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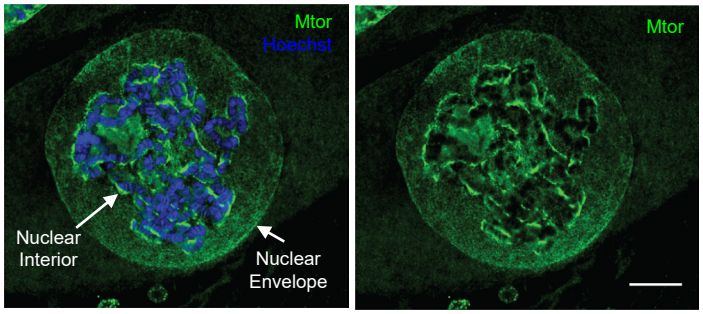
Supplemental information

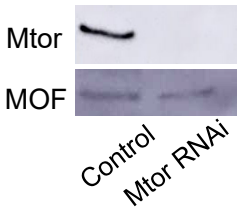
Correct dosage of X chromosome transcription is controlled by a nuclear pore component

Jennifer R. Aleman, Terra M. Kuhn, Pau Pascual-Garcia, Janko Gospocic, Yemin Lan, Roberto Bonasio, Shawn C. Little, and Maya Capelson

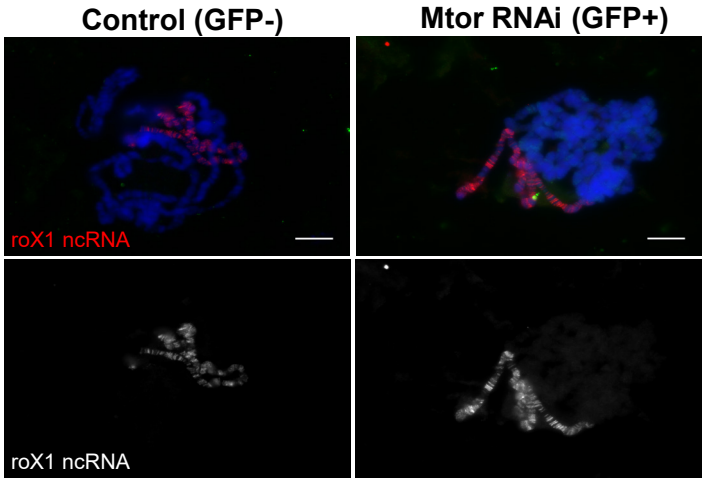
A.

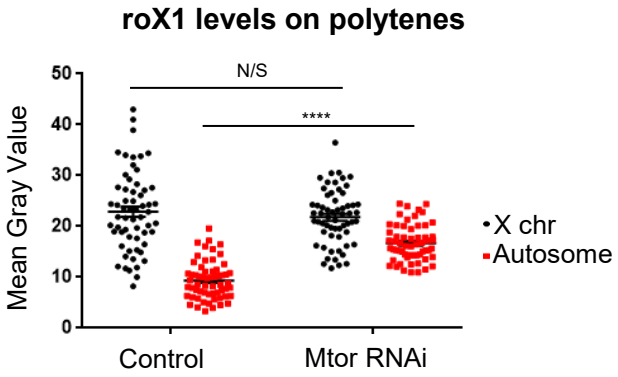

Mtor
Hoechst
Nuclear Interior
Nuclear Envelope
Mtor

B.

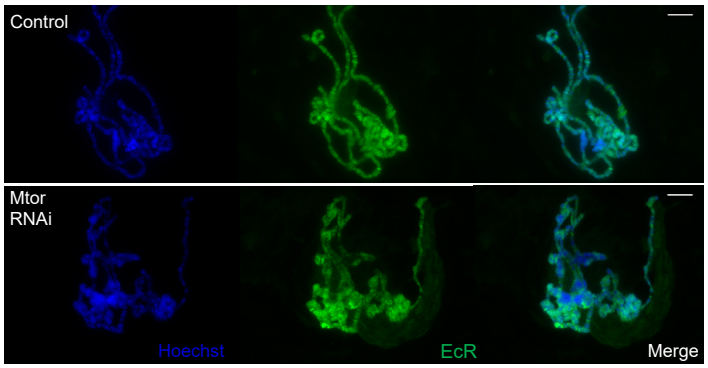

Mtor
MOF
Control
Mtor RNAi

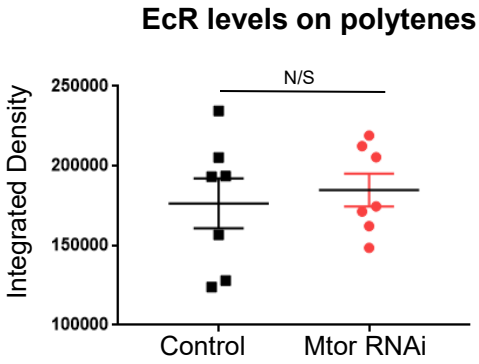
C.


Control (GFP-)
Mtor RNAi (GFP+)
roX1 ncRNA
roX1 ncRNA

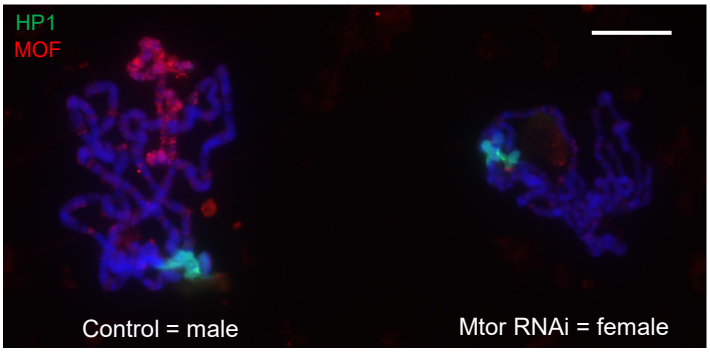

roX1 levels on polytenes
N/S
****
Mean Gray Value
50
40
30
20
10
0
Control
Mtor RNAi
X chr
Autosome

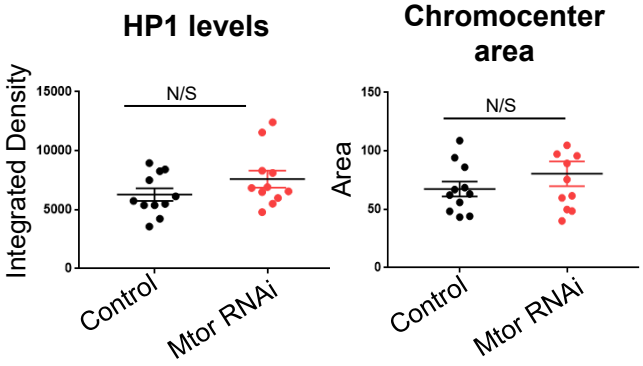
D.


Control
Mtor RNAi
Hoechst
EcR
Merge

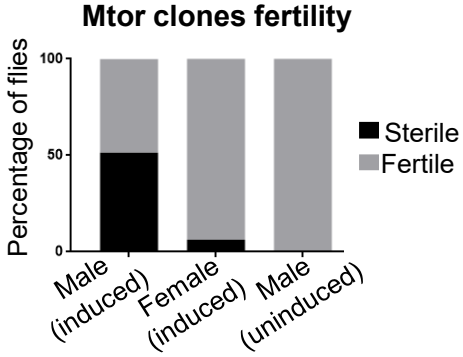

EcR levels on polytenes
N/S
Integrated Density
250000
200000
150000
100000
Control
Mtor RNAi

E.


