Long non-coding RNA regulation of spermatogenesis via endo/lysosome activity and cytoskeletal elements in *Drosophila*

Mark J Bouska [1,*], Hua Bai [1,*]

[1]Department of Genetics, Developmental, and Cellular Biology, Iowa State University, Ames, Iowa 50011, USA

The Allied Genetics Conference 2020

ABSTRACT

The spectrin cytoskeleton has been shown to be critical in diverse processes such as axon development and degeneration, myoblast fusion, and spermatogenesis. Spectrin can be modulated in a tissue specific manner through junctional protein complexes, however, it has not been shown that lncRNAs interact with and regulate spectrin during varied processes. Here we provide evidence of a lncRNA that interacts with α and β Spectrin to regulate spermatogenesis and endosomal related activity in fat bodies of *Drosophila*. Protein-RNA and Protein-Protein biochemical analysis indicated the interaction between α and β Spectrin is modulated by the lncRNA CR45362. Immunocytochemistry revealed CR45362 is highly expressed in the basal testis while α and β Spectrin are clearly disrupted in this same region of CR45362 mutants. We genetically demonstrate α Spectrin and CR45362 deficiencies cause spermatid bundling defects with congruous reduction of cortical lipid droplet size and Lysotracker staining in the fat body. Our data suggests lncRNA regulation of spectrin could provide cells with a repertoire of modulatory molecules to manipulate cell type specific cytoskeletal and endosomal requirements.

Discussion and Future Work

This work provides unique data indicating that the spectrin structures in the testes and fat bodies can be organized by lncRNA. This creates several new questions that provide interesting opportunities for future work. First, is spectrin regulated by lncRNAs during mammalian spermatogenesis, and what RNA motifs regulate their interaction? Does the spectrin cytoskeleton have a diversity of tissue specific regulatory lncRNAs, and how do these lncRNAs influence endocytic processes in those tissues? With humans having many testis specific lncRNAs, spectrin related lncRNAs could be viable targets for contraceptive development or infertility treatment, particularly with the advent of successful Antisense Oligonucleotides by Ionis Pharmaceuticals and other promising paths in the oligonucleotide field.

1. The lncRNA CR45362 has positive effect on endo/lysosomal activity and fertility.

(A) Diagram of CR45362 CRISPR deletion, SNP's identified from our lysosomal GWAS, verification primers (p473 & 474), and gRNAs. (Image: SnapGene 4.3.4)

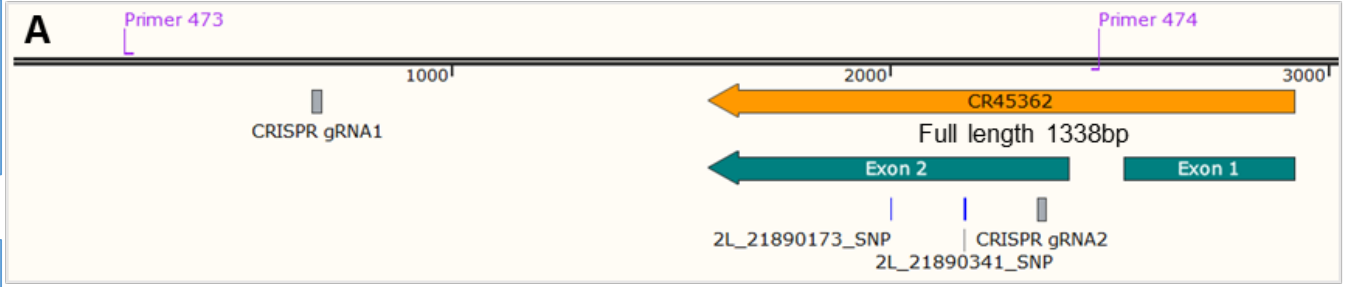
(B) DNA gel using primers flanking deletion target segment. WT has band at 2182 demonstrating the primer targeted segment has no deletion, heterozygous CR45362 KO (+/-) has the 2182bp band but also the expected 508bp band for the deletion on the homologous chromosome, and KO has only the 508bp band indicating successful deletion in the CR45362 gene. The 508bp band was excised for sequencing and alignment.

