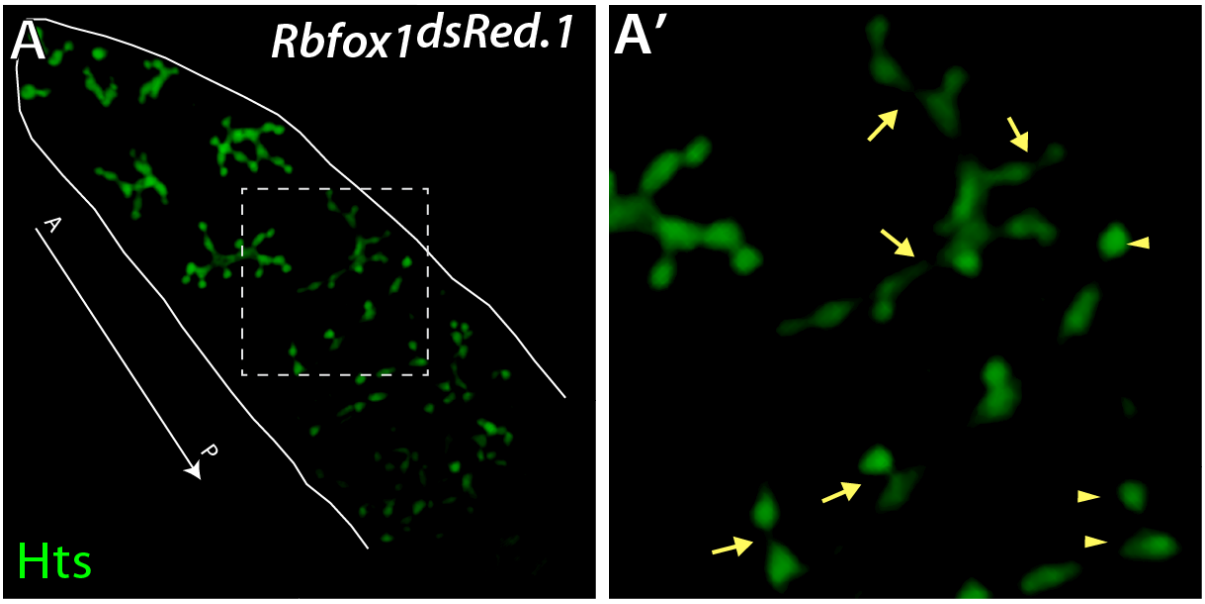


Fig 4.13 Dedifferentiating cyst in Rbfox1 mutant. A 3D reconstruction of a *Rbfox1*$^{dsRed.1}$ homozygous mutant germarium stained for Hts (green). A white arrow marks the anterior (A) to posterior (P) axis. The boxed region in (A) is magnified in (A'). Yellow arrows mark fusomes in the process of breaking down. Yellow arrowheads mark round fusomes contained within single cells.

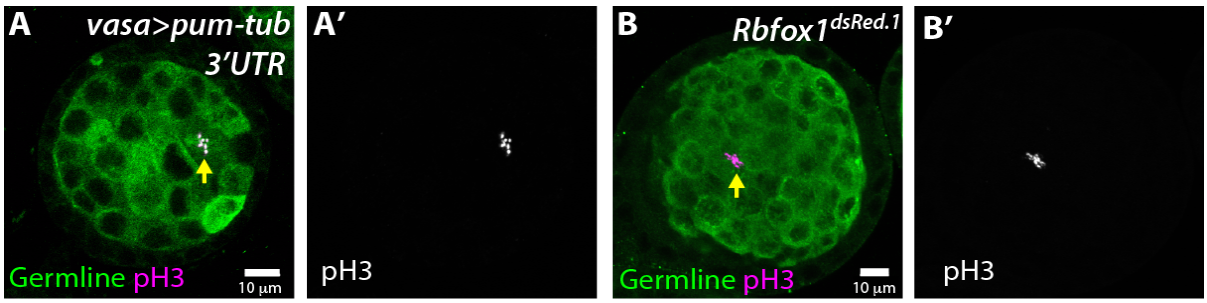


Fig 4.14 Pum overexpressing tumors and *Rbfox1* mutant tumors contain single mitotically active cells. (A-B') Single cells divide (arrows), as denoted by presence of phospho Histone 3 (pH3) (magenta), within tumorous pseudo-egg chambers of ovaries overexpressing Pum (A-A') or mutant *Rbfox1*$^{dsRed.1}$ (B-B') ovaries.