HP1
MOF
Control = male
Mtor RNAi = female


HP1 levels
N/S
Integrated Density
15000
10000
5000
0
Control
Mtor RNAi
Chromocenter area
N/S
Area
150
100
50
0
Control
Mtor RNAi

F.


Mtor clones fertility
Percentage of flies
100
50
0
Sterile
Fertile
Male (induced)
Female (induced)
Male (uninduced)

G.

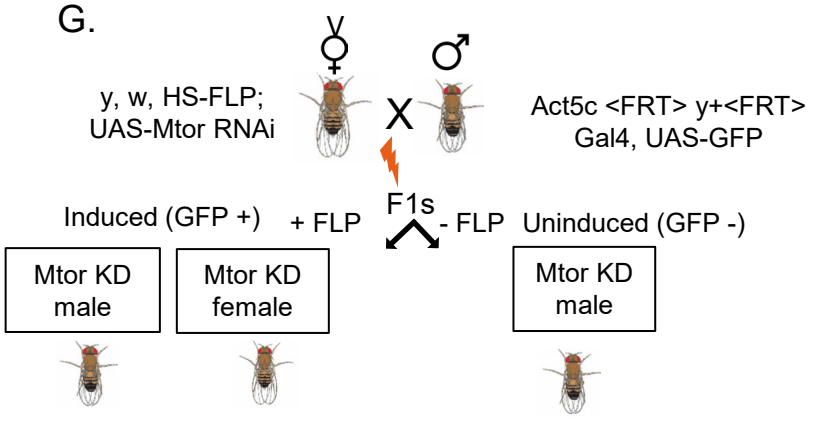
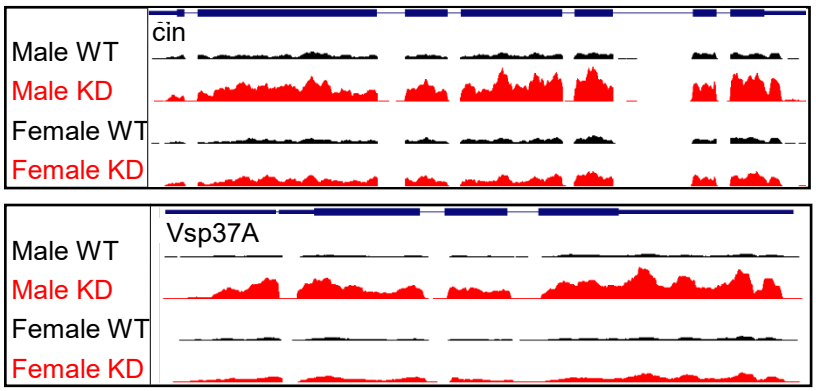

y, w, HS-FLP;
UAS-Mtor RNAi
X
Act5c <FRT> y+<FRT>
Gal4, UAS-GFP
F1s
Induced (GFP +)
+ FLP
- FLP
Uninduced (GFP -)
Mtor KD male
Mtor KD female
Mtor KD male

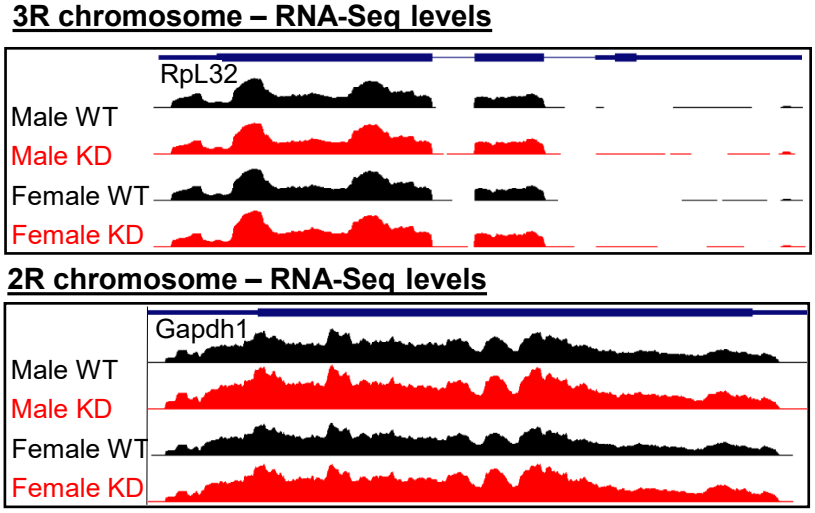
Figure S1. Depletion of Mtor does not affect nuclear integrity and results in male-specific phenotypes at an organismal level. Related to Figure 1.

(A) Semi-squash preparation of a WT (OreR) larval salivary gland nucleus, stained with antibodies to Mtor (green), showing Mtor localizing in two distinct pools – as part of the nuclear pore complex at the nuclear periphery as well as in the nuclear interior as what appears to be filaments along chromatin. Hoechst is shown in blue here and thereafter in IF images. (B) Western blot verification of Mtor depletion in larval salivary glands (as in Figure 1D) when UAS-Mtor RNAi is driven using the Nub-Gal4 driver, immuno-blotted as indicated. (C) Single molecule RNA FISH for roX1 in polytene chromosome squashes carried out in salivary glands mosaic for Mtor RNAi. Mtor RNAi nuclei were identified by strong, visible presence of GFP (not shown). Scale bar = 14 uM. Graph shows quantification of fluorescence intensity by mean gray value, on the X chromosome vs. the autosomes. N= 2 animals per condition, 4 images of l nuclei per condition quantified. Error bars represent SEM. **** is $P<0.0001$, N/S is $P>0.05$. P-values were obtained using a one-way ANOVA, followed by the Holm-Sidak test for multiple comparisons. (D) Polytene chromosome squashes, stained with antibodies to EcR (green) in control and Mtor depleted conditions, and quantification of EcR levels on chromosomes (plot at right). Scale bar = 14uM. EcR levels were quantified and represented in control (black) and Mtor RNAi conditions (red) as integrated density. N=7 nuclei from 3 animals per genotype. Error bars represent SEM. N/S is $P>0.05$. P-value was obtained using an unpaired t-test. (E) Polytene chromosome squashes, stained with antibodies to HP1 (green) in control and Mtor depleted conditions, and quantification of HP1 fluorescence signal (integrated density) and chromocenter size (area). Scale bar = 23 uM. In this experiment, as an approach to reduce the variability in IF staining between slides, a male control salivary gland and female Mtor KD salivary gland were squashed on the same cover slip. MOF co-staining (in red) was used here only to identify males, which are of the control genotype. N= 11 nuclei from 3 animals per genotype. Error bars = SEM. N/S is $P>0.05$. P-values obtained using an unpaired t-test. (F) Graph showing male and female fertility of adults made to be mosaic for Mtor RNAi and controls. Percentage of flies that were sterile or fertile for each category is reported. See methods for scoring details. N=3 independent rounds of clone generation and matings. Total scored breakdown: male (induced) – sterile = 19, fertile = 18, female (induced) – sterile=1, fertile = 18, male (uninduced) sterile = 0, fertile = 5. (G) Schematic of genotypes of P0, which are mated to generate Mtor mosaics. The F1 larvae are heat shocked at 37C for 30 mins on day 3 after initial mating day, to induce Mtor RNAi in tissues in a mosaic manner. Uninduced denotes non-heat shocked F1 larvae where Mtor was not knocked down.