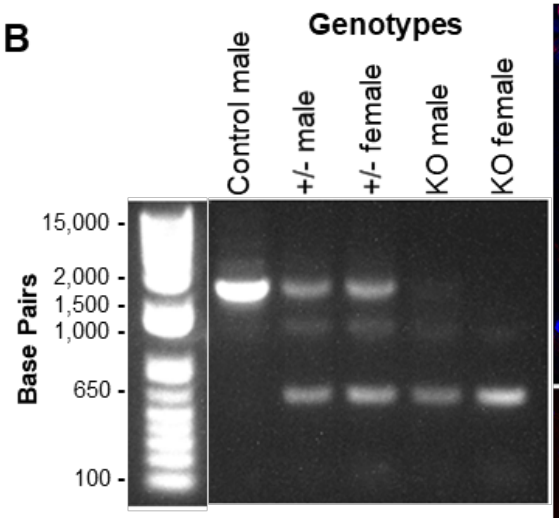
(C) Lysotracker staining of WT and CR45362KO fed and fasted fat bodies indicates reduced endo/lysosome activity in CR45362KO.

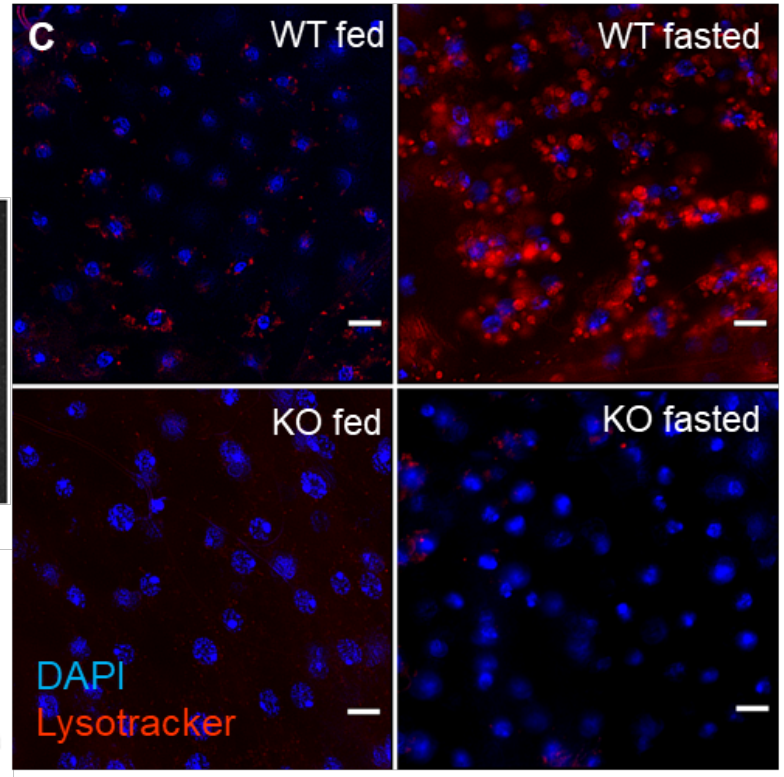
(D) Knockout of the lncRNA CR45362 results in lowered Lysotracker staining under fed and fasted conditions. One replicate consists of the average of three circular areas of 100µm per fly fat body measured by Cell Sens software for percentage of measured area containing fluorescence. From left to right n=7,12,6,7,6,8 Fed/fasting interaction calculated using Two-way Anova column factor (** p = 0.0034) , WT vs KO fed two tailed unpaired t-test (*** p = 0.001) mean+/-s.e.m

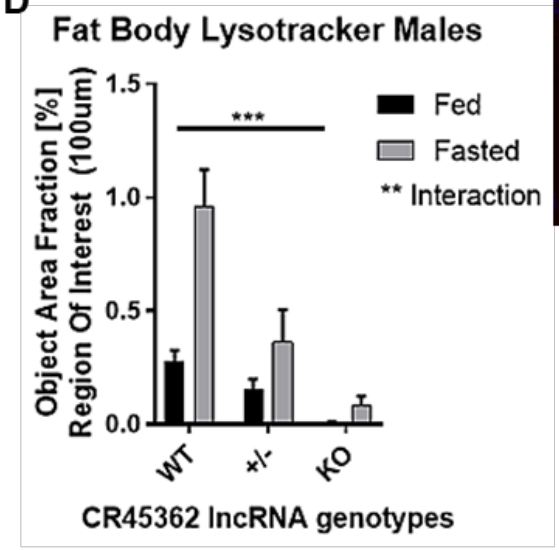
(E) Fertility was tested by crossing individual CR45362KO male flies with KO, heterozygotes for the KO allele, or WT virgin females, but these crosses were unable to produce offspring. However, KO and heterozygous females produced adult progeny and were fully fertile indicating a male homozygous KO defect.