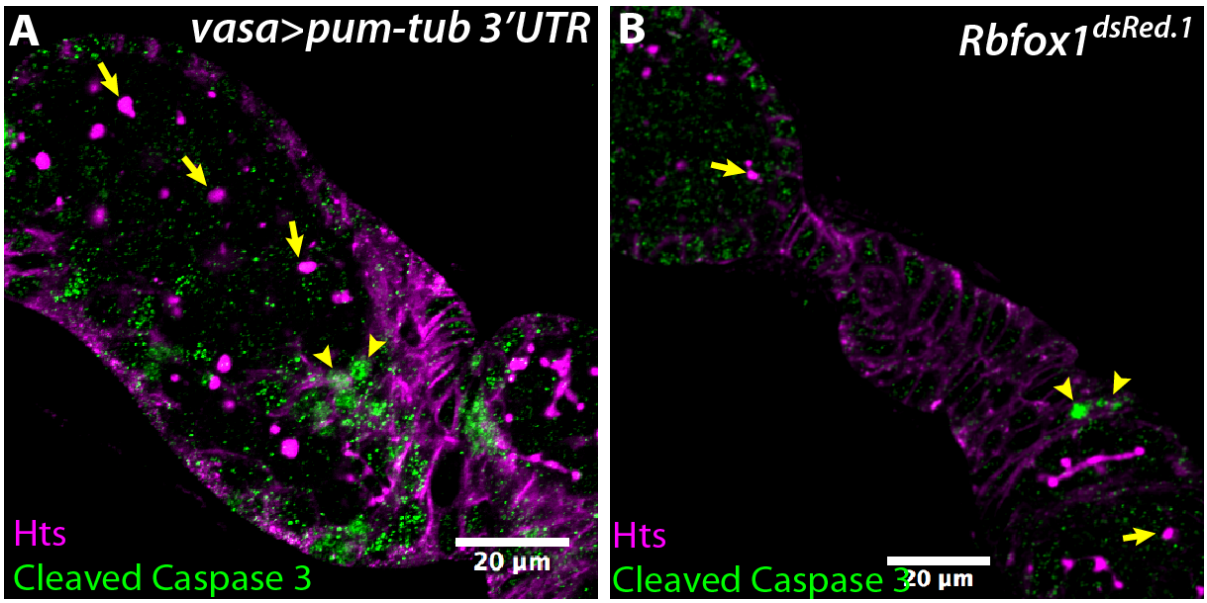


Fig 4.15 Loss of Rbfox1 mutant or overexpression of Pum does not lead to abnormal cell death. (A-B) Cell death analysis assayed with cleaved caspase 3 antibody (green) in *vasa> pum-tub 3'UTR* (A) and *Rbfox1*$^{dsRed.1}$ mutant (B) ovaries. We did not detect any abnormal amount of cell death. Arrows point to cells that presumably underwent dedifferentiation as denoted by the presence of the rounded fusome while arrowheads highlights somatic cells positive for cleaved caspase 3 as a positive control.

Discussion

Is repression of Pumilio by Rbfox1 a conserved genetic module?

Pum proteins play crucial roles in GSC maintenance, and once a germline cyst reaches the 4-cell stage, Pum expression declines. Using several approaches, we have demonstrated that Pum expression is regulated through its 3'UTR, and that Rbfox1 binding sites are essential for normal expression of Pum. There are several reasons to think that the negative regulation of Pum by Rbfox proteins is conserved across species. RBFOX1, 2 and 3 all associate with the 3'UTRs of Pum1 and Pum2 in murine neuronal tissue (Weyn-Vanhentenryck et al., 2014). Two independent studies have shown that ectopic expression of Pum mRNA diminishes dendritic growth in mouse neurons (Vessey et al., 2010), and down-regulation of cytoplasmic Rbfox1 leads to a very similar phenotype (Hamada et al., 2015) **(Fig 4.17)**. Pum proteins arose with the evolution of eukaryotic unicellular life and play an important role in the maintenance of a proliferative undifferentiated state of stem cells across metazoan organisms (Wickens et al., 2002). In contrast, Rbfox homologs are present in all metazoans and absent in eukaryotic unicellular organisms (Venables et al., 2012). Thus, we propose that repression of Pum by Rbfox1 represents an ancient genetic module, which dictates multiple processes including a proliferative, undifferentiated state versus a differentiated state in animals.