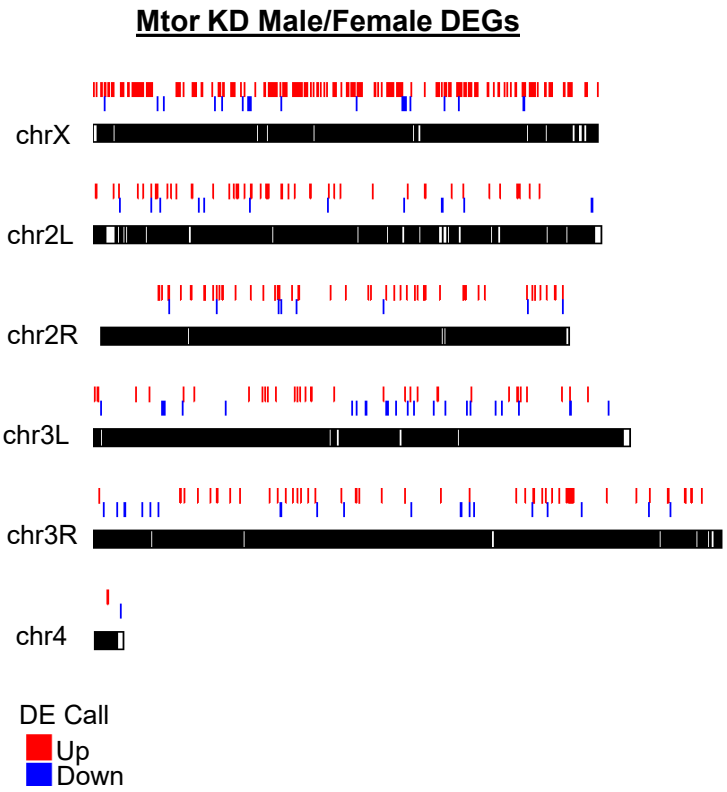
A.


X chromosome – RNA-Seq levels (averaged)
cin
Male WT
Male KD
Female WT
Female KD
Vsp37A
Male WT
Male KD
Female WT
Female KD

B.


3R chromosome – RNA-Seq levels
RpL32
Male WT
Male KD
Female WT
Female KD
2R chromosome – RNA-Seq levels
Gapdh1
Male WT
Male KD
Female WT
Female KD

C.


Mtor KD Male/Female DEGs
chrX
chr2L
chr2R
chr3L
chr3R
chr4
DE Call
Up
Down

D.

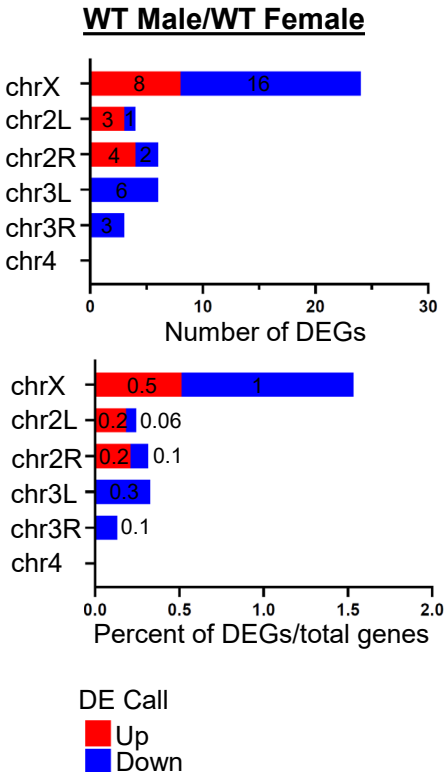
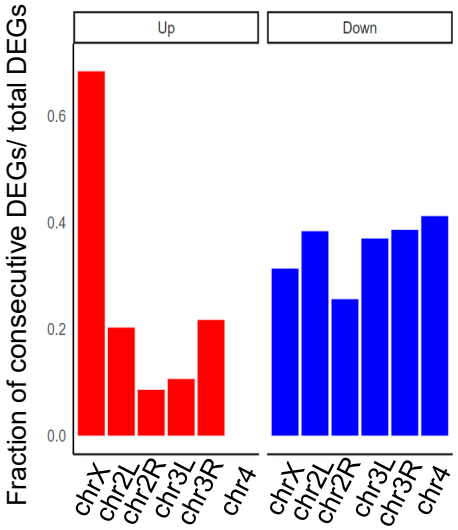

WT Male/WT Female
chrX
chr2L
chr2R
chr3L
chr3R
chr4
8
16
3
1
4
2
6
3
0
10
20
30
Number of DEGs
0.5
1
0.2
0.06
0.2
0.1
0.3
0.1
0.0
0.5
1.0
1.5
2.0
Percent of DEGs/total genes
DE Call
Up
Down

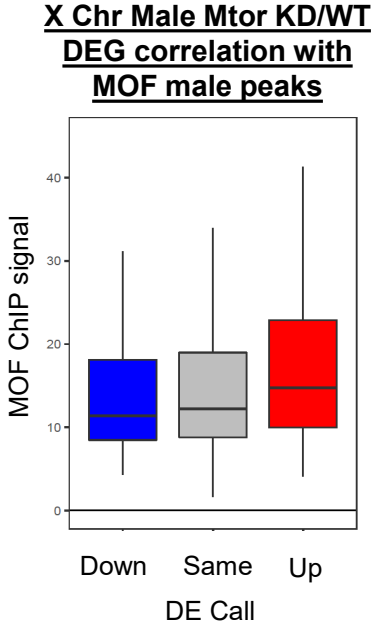
Figure S2. Upon Mtor depletion, the most prevalent change is the upregulation of male X chromosome genes. Related to Figure 2.

(A) Two genome browser snapshots of X chromosome genes cin and Vsp37A, showing RNA-Seq tracks displayed as averages of replicates per sex, from male and female salivary glands in control (WT) and Mtor depleted conditions. For cin, the displayed region is 2.8 kb and the scale is from 0-30. For Vsp37A, the displayed region is 1.7 kb and the scale is from 0-150. (B) Genome browser snapshots of RNA-Seq reads at two autosomal genes, RpL32 and Gapdh1, whose expression profiles are unaffected by Mtor depletion in both sexes. Note that RpL32 is used as our housekeeping gene control for qRT-PCR experiments. Tracks displayed are averages of replicates per sex. For RpL32, the displayed region is 1.2 kb and the scale is from 0-2000. For Gapdh1, the displayed region is 1.3 kb and the scale is from 0-300. (C) Genomic plot showing DEGs along each chromosome when comparing RNA-Seq data from Mtor KD males to Mtor KD females. DE call shows upregulation in red and downregulation in blue. (D) Breakdown of number of DEGs per chromosome when comparing WT males to females (top row) and breakdown of percentage of DEGs out of total number of genes, per chromosome (bottom row). DE call is red for upregulation and blue for downregulation. Differential gene analysis was performed using DESeq2. Genes with an FDR less than 0.05 and fold-change more than 1.5 fold were defined as differentially expressed genes (DEGs).