*All scale bars represent 10 µm *For all figures: WT = (w1118), +/- = Heterozygous, KO = CR45362 mutant.









E

Fertility Assay

Cross Genotypes Female	X Male	Crosses producing offspring/ Attempts
KO	KO	0/4
WT	KO	0/13
+/-	KO	0/27
+/-	WT	3/3
KO	WT	2/2
+/-	+/-	5/5
WT	+/-	2/3
KO	+/-	6/6

Figure 2. The lncRNA CR45362 localizes to endosomes and cell cytoplasm in the terminal epithelial region of testis

(A) Fluorescent in-situ hybridization (FISH) probes for the lncRNA CR45362 localize to the basal testis of WT flies.
(B) Enlarged inset from Figure 2A reveals probes localize to the cytoplasm of somatic cells and a bundle of elongated spermatids can be seen infiltrating these same somatic cells.
(C) Basal testis in KO for same region as Figure 2A shows no probes, and indicates successful knockout.
(D) CR45362 probes co-localized (white arrows) with antibodies against the early endosome marker Hrs but were less often with lysosome marker arl8.
(E) (Probes did not colocalize with autophagy marker GABARAP but were occasionally near this marker (yellow arrows) (F).

*All scale bars represent 10 µm.

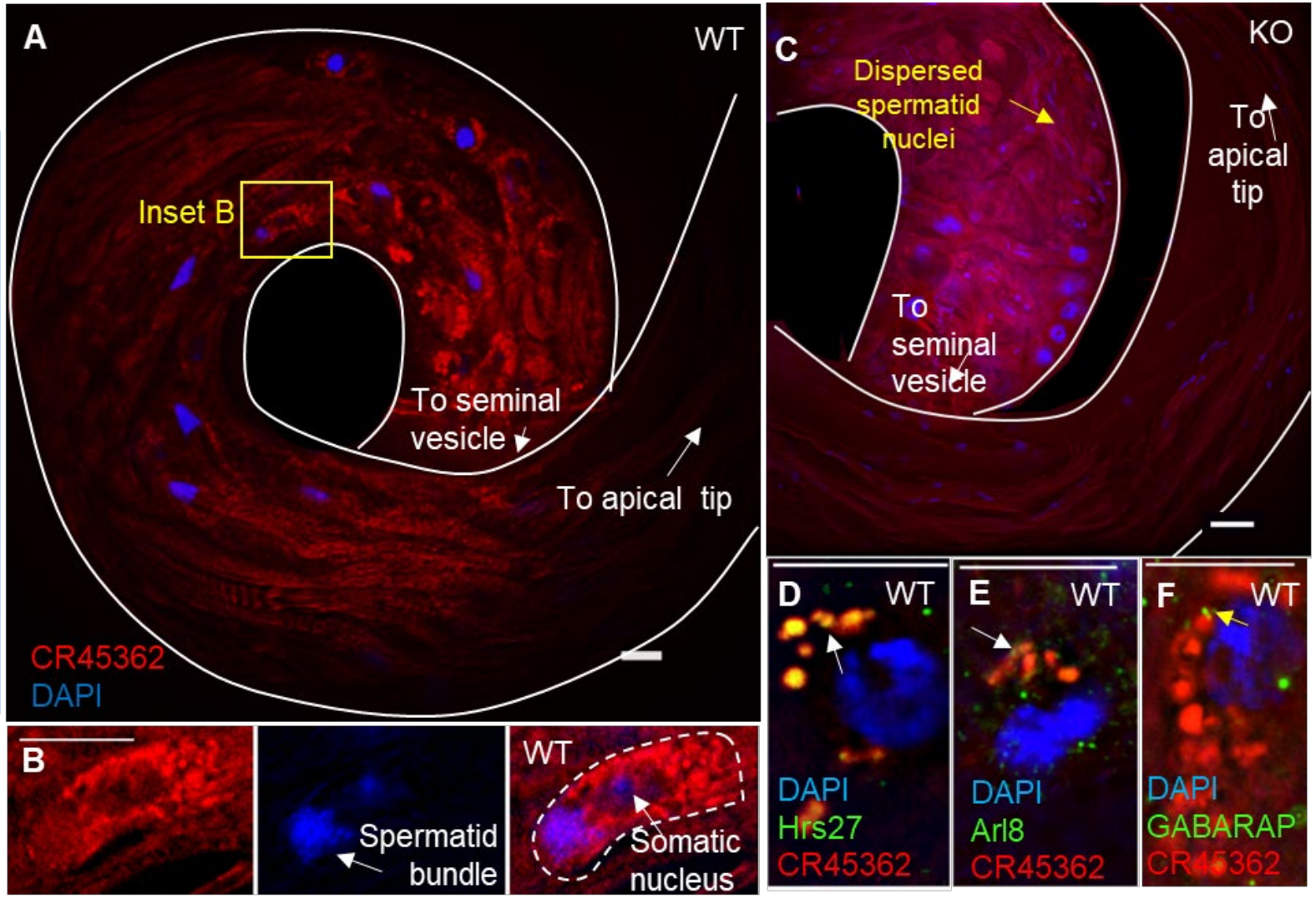


Figure 3. Spermatid bundling process impaired in CR45362KO lines

(A) Spermatid stage nuclei (DAPI) are bundled in WT but dispersed in KO. Phalloidin staining for actin cones reveals cones form and are bound by Tm1 in WT and KO spermatids. Indicating the defect occurs independent of individualization, and spermatids are dispersed before actin cone formation.
(B) Spermatids (indicated by dj-GFP) fail to enter the seminal vesicle (SV) in KO males. (WT testis were manually uncoiled for imaging)
*All scale bars represent 10 µm.