Germ cells first need to repress the previous program and can then move on

Rbfox1 mutants exhibit increased Pum protein levels, and genetic perturbation of Pumilio suppresses the Rbfox1 tumorous phenotype. In addition, we have shown that ectopic

expression of Pumilio in an otherwise wild type background blocks differentiation. Furthermore, our study strongly argues that this regulation is dependent on the pum 3'UTR, since the pum-pum transgene leads to a very mild phenotype as compared to pum-tub. This observation together with our data showing that *pum* 3'UTR is sensitive to Rbfox1 led us to propose a model, whereby Rbfox1 binds to *pum* 3'UTR to repress its translation. When cells fail to down-regulate Pum, the differentiation program is blocked (**Fig 4.16**). Although we cannot rule out the possibility that Rbfox1 might also promote translation of different targets, our study strongly suggests that repression of Pum by Rbfox1 is a critical process in germline differentiation. A similar case has been reported during the GSC to CB transition, where Bam-Bgcn complex represses Nos protein to promote cyst commitment (Li et al., 2009). Hence, we propose a model where germ cells reiteratively turn off the previous genetic program at the post-transcriptional level to progress through cyst differentiation. This significantly differs from the current model of how somatic cells differentiate. Somatic cells exploit regulation at the transcriptional level while germ cells at the post-transcriptional level. In addition, somatic cells turn on genetic programs while germ cells turn off genetic programs to progress through differentiation (**Fig. 4.18**).

Intermediate differentiation steps are unstable

Unexpectedly, we observed that ovaries ectopically expressing Pum tend to dedifferentiate. Analysis of Rbfox1 mutants also revealed that cysts revert back to single, mitotically active cells. Previous studies using genetic manipulations showed that cysts have the ability to dedifferentiate. In those experiments, GSCs were depleted before dedifferentiation was

observed. The authors suggested that the developing cysts perhaps serve as a pool to replenish the stem cells that can be lost under various types of stress (Kai and Spradling, 2004). In our experiments, GSCs are present and yet, we still observe dedifferentiation. We have also observed that this occurs in *mei-p26* mutant ovaries (data not shown). We propose that the developing 2 to 8-cell cyst exists in an "unstable" state flanked by two more stable states in time; the CB in the past and the 16-cell cyst with the specified oocyte in the future. In an unperturbed ovary, germ cells develop into 16-cell cyst. However, if the normal differentiation process is blocked, such as in the Rbfox1 mutants, germ cells tend to revert back to a CB-like state. These results may also have implications for cancers associated with Rbfox1 loss. Failure to differentiate has recently been demonstrated to occur in several types of cancers including glioblastomas where Rbfox1 levels are reduced (Hu et al., 2013b). Our study raises the possibility that these cells might revert back to a stem cell-like state, which in turn can enhance their malignancy.

Again, germ cells seem to deviate from the Waddington model, which proposes that cells move downhill in an irreversible process as they advance through differentiation. Although certain insults are able to force the cells uphill in adult tissues with regenerative capacity (Rajagopal and Stanger, 2016), it seems that for germ cells the path is not so hilly and rather flat. In germ cells, active mechanisms move cells forward; but if those mechanisms fail, the cells can easily go backwards (**Fig. 4.18**).

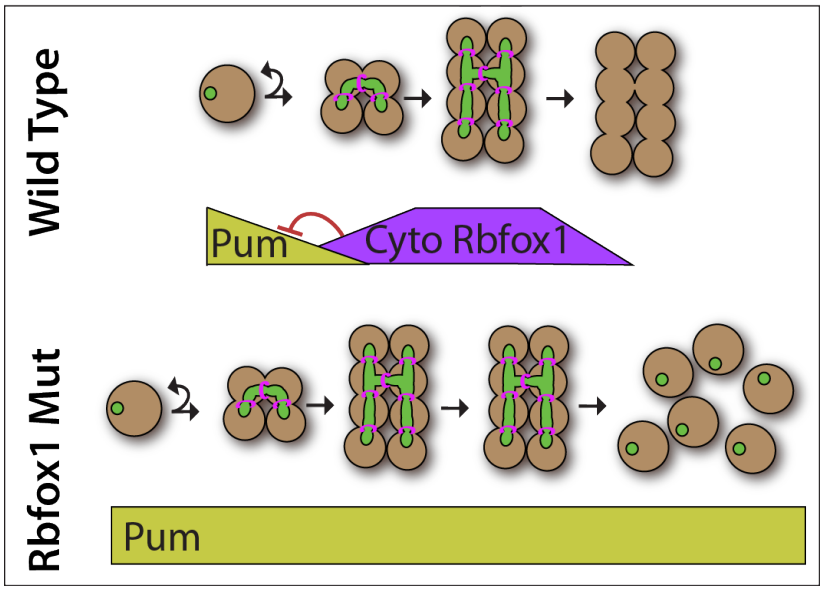


Fig 4.16 Model of how Rbfox1 promotes germline cyst differentiation. Pum is expressed in GSCs and stays on until the 2-cell cyst stage, where it promotes a proliferative undifferentiated state. In 4-cell cysts, Pum levels dramatically decrease and cytoplasmic Rbfox1 levels start to increase. During this phase, Rbfox1 directly represses Pum expression through its 3'UTR. This promotes germline differentiation. Rbfox1 levels then decrease and the differentiation program continues, marked by disappearance of branched fusomes. When cells attempt to undergo differentiation in the absence of Rbfox1 (lower scheme), Pum levels fail to decrease. In turn, these germ cell cysts never reach a terminally differentiated state and revert back to single cells.