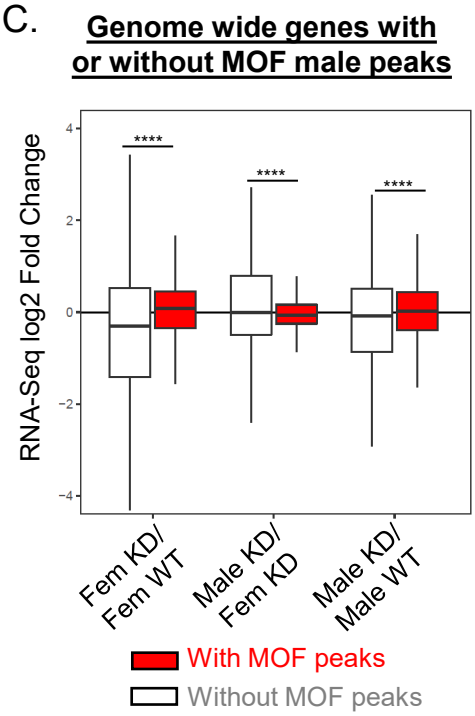
A.


Up
Down
Fraction of consecutive DEGs/ total DEGs
0.0
0.2
0.4
0.6
chrX
chr2L
chr2R
chr3L
chr3R
chr4
chrX
chr2L
chr2R
chr3L
chr3R
chr4

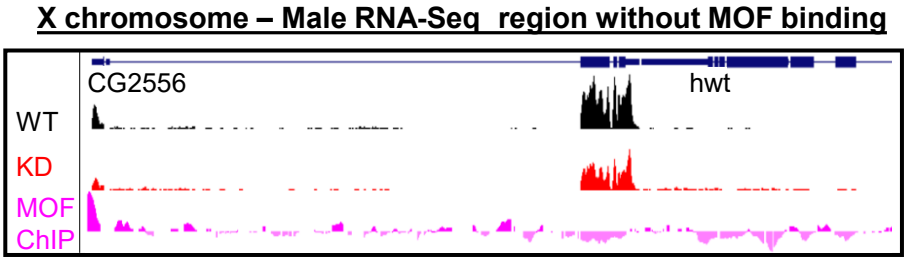
B.


X Chr Male Mtor KD/WT DEG correlation with MOF male peaks
MOF ChIP signal
0
10
20
30
40
Down
Same
Up
DE Call

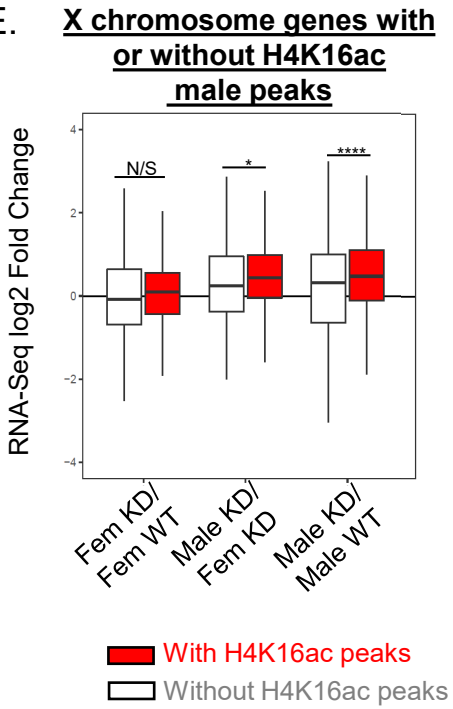
C.


Genome wide genes with or without MOF male peaks
RNA-Seq log2 Fold Change
****
****
****
Fem KD/ Fem WT
Male KD/ Fem KD
Male KD/ Male WT
With MOF peaks
Without MOF peaks

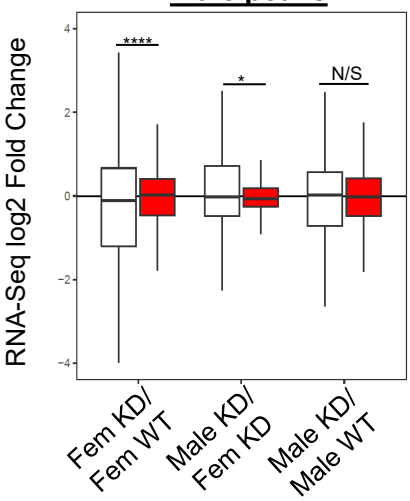
D.


X chromosome – Male RNA-Seq region without MOF binding
CG2556
hwt
WT
KD
MOF ChIP

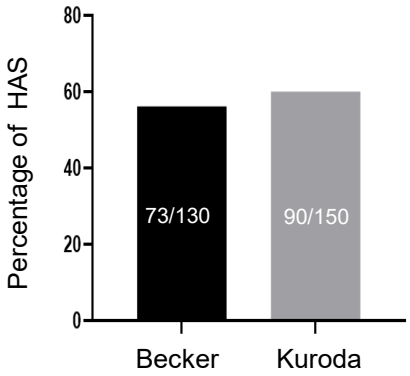
E.


X chromosome genes with or without H4K16ac male peaks
RNA-Seq log2 Fold Change
N/S
*
****
Fem KD/ Fem WT
Male KD/ Fem KD
Male KD/ Male WT
With H4K16ac peaks
Without H4K16ac peaks

F.


Genome wide genes with or without H4K16ac male peaks
RNA-Seq log2 Fold Change
****
*
N/S
Fem KD/ Fem WT
Male KD/ Fem KD
Male KD/ Male WT

G.


Percentage of HAS that overlap with DEGs upregulated in male Mtor KD
Percentage of HAS
0
20
40
60
80
73/130
90/150
Becker
Kuroda

H.

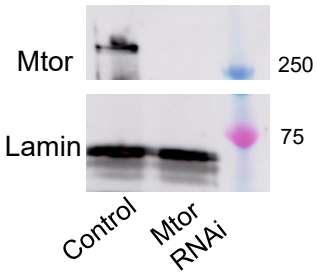
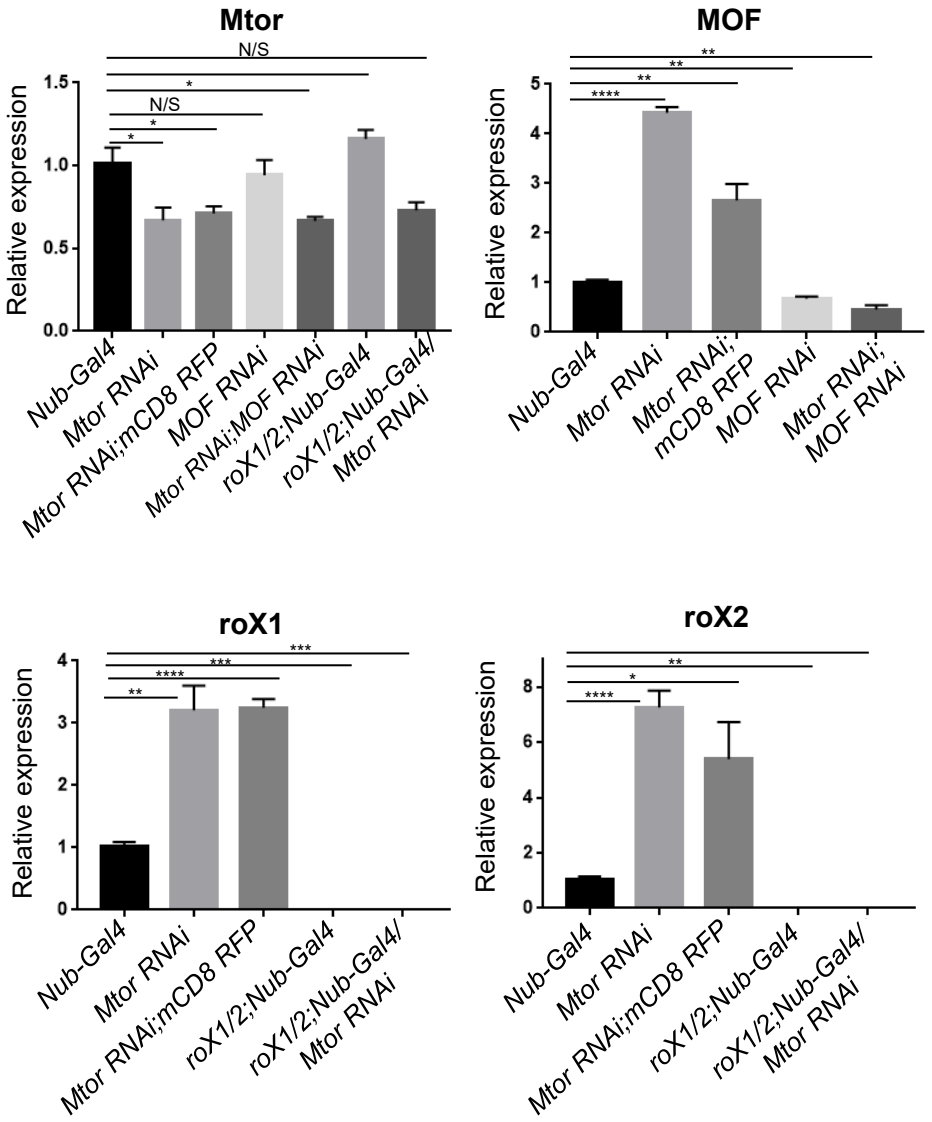