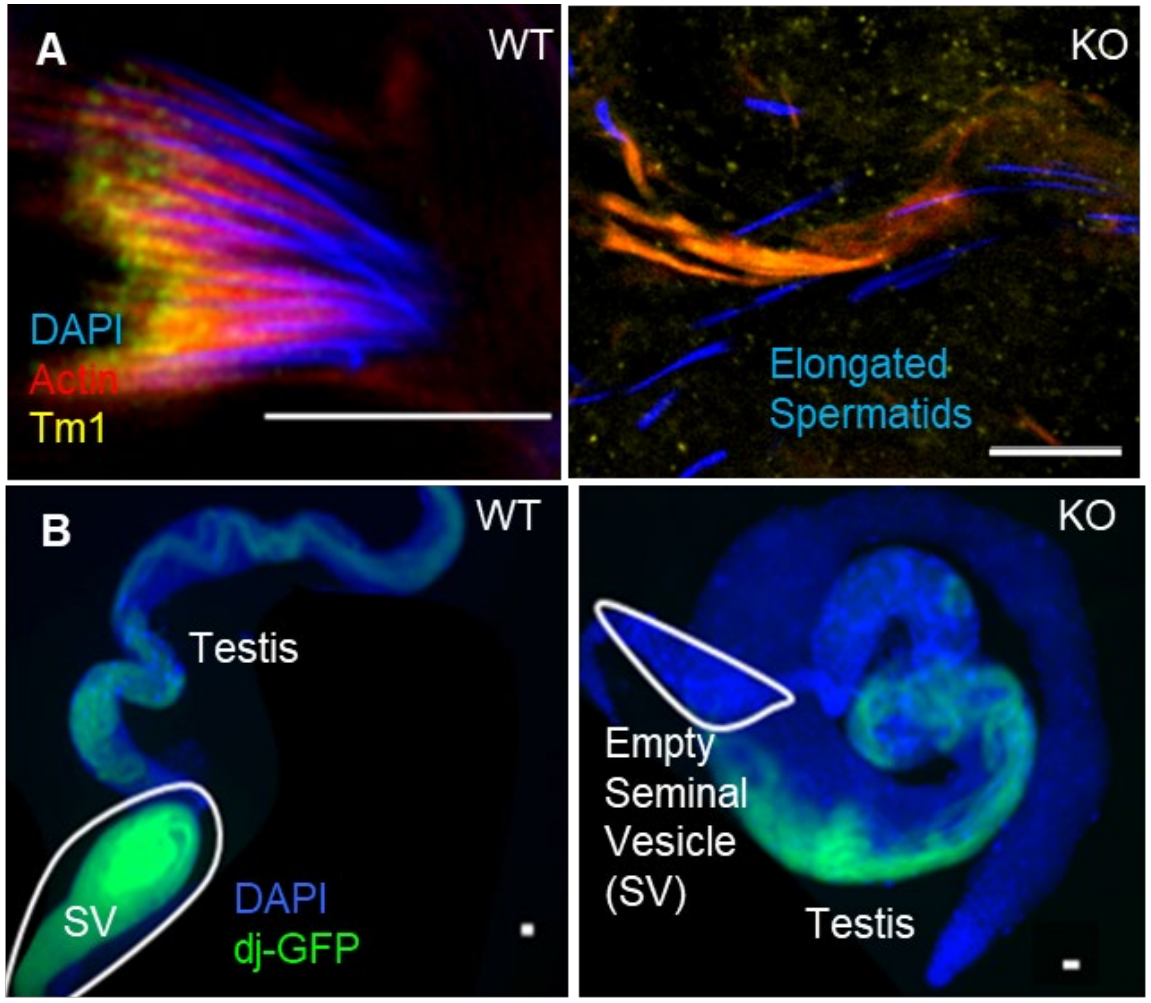


Figure 4. The lncRNA CR45362 biochemically and Genetically interacts with cytoskeletal and endosomal related proteins

(A) Molecular Function Gene Ontology for top CR45362 RNA-protein binding assay “Chromatin Isolation by RNA Precipitation- Mass Spectrometry” (ChIRP-MS) indicates cytoskeletal proteins bind CR45362.

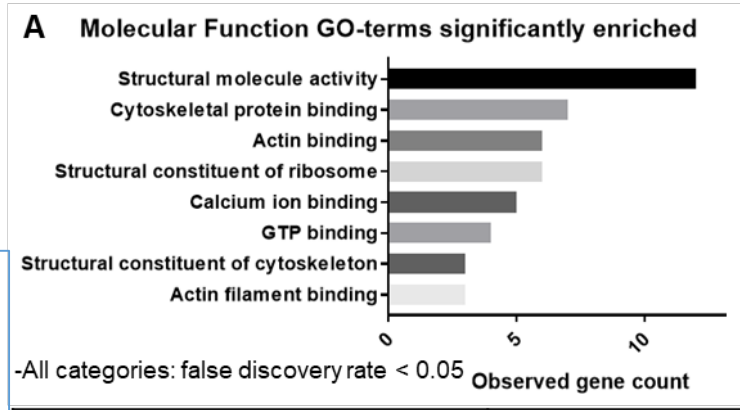
(B) Driver test indicates Ptc-Gal4 x UAS-GFP expresses GFP in the same region of the basal testis as is indicated by CR45362 FISH (see Fig. 2A).

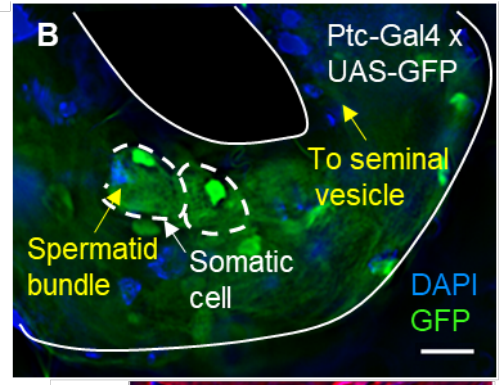
(C) RNAi screen of top ChIRP-MS candidate proteins. Five pairs of testis from 3-5 day old males from each UAS-Gal4 cross were dissected of which we scored 3. If defects were seen, we repeated this screening for a second round of crosses. If both testis of a pair had the same phenotype, they were scored according to the following criteria: normal bundling, one or more scattered nuclear bundles, no nuclear bundles present, one or more individualization complexes (actin) scattered, no actin cones present, or testis deformed.

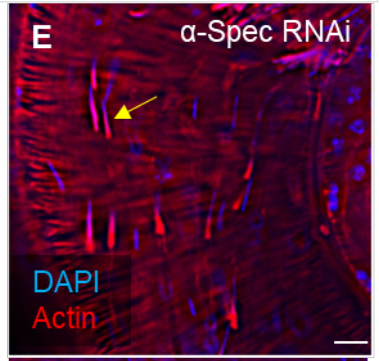
(D) Results from ChIRP-MS. Columns are first listed by unique MS-hits and total hits in KO control samples. The following columns are ordered by the tiled probes use to pull down the RNA-protein complex in WT samples. Pools of every other probe (even or odd numbered along the length of the lncRNA) or all probes were used. The “All probes” trial had increased diversity of probes causing numbers of protein species to increase, which in MS, results in fewer hits detected per protein species.