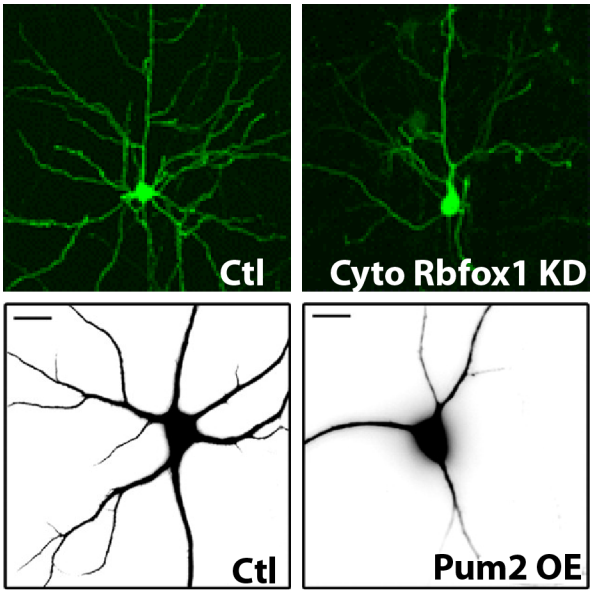


Fig 4.17 Cytoplasmic Rbfox1 knockdown or Pum overexpression phenotypes in mammalian neurons. Top panels show cortical neurons treated with control or cytoplasmic Rbfox shRNA. (Images taken from (Hamada et al., 2015)). Bottom panels show control vector or Pum2 overexpressing hippocampal neurons. (Images taken from(Vessey et al., 2010))

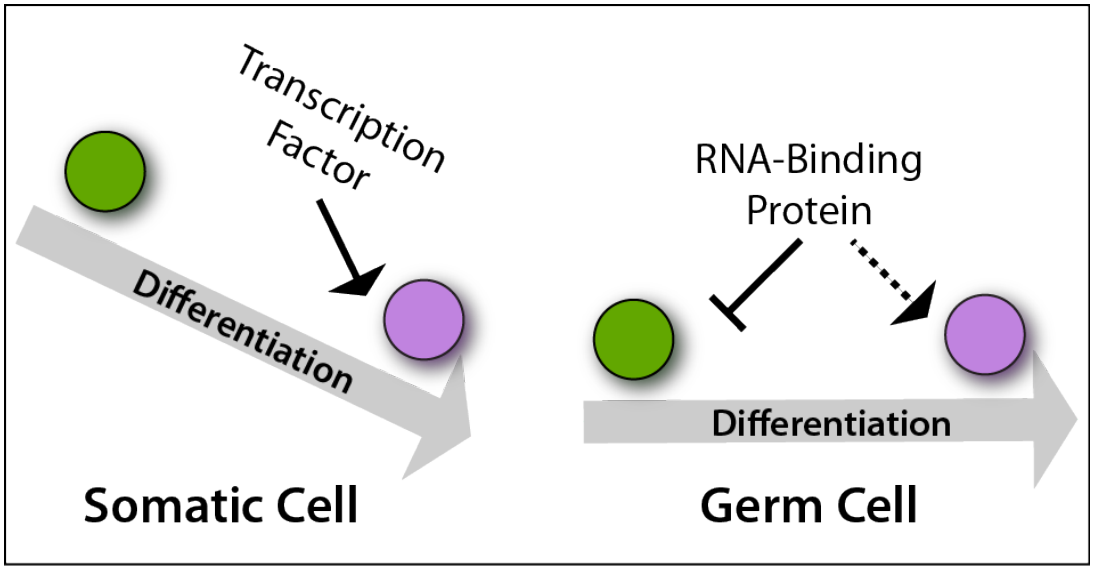


Fig 4.18 Comparison of somatic and germ cell differentiation. (Left) Schematic of a somatic cell undergoing differentiation. Normally somatic cells turn on transcription factors that will directly activate expression of genes involved in differentiation (Solid arrow). This process is irreversible and therefore depicted as a downhill process. **(**Right) Schematic of a germ cell undergoing differentiation. RNA-binding proteins directly repress expression of genes essential for the undifferentiated states at the post-transcriptional level (Solid line). This in turns promotes differentiation (Dashed arrow). This process is highly reversible and thus differentiation process is depicted as flat.