Mtor
250
Lamin
75
Control
Mtor RNAi

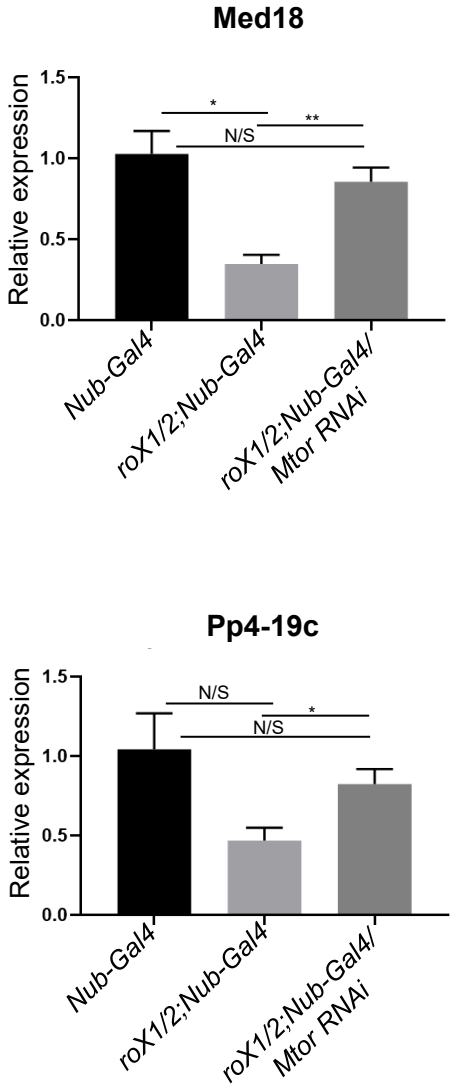
Figure S3. Groups of genes on the male X chromosome that are upregulated with Mtor depletion correlate with MOF binding. Related to Figure 3.

(A) Verification that the grouped upregulation of X chromosome genes upon Mtor depletion is not explained by simply the number of affected genes per chromosome. The fraction of consecutive (2 or more) DEGs/total DEGs was plotted for each chromosome. (B) Boxplot showing the correlation of male MOF ChIP-Seq peak intensity with differential expression (DE) call on the male X chromosome in Mtor KD/WT conditions. MOF ChIP-Seq data sets used in comparisons were from larval salivary glands and obtained from Conrad et al., 2012. Error bars represent SEM. P-values were obtained with two-sample Wilcoxon tests. **** is P<0.0001. (C) Boxplot showing the correlation of MOF ChIP-Seq peaks with RNA-Seq log2 fold change in different sex comparisons, genome wide. Red boxes denote correlation with MOF peaks and white box denote correlation without MOF peaks. MOF ChIP-Seq data sets used in comparisons were from larval salivary glands and obtained from Conrad et al., 2012. Error bars represent SEM. P-values were obtained with two-sample Wilcoxon tests. **** is P<0.0001. (D) Genome browser snapshot of an area on the male X chromosome that is not upregulated upon Mtor depletion. Snapshot is displaying averages of male replicates. In this area of downregulation with Mtor depletion, MOF ChIP-Seq peaks are also absent. The displayed region is 33.2kb and the scale is from 0-15 for RNA-Seq tracks and for ChIP-Seq is -6.18162 – 12.659. (E) Boxplot showing the correlation of H4K16ac ChIP-Seq peaks (from male larval salivary glands) with RNA-Seq log2 fold change for X chromosome genes in Mtor KD male and female salivary glands relative to WT and each other. Red boxes denote correlation with H4K16ac peaks and white box denote correlation without H4K16ac peaks. H4K16ac ChIP-Seq peaks were obtained from Conrad et al., 2012. Error bars represent SEM. P-values were obtained with two-sample Wilcoxon tests. **** is P<0.0001, * is P<0.05 and N/S is P>0.05. (F) Boxplot showing the correlation of H4K16ac ChIP-Seq peaks with RNA-Seq log2 fold change genome-wide. Red boxes denote correlation with H4K16ac peaks and white box denote correlation without H4K16ac peaks. H4K16ac ChIP-Seq peaks were obtained from Conrad et al., 2012. Error bars represent SEM. P-values were obtained with two-sample Wilcoxon tests. **** is P<0.0001, * is P<0.05 and N/S is P>0.05. (G) Bar graph showing the percentage of HAS that overlap with upregulated X chromosome DEGs in Male Mtor KD/WT conditions. Shown are the values for the two independent studies from Becker (Straub et al., 2008) and Kuroda (Alekseyenko et al., 2008) defining HAS. Inside the bars in white are the number of HAS that overlap with upregulated X chromosome DEGs out of the total number of HAS defined in each corresponding study. (H) Western blot verification of Mtor knock down in S2 cells via RNAi treatment for 6 days. S2 cells were treated with dsWhite (control) or dsMtor (Mtor RNAi), see methods for RNAi procedure. Blots were stained with antibodies as indicated (Lamin antibody blotting was used as loading control).

A.



B.



C.

	roX1/2; Nub-Gal4	*roX1/2; Nub-Gal4/ Mtor RNAi*
Adult Male	5	80
Half Eclosed Male	15	118
Pharate Male	79	169
Adult Female	1518	1359
Total Flies	**1617**	**1726**

D.

	roX1/2; NubGal4/ Mtor^{K03905}	*roX1/2;Nub-Gal4/CyO RFP*
Male	28	0
Female	112	95
Total Flies	**140**	**95**

Figure S4. Rescue of male-specific phenotypes of MSL by loss of Mtor. Related to Figure 4.