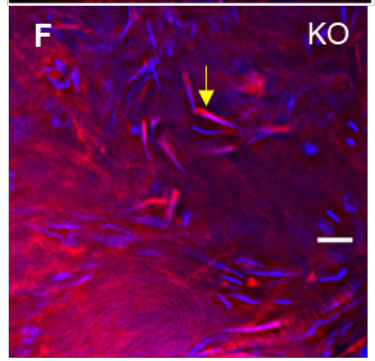
(E) Ptc-Gal4 x α-spec RNAi line has dispersed spermatid nuclei (yellow arrows) and actin cone (indicated by phalloidin staining) phenotypes seen in CR45362KO (F).

*All scale bars represent 10 µm.









Normal bundling
Bundling defects in 1 round of screening
One or more scattered nuclear bundle
No nuclear bundles present
One or more actin complexes scattered
No Actin cones present

C Genetic RNAi screen of ChIRP candidate proteins		RNAi Screen Dispersion Phenotypes		D ChIRP-MS hits							
Ptc-Gal4 x UAS Lines	BL Stock ID# 2017	Nuclei Bundles	Actin Bundles	Unique hits KO	Total hits KO	Unique Even probes	Total Even probes	Unique Odd probes	Total Odd probes	All probes Unique	All probes Total
Act88F-GFP	9253	Control	Control								
alpha-Spec	56932			1	1	25	25	5	5	2	2
Prm RNAi	31549			0	0	24	29	11	11	13	14
Mfe2 RNAi	51854			0	0	19	19	7	7	3	3
CT9894 RNAi	55962			0	0	16	17	4	4	5	5
beta-Spec RNAi	42802			0	0	16	16	4	4	3	3
Tudor-SN RNAi	34865			0	0	16	16	3	3	7	7
Tm1 RNAi	77376			2	3	15	29	7	20	3	5
chc RNAi	34742			0	0	15	15	3	3	5	6
Aldh-III RNAi	51820			0	0	12	14	2	3	6	7
Obp44a RNAi	28794			1	1	10	34	4	4	2	2
Dip-B RNAi	54045			0	0	8	8	5	5	3	3
Mitofilin RNAi	63994			0	0	8	8	4	4	1	3
betaTub97EF	64858			0	0	7	7	4	4	1	1
Rab1 RNAi	34670			0	0	7	13	2	3	2	3
RpL14 RNAi	80419			0	0	6	7	3	3	3	3
Got1 RNAi	43194			0	0	6	8	3	3	3	3
Mp20 RNAi	34963			0	0	5	5	4	4	4	6
Act88F RNAi	60347			0	0	5	76	3	22	3	21

Figure 5. Alpha & Beta spectrin disrupted in basal testis in CR45362 mutant

(A,C) Under immunostaining α-spectrin localizes to the exterior of the somatic cells in the basal testis, while Beta Heavy spectrin accumulates in the somatic cell at the location where the spermatid bundles are anchored.

(B,D) Alpha spectrin is dispersed throughout somatic cells in KO with enhanced cyst nuclear membrane localization.

(E) Beta spectrin in basal testis of KO has dispersion pattern similar to α-spectrin.

(F) Inset of enlarged somatic cells show dispersion of Beta spectrin in KO vs WT (G).

(H) Western blots for α-spectrin antibody verification. First 2 columns are WT replicates, columns 3 and 4 are from an α-spectrin fused GFP line. Two α-spectrin antibodies were tested (rows) and have bands at the same MW as the α-GFP antibody. The image is of same location after stripping the membrane between each separate antibody incubation. ~Size estimates based on 250kDa standards band and 278 kDa MW of α-Spectrin.

(I) Co-immunoprecipitation for α-spectrin indicates the ratio of α-spectrin to β-spectrin increases in WT testis. n=2 Unpaired t-test (p = 0.1678, ns = not significant)
*All scale bars represent 10 µm.