CHAPTER FIVE
Conclusions and Future Directions

Conclusions

In summary, work presented throughout my dissertation advances our understanding of how Rbfox proteins function in the context of differentiation. Our discovery of a new molecular function for Rbfox proteins significantly expands the existing knowledge on Rbfox biology. In the *Drosophila* germline cytoplasmic Rbfox1 isoforms function to prevent the translation of mRNAs that contain (U)GCAUG elements within the 3' UTR. Furthermore, we demonstrate that Rbfox1 silences *pum* mRNA which in turn promotes differentiation. Repression of this key stem cell maintenance factor by Rbfox1 protein represents a previously unknown step, critical for cyst differentiation. Together with previous studies, these results also suggest that germ cells must turn off previous differentiation programs in order to progress to the next step in differentiation. Notably, overexpression of Pum or loss of Rbfox1 causes germ cells to dedifferentiate, suggesting that germ cells can revert back to a previous differentiation state when their normal developmental path is blocked. Overall, this work advances our understanding of female germline development, cellular differentiation in general and the molecular function of Rbfox proteins. My hope is that in the future this knowledge will contribute towards the improvement of technologies to generate human oocytes *in vitro* and in the design of therapeutics to treat Rbfox-related disorders.

Future directions

Identification of additional targets may uncover new aspects of germline cyst biology

While *Pum* is a key Rbfox1 target gene, Rbfox1 most likely alters the expression of other genes during cyst differentiation. In line with this idea, bioinformatics analysis identified hundreds of potential target genes in addition to *pum*. Ideally, unbiased approaches, such as CLIP-Seq or ribosome profiling will identify genes associated with and affected by Rbfox1. Combined with genetic analysis of target genes, this data could lead to the discovery of new genes and pathways important for cyst development. In addition, these experiments will provide other information, such as additional cis-elements within the target 3'UTRs, that may lead to mechanistic insights into how Rbfox1 represses translation. Similar approaches to identify target genes of other RNA-binding proteins necessary for cyst formation will help us understand the developmental transitions that occur during cyst differentiation.

How is Rbfox1 expression regulated?

Rbfox1 exhibits a very dynamic expression pattern in the *Drosophila* germline. Given the crucial role of Rbfox1 in cyst development, understanding what regulates the expression of Rbfox1 becomes an important question. Different approaches could be taken to answer this question. Using reporter-based experiments combined with bioinformatics analysis may uncover cis-acting elements on Rbfox1 regulatory regions. This will then help to identify trans-acting factors regulating Rbfox1. A modifier screen using weak *Rbfox1* alleles could also unravel positive and negative regulators of Rbfox1. Regardless of the approach or the

answer, identifying regulators of Rbfox expression will shed light into how cyst differentiation occurs.

What are the extrinsic mechanisms regulating cyst formation?

During differentiation germline cysts are constantly wrapped by somatic cells. Disruption of the hedgehog or hippo pathway in these somatic cells leads to cystic tumors in *Drosophila* (Li et al., 2015; Narbonne-Reveau et al., 2006). The existent protocol to produce mouse oocytes *in vitro* relies on co-culturing of primordial germ cells with fetal ovarian somatic cells (Hayashi et al., 2012). These observations highlight the crucial role of somatic cells during germline cyst formation, yet the vast majority of genes known to participate in cyst development act in a germ cell-autonomous manner. An escort cell specific knockdown screen may yield new candidate factors important for proper cyst development.

Cyst development in mammals

Germ cell cyst formation represents a critical stage in oocyte production and has started to garner more attention within the research community. Recent papers have demonstrated that developing female murine germ cells indeed form functional cysts (Lei and Spradling, 2013, 2016). Nanos and Pum, first characterized in flies, also play essential roles in germline maintenance in mammals, including humans (Miller and Olivas, 2011; Tsuda et al., 2003). In addition, there are clear homologs in humans for most key genes involved in cyst formation, with the exception of *bam*. I predict that most differentiation factors important for *Drosophila* cyst formation also play important roles in this same process in mammals.

Recently, several labs have improved the ability to observe cyst formation in fetal gonads of mice by induction of genetic mosaic clones. Similar techniques combined with loss-of-function analysis will allow us to determine whether other differentiation factors important in *Drosophila* are also relevant in mammalian cyst formation. These experiments may improve our understanding of how oocytes are formed in humans and will further justify our efforts of research using simpler yet more tractable systems such as *Drosophila*.