(A) Mtor, MOF, roX1 and roX2 expression levels via RT-qPCR in adults of genotypes used in genetic rescue experiments in Figure 4. N=4 biological replicates for control and Mtor KD conditions and 3 biological replicates for all other genotypes. Each replicate consisted of 10 male salivary glands. Error bars represent SEM. **** is P<0.0001, *** is P<0.001, ** is P<0.01, * is P<0.05 and N/S is P>0.05. P-values obtained using an unpaired t-test. (B) Normalized expression levels of two X chromosome targets, Med18 and Pp4-19c, via RT-qPCR on male salivary glands of larvae obtained from genetic rescue experiments with Mtor RNAi and roX1/roX2 null mutants. N=4 biological replicates for control and Mtor KD conditions and 3 biological replicates for all other genotypes. Each replicate consisted of 10 male salivary glands. Error bars represent SEM. ** is P<0.01, * is P<0.05 and N/S is P>0.05. P-values obtained using an unpaired t-test. (C) Numbers of adults scored in each phenotypic category for both genotypes – the roX1/roX2 mutation alone and the roX1/roX2 mutation in combination with Mtor RNAi, in the male-specific lethality rescue experiment. Also see Figure 4D. (D) Numbers of pharate male or live female adults scored for both genotypes – the roX1/roX2 mutation alone and the roX1/roX2 mutation in combination with MtorK03905, in the male-specific lethality rescue experiment. Also see Figure 4E.

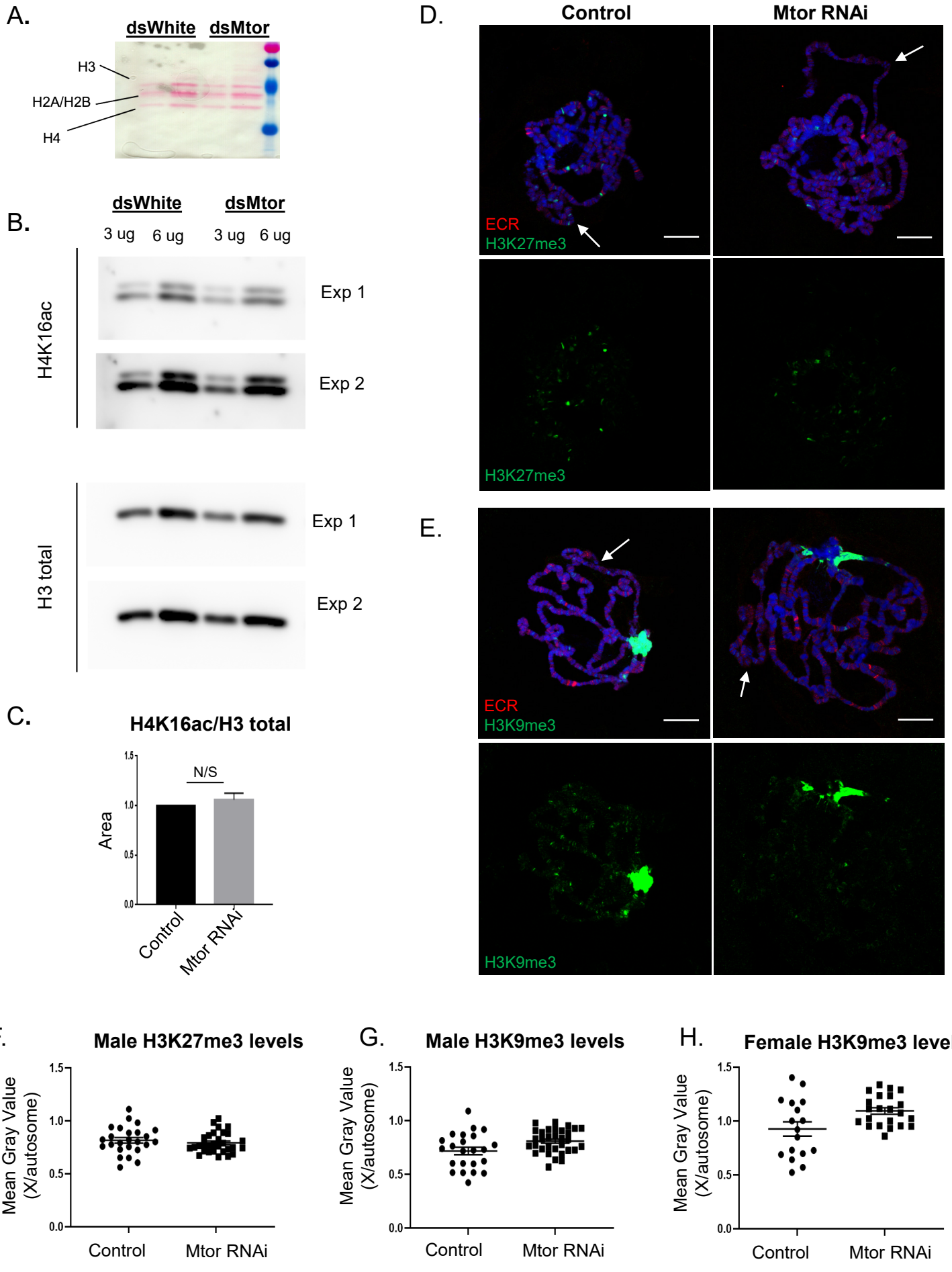
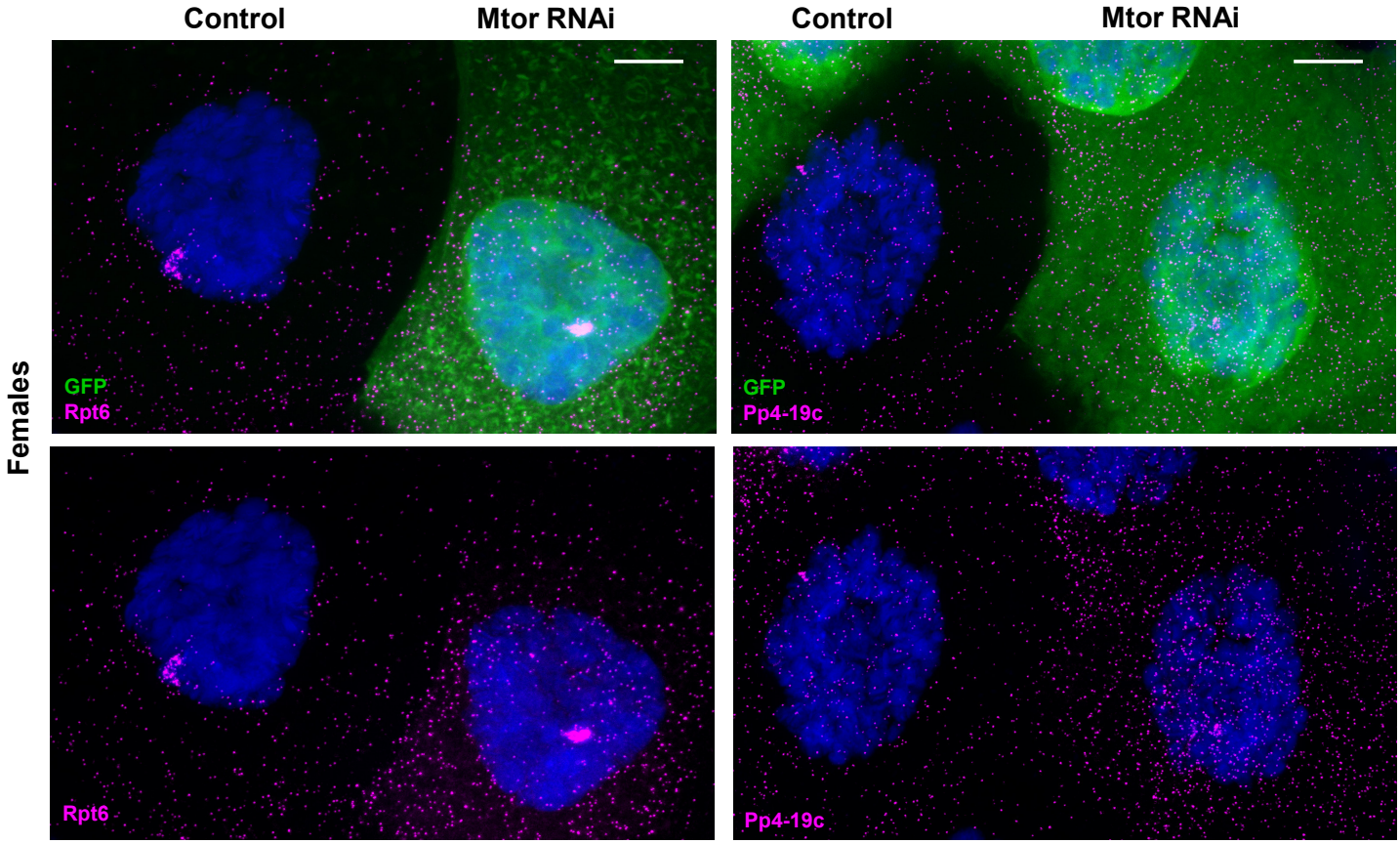