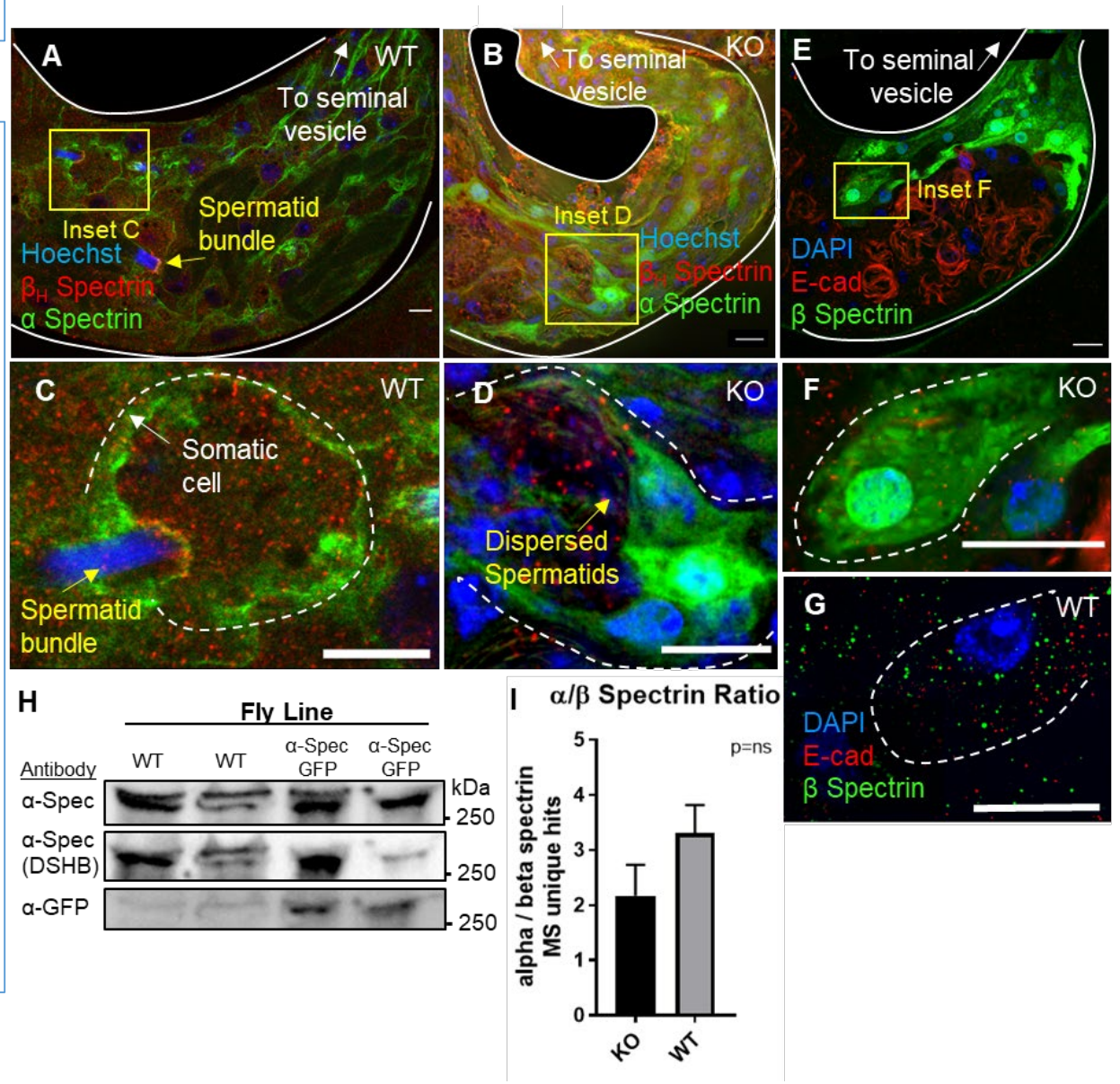


Figure 6. Alpha spectrin and CR45362 regulate endo/lysosomes and lipid droplets in fat bodies

(A) Lysotracker assay comparing 5 day old fat bodies in 2 KO lines (KO1, and KO backcrossed into YwR control line = KO_YwR), fat body specific α-spectrin RNAi, and 2 WT lines (w1118 & YwR).

(B) Cortical lipid droplet numbers in 2 WT and 2 KO lines indicate that cortical lipid droplets are more numerous and reduced in size in the L3 larval fat body of mutants. One replicate consist of the average of three circular areas of 100µm per fly fat body measured by Cell Sens software for percentage of measured area containing fluorescence for Lysotracker or manually counting for droplets. Two-tailed unpaired t-test (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant) mean+/-s.e.m

(C) (Fat body light microscopy images from the larval wandering stage reveal a consistent pattern of smaller cortical lipid droplets. *All scale bars represent 10 µm.