APPENDIX A
Primers Used

Primer	Sequence	Description
Rbfox1unique exon-R	TCTCTTCAGGTTTCGGAGTTAGG	Clone Rbfox1-RF/RN
CACC_Rbfox1-F	CACCATGTATTATCCGCACATGGTGC	Clone Rbfox1-RF/RN
KpnIHA-F	GCATGGTACCATGGATCTCCACCGCGGTGG	Clone HA::Rbfox1-RF/RN into pJFRC28
XbaI-Rbfox1-R	GCATTCTAGATTAATAAAAATTATTTAATTTACACAATGTCTTGCAAAAGTGTTCAATCGACTG	Clone HA::Rbfox1-RF/RN into pJFRC28
Tub3UTR-NotI F	ATAAGAATGCGGCCGC ATAAAAGCACCGACCATCGG	SOE PCR alphaTubulin with nested RRE
Tub 3UTR-KpnI R	GTGGTACCCAAAAATATTTTATTTGTATTTAGCC	SOE PCR alphaTubulin with nested RRE
UGCAUA-Tub F	TGCATAGAGGATACACTACTGCATAGATATGCAGTGACTGCATAGATGAAGATTGTACGAGAAACCAT	SOE PCR alphaTubulin with nested RRE
UGCAUA-Tub R	TATGCAGTCACTGCATATCTATGCAGTAGTGTATCCTCTATGCAGTTTTCGTATGCTTTTCAGTGTTG	SOE PCR alphaTubulin with nested RRE
UGCAUG-Tub F	TGCATGGAGGATACACTACTGCATGGATATGCAGTGACTGCATGGATGAAGATTGTACGAGAAACCAT	SOE PCR alphaTubulin with nested RRE
UGCAUG-Tub R	CATGCAGTCACTGCATATCCATGCAGTAGTGTATCCTCCATGCAGTTTTCGTATGCTTTTCAGTGTTG	SOE PCR alphaTubulin with nested RRE
BamHI-VasP-F	gcatGGATCCatcgttgatggcctccttgacg	generation of sensors
Tub5'UTR-VasP-R	CGATTTCGAGGTCTGACTCAGAGAGCGTATACGTTCGAACTGCCGGCTATGAGGCTTGACAAACGTAAAACGAATATGAgtggaatttccattgtgctatcgc	generation of sensors
Tub5'UTR-Venus-F	CCTCGAAATCGTAGCTCTACACAATTCTGTGAATTTTCCTTGTCGCGTGTGAAACACTTCCAATAAAAACTCAATATGGTGAGCAAGGGCGAGG	generation of sensors
KpnI-Tub3'UTR-R	GCATGGTACCAAAGAAAAACAGTGGGGTTTTCTTATTTCTG	generation of sensors
Top_Oligo_R	ctagcagtAGACATTGTGTAAATTAAATAtagt	inserted fragment into

bfox1-RF_shRNA-1	tatattcaagcataTATTTAATTTACACAATGTCTgcg	Valium22
Bot_Oligo_Rbfox1-RF_shRNA-1	aattcgcAGACATTGTGTAAATTAAATAtatgcttgaatataactaTATTTAATTTACACAATGTCTactg	inserted fragment into Valium22
Top_Oligo_Rbfox1-RF_shRNA-2	ctagcagtGAAGAGATCCGACAGTCGATTagttatattcaagcataAATCGACTGTCGGATCTCTTCgcg	inserted fragment into Valium22
Bot_Oligo_Rbfox1-RF_shRNA-2	aattcgcGAAGAGATCCGACAGTCGATTatgcttgaatataactaAATCGACTGTCGGATCTCTTCactg	inserted fragment into Valium22
Rbfox1-Zeo_F	tggcgaaagaatgagtggagagcgtggcaggcaggcgagggaagtggcgTGTTGACAATTAATCATCGGCATAG	[FF>AA] RRM and right homology arm Zeo cassette
F158A,F160A_Rbfox1-Zeo_R	CGTGCTCGTTCTGCATCGTTGCTGTTAGCGAATGTTACAGCACCGGCTCCctgccattcgcaatcaaacatgcaattaaagaaacatatagagtatatagTCAGTCCTGCTCCTCGGC	[FF>AA] left homology arm Zeo cassette
F158A,F160A_Ctl_ Rbfox1-Zeo_R	CGTGCTCGTTCTGCATCGTTGCTGTTAGCGAATGTTACAAAACCGAATCCctgccattcgcaatcaaacatgcaattaaagaaacatatagagtatatagTCAGTCCTGCTCCTCGGC	Control Dead RRM left homology arm Zeo cassette
5'Targ RF-Sense	CTTCGaacaataattgtgtgtaatt	To create guide RNA
5'Targ RF-AntiSense	AAACaattacacacaattattgttC	To create guide RNA
3'Targ RF-Sense	CTTCGaatcataacataaagccaac	To create guide RNA
3'Targ RF-AntiSense	AAACgttggctttatgttatgattC	To create guide RNA
RF-LeftHA-F	gcatCACCTGCtgacTCGCcacactactccactgtttgcac	To generate Donor plasmid
RFleft-HA-R	gcatCACCTGCtgacCTACtacacacaattattgttaaaaagtttcaaaaagaag	To generate Donor plasmid
RF-RightHA-F	gcatGCTCTTCgTATAACTGGAATCATCAAACACCATTTAAC	To generate Donor plasmid
RF-RightHA-R	gcatGCTCTTCgGACGCTGCATTGTTACTTTCCCTTAC	To generate Donor plasmid
PCRCheck1 Rbfox1 del_F	CTCTTCTTTTTGAAACTTTTTAACAATAATTGTGTG	Molecular Characterization Rbfox1Del1
PCRCheck1 Rbfox1 de_R	CTCAATGGCGGCATTTGAAA	Molecular Characterization Rbfox1Del1
PCRCheck2	caccGACTACACCATCGTGGAGCAG	Molecular Characterization