A.
dsWhite
dsMtor
H3
H2A/H2B
H4
B.
dsWhite
dsMtor
3 ug
6 ug
3 ug
6 ug
H4K16ac
Exp 1
Exp 2
H3 total
Exp 1
Exp 2
C.
H4K16ac/H3 total
N/S
Area
1.5
1.0
0.5
0.0
Control
Mtor RNAi
D.
Control
Mtor RNAi
ECR
H3K27me3
H3K27me3
E.
ECR
H3K9me3
H3K9me3
F.
Male H3K27me3 levels
Mean Gray Value (X/autosome)
1.5
1.0
0.5
0.0
Control
Mtor RNAi
G.
Male H3K9me3 levels
Mean Gray Value (X/autosome)
1.5
1.0
0.5
0.0
Control
Mtor RNAi
H.
Female H3K9me3 levels
Mean Gray Value (X/autosome)
1.5
1.0
0.5
0.0
Control
Mtor RNAi

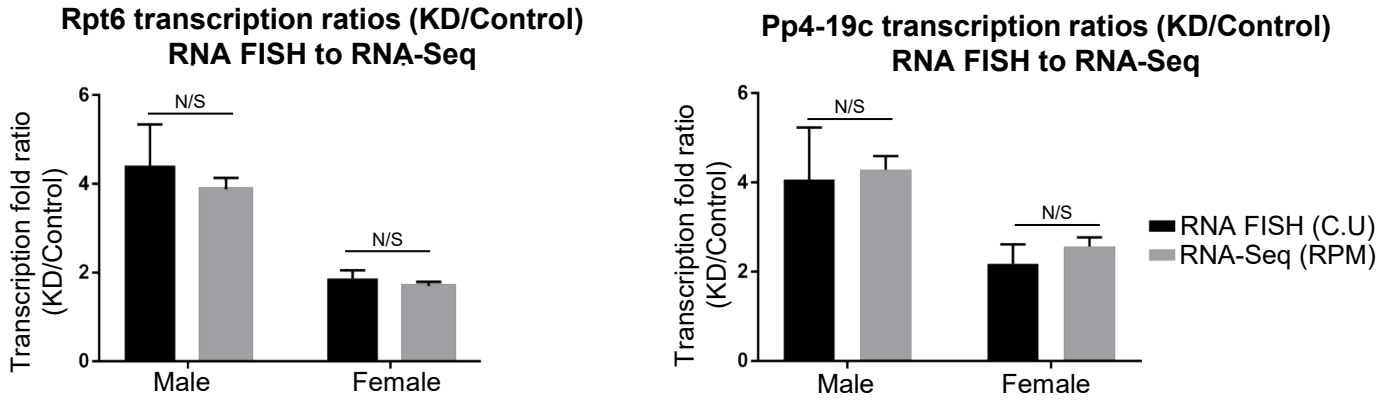
Figure S5. H4K16ac levels are unchanged upon Mtor depletion. Related to Figure 5.

(A) Ponceau staining of membrane from western blots in Figure 5C, showing successful histone extraction as seen by the presence of all 4 core histone proteins. (B) Western blot showing total H4K16ac levels relative to total H3 levels in control (dsWhite treated) and Mtor KD (dsMtor treated) S2 cells (see methods for biochemical details). Samples were loaded at two concentrations, 3ug and 6ug. Exp1 and Exp2 show the same loading at two different exposures. (C) Quantification of total H4K16ac levels, as normalized to total H3 levels, from western blots in S5B. N=3 loading trials per condition, averaged. Error bars represent SEM. N/S is P>0.05. P-value obtained with an unpaired t-test. (D) Confocal images of male polytene chromosome squashes, stained with antibodies to H3K27me3 and EcR (EcR was used to facilitate identification of autosome 3R). White arrow denotes X chromosome. Antibodies are as labeled, Hoechst staining shown in blue. Staining done in control and Nub-Gal4-driven Mtor RNAi males. Single Z-slice shown. Scale bar = 19 uM. (E) Confocal images of male polytene chromosome squashes, stained with antibodies to H3K9me3 and EcR (EcR was used to facilitate identification of autosome 3R). White arrow denotes X chromosome. Antibodies are as labeled, Hoechst staining shown in blue. Staining done in control and Nub-Gal4-driven Mtor RNAi males. Single Z-slice shown. Scale bar= 19 uM. (F) Quantification of male H3K27me3 levels in polytene chromosome squashes. Mean gray value was represented as a ratio of signal on the X chromosome to signal on the 3R autosome. Images analyzed were: N=25 nuclei for control males and 30 nuclei for Mtor RNAi males from 4 biological replicates. Error bars represent SEM. N/S is P>0.05. P-value obtained with an unpaired t-test. (G) Quantification of male H3K9me3 levels in polytene chromosome squashes. Mean gray value was represented as a ratio of signal on the X chromosome to signal on the 3R autosome. Images analyzed were: For H3K9me3, N= 29 nuclei for control males and 32 nuclei for Mtor RNAi males from 6 biological replicates. Error bars represent SEM. * is P<0.05. P-value obtained with an unpaired t-test. (H) Quantification of female H3K9me3 levels in polytene chromosome squashes (images not shown). Mean gray value was represented as a ratio of signal on the X chromosome to signal on the 3R autosome. Images analyzed were: N=17 nuclei for control females and 22 for Mtor RNAi females from 3 biological replicates. Error bars represent SEM. * is P<0.05. P-value obtained with an unpaired t-test.

A.


Control
Mtor RNAi
Control
Mtor RNAi
Females
GFP
Rpt6
GFP
Pp4-19c
Rpt6
Pp4-19c

B.