Rbfox1 del_F		Rbfox1Del1
PCRCheck2 Rbfox1 del_R	gagcaattcgaattgagtcgtgag	Molecular Characterization Rbfox1Del1
UGCAUG-F_T7	taatacgactcactatagggGATCTGCATGGATCTGCATGGATATGCATGACGTAATAAAGCAT	to generate template for DIG probe generation used in RNA-RMSA
UGCAUG-R_T7	ATGCTTTATTACGTCATGCATATCCATGCAGATCCATGCAGATCccctatagtgagtcgtatta	to generate template for DIG probe generation used in RNA-RMSA
UGCAUA-F_T7	taatacgactcactatagggGATCTGCATAGATCTGCATAGATATGCATAACGTAATAAAGCAT	to generate template for DIG probe generation used in RNA-RMSA
UGCAUA-R_T7	ATGCTTTATTACGTTATGCATATCTATGCAGATCTATGCAGATCccctatagtgagtcgtatta	to generate template for DIG probe generation used in RNA-RMSA
Venus F1 (PAT)	CACCGGTCCACGTGAGC	For PAT assay
Venus R1 (PAT)	CTTGTGTACACAACTTATCGCCGAG	For PAT assay
pum RT-F2	GCCTGATGACCGATGTCTTT	pum RT-qPCR
pum RT-R2	CAGTTCGTGGACGATTTCCT	pum RT-qPCR
1RRE	GCATGCGGCCGCGCGTCACGCCACTTCAACGCTCGATGGGAGCGTCATTGGTGGGCGGGGTAACCGTCGAAATCAGTGTTTACGCTTCCAATCGCAACAAAAAATTCACTGCAACACTGAAAAGCATACGAAAACTGCATaGAGGATACACTACTGCATGGATATGCAGTGACTGCATaGATGAAGATTGTACGAGAAACCATAAAGTATTTTATCCACAAAGACACGTATAGCAGAAAAGCCAAGTTAACTCGGCGATAAGTTGTGTACACAAGAATAAAATCGGCCAGATTCAGTGTTGTCAGAAATAAGAAAACCCCACTGTTTTTCTTTGGTACCAC	Generation of 1X Rbfox1 sensor
2RRE	GCATGCGGCCGCGCGTCACGCCACTTCAACGCTCGATGGGAGCGTCATTGGTGGGCGGGGTAACCGTCGAAATCAGTGTTTACGCTTCCAATCGCAACAAAAAATTCACTGCAACACTGAAAAGCATACGAAAACTGCATaGAGGATACACTACTGCATGGATATGCAGTGACTGCATGGATGAAGATTGTACGAGAAACCATAAAGTATTTTATCCACAAAGACACGTATAGCA	Generation of 2X Rbfox1 sensor

	GAAAAGCCAAGTTAACTCGGCGATAAGTT GTGTACACAAGAATAAAATCGGCCAGATT CAGTGTTGTCAGAAATAAGAAAACCCCAC TGTTTTTCTTTGGTACCAC	
PumWTFrag1	caccGCATGCGGCCGCAGGAAATAACAAAT TAAGCCAGGCAGTCAAAGGAAACTTCCTT CTCGAATCGCAGTATAGTTTTTAGAAGCTG TAGAGCTTAACATAAACAACAAGTACATAT AAATGTAATCTTATTTATTGGAAAAGCAGC GATAAATGGAGCTGCACTCGAAGATTTGC AAAGAGGATAGTAAAACACACATGCGCCA ATCTAGAGAAACAAATAGCAAACAAAGAA GCACACTGGCAAGCAAAAAAGCAAAAGA GCTTAACAGCTAAAACTAAAAGAAATTTGT ATTTTTACGAACAAAACTAATAACGTTCTC ATGAAAAAAGATTTCAAAATATTTGTAAAA TGCGCTCGCATAATTAATTTGTAAAAAAAA GGCATGAACCGCAAAGATGAAAGAAAAC AAAAATGCGTAGTAAATCGCGATCAAGAA AAAATAATG	Generation of PUM GCAUG sensor
PumWT Frag2	CGATCAAGAAAAAATAATGAATGTAATGT AAAATGTCAATGAAACAGATTTGTCTGCGT ACATTTTCGTTGTAACTTTGTATAAATTAAT TATTATATAGCAAGTCTATCTGTAAATGAT TAATGTTTCGACTGTAAATTAATAAGAAGA CAACTGAAGAGCCGGCGAGCTGAAAAAA AAGAAAGTAAAAAGAGCGGGCTGCATGA ATTAGCCTACGATTTATAAGTTCAGACAGA GGAACCATTTCTAATATACAAACATATATA CGAGGGATAACAGCAGAAGCCGCACTTAG TGTAGAATGTAGAGTAATAATGTTTTTGGA GCCAGCAGCTACAAAGACACAATGAAAAC AGAGACACACGAGACACGCCCACGCCCCC TCACGCACACTCGGTTGCATACACCCACAC AATGAACGACTCTTCAGCCCATTCACGTTG CTTTTGCACTATGTAAAAATTTTGTATAAA AAAAAACCCCAAACAACAAACCATGTAAA CCATGTAATTTTCAAATGTTTCACTGTAAA ATGTATACATACTTTATTTTGTAAATTTTTT TTAAGTCGCAAGTAACTCATACATATTCTA TTCTAAACCTCACGCATGTATTTATAATTTT	Generation of PUM GCAUG sensor

	ATACACATTAGCTGGTGACCACCGATCGAC GATCTGCATGGATATTGGTCAGCTGGTGG CCAGCTAAAAGAACCTGTTAGCCAAGTAA GCCAAAAATGATAATAATTGGATTTTAAAA CAATAACCATCAAAATAAACCAATTTTTTTC GGTACCACGATAGCAGCTAGC	
PumMutFrag 1	caccGCATGCGGCCGCAGGAAATAACAAAT TAAGCCAGGCAGTCAAAGGAAACTTCCTT CTCGAATCGCAGTATAGTTTTTAGAAGCTG TAGAGCTTAACATAAACAACAAGTACATAT AAATGTAATCTTATTTATTGGAAAAGCAGC GATAAATGGAGCTGCACTCGAAGATTTGC AAAGAGGATAGTAAAACACACATGCGCCA ATCTAGAGAAACAAATAGCAAACAAAGAA GCACACTGGCAAGCAAAAAAGCAAAAGA GCTTAACAGCTAAAACTAAAAGAAATTTGT ATTTTTACGAACAAAACTAATAACGTTCTC ATGAAAAAAGATTTCAAAATATTTGTAAAA TGCGCTCGCATAATTAATTTGTAAAAAAAA GaCATaAACCGCAAAGATGAAAGAAAACA AAAATGCGTAGTAAATCGCGATCAAGAAA AAATAATG	Generation of PUM ACAUA sensor
PumMut Frag2	CGATCAAGAAAAAATAATGAATGTAATGT AAAATGTCAATGAAACAGATTTGTCTGCGT ACATTTTCGTTGTAACTTTGTATAAATTAAT TATTATATAGCAAGTCTATCTGTAAATGAT TAATGTTTCGACTGTAAATTAATAAGAAGA CAACTGAAGAGCCGGCGAGCTGAAAAAA AAGAAAGTAAAAAGAGCGGGCTaCATaAA TTAGCCTACGATTTATAAGTTCAGACAGAG GAACCATTTCTAATATACAAACATATATAC GAGGGATAACAGCAGAAGCCGCACTTAGT GTAGAATGTAGAGTAATAATGTTTTTGGA GCCAGCAGCTACAAAGACACAATGAAAAC AGAGACACACGAGACACGCCCACGCCCCC TCACGCACACTCGGTTGCATACACCCACAC AATGAACGACTCTTCAGCCCATTCACGTTG CTTTTGCACTATGTAAAAATTTTGTATAAA AAAAAACCCCAAACAACAAACCATGTAAA CCATGTAATTTTCAAATGTTTCACTGTAAA ATGTATACATACTTTATTTTGTAAATTTTTT	Generation of PUM ACAUA sensor

TTAAGTCGCAAGTAACTCATACATATTCTA
TTCTAAACCTCACaCATaTATTTATAATTTT
ATACACATTAGCTGGTGACCACCGATCGAC
GATCTaCATaGATATTGGTCAGCTGGTGGC
CAGCTAAAAGAACCTGTTAGCCAAGTAAG
CCAAAAATGATAATAATTGGATTTTAAAAC
AATAACCATCAAAATAAACCAATTTTTTTC
GGTACCACGATAGCAGCTAGC

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