Rpt6 transcription ratios (KD/Control)
RNA FISH to RNA-Seq
Transcription fold ratio (KD/Control)
N/S
N/S
Male
Female
Pp4-19c transcription ratios (KD/Control)
RNA FISH to RNA-Seq
Transcription fold ratio (KD/Control)
N/S
N/S
Male
Female
RNA FISH (C.U)
RNA-Seq (RPM)

C.

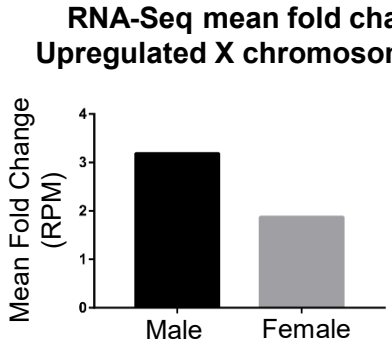

RNA-Seq mean fold change of
Upregulated X chromosome genes
Mean Fold Change (RPM)
Male
Female

Figure S6. Transcriptional upregulation of X chromosome targets upon Mtor RNAi is consistently more pronounced in males. Related to Figure 6.

(A) Single molecule RNA FISH for two X chromosome targets, Rpt6 and Pp4-19c, in intact salivary gland nuclei of female larvae with Mtor RNAi mosaic system. Mtor depleted nuclei are marked with GFP in the top panel. Bottom panel displays only RNA channel in magenta. Images are maximum projections spanning 5 microns centered on the nascent transcription sites. Scale bars = 12 uM. (B) Transcription fold change in males and females represented as a ratio of Mtor KD/Control for both Rpt6 and Pp4-19c from RNA-FISH and RNA-Seq methods. RNA FISH measurements are transcriptional activity measurements obtained via quantification of nascent transcription site activity, represented in cytoplasmic units (C.U). RNA-Seq measurements are in the units of reads per million (RPM). For RNA FISH, images analyzed were as follows: N=8 males and 7 females for Rpt6, 7 males and 7 females for Pp4-19c. For RNA-Seq, N= 3 replicates of 10 salivary glands per sex. Error bars represent SEM. N/S is $P>0.05$. P-values obtained with unpaired t-tests. (C) RNA-Seq mean fold change of upregulated X chromosome genes in males and females upon Mtor KD. Mean fold change is represented in reads per million (RPM). Actual values for mean fold change above bars. N= 3 replicates of 10 salivary glands per sex.

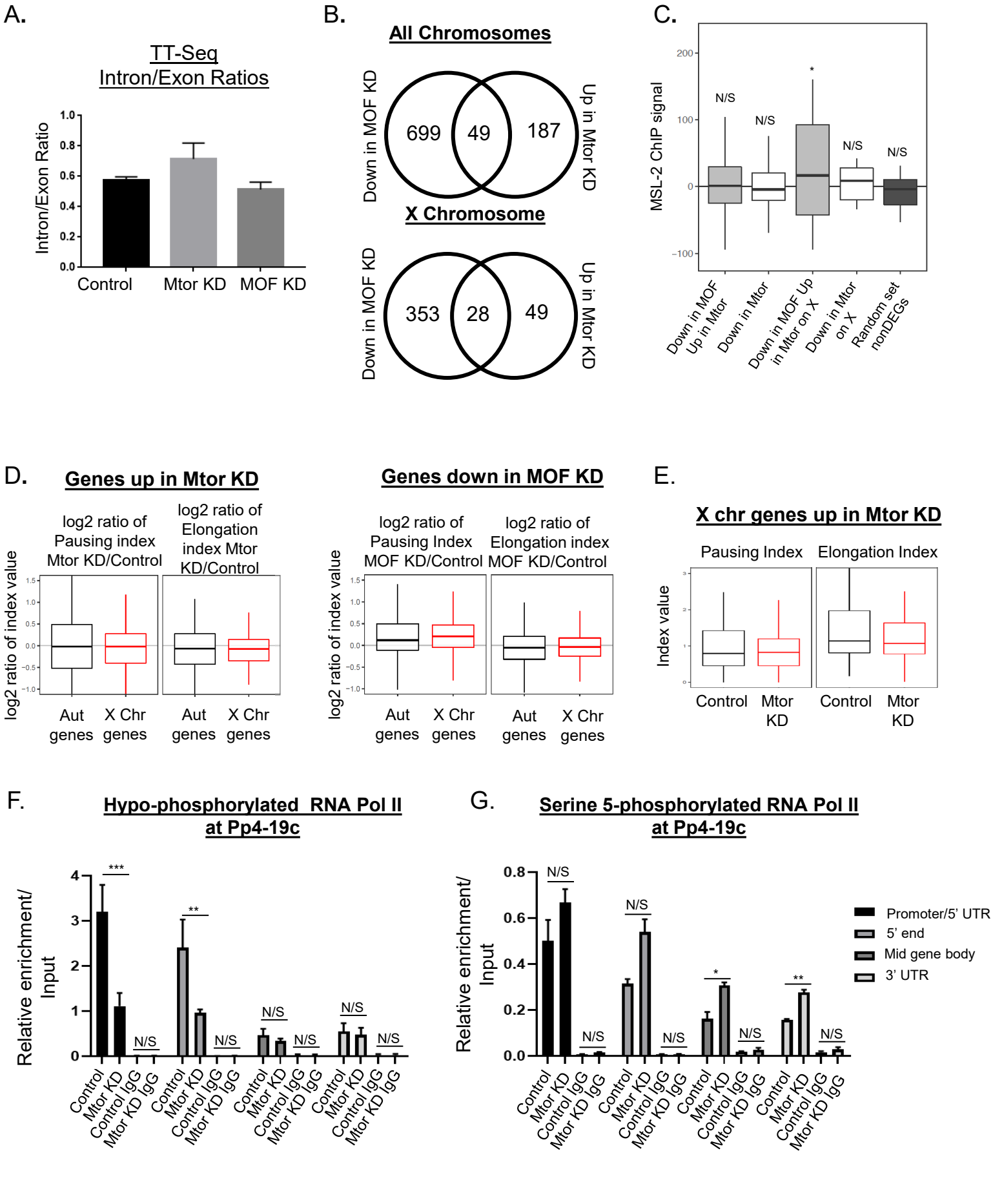

A.
TT-Seq
Intron/Exon Ratios
Intron/Exon Ratio
1.0
0.8
0.6
0.4
0.2
0.0
Control
Mtor KD
MOF KD
B.
All Chromosomes
Down in MOF KD
699
49
187
Up in Mtor KD
X Chromosome
Down in MOF KD
353
28
49
Up in Mtor KD
C.
MSL-2 ChIP signal
200
100
0
−100
N/S
N/S
*
N/S
N/S
Down in MOF Up in Mtor
Down in Mtor
Down in MOF Up in Mtor on X
Down in Mtor on X
Random set nonDEGs
D.
Genes up in Mtor KD
log2 ratio of Pausing index Mtor KD/Control
log2 ratio of Elongation index Mtor KD/Control
log2 ratio of index value
Aut genes
X Chr genes
Aut genes
X Chr genes
Genes down in MOF KD
log2 ratio of Pausing Index MOF KD/Control
log2 ratio of Elongation index MOF KD/Control
log2 ratio of index value
Aut genes
X Chr genes
Aut genes
X Chr genes
E.
X chr genes up in Mtor KD
Pausing Index
Elongation Index
Index value
Control
Mtor KD
Control
Mtor KD
F.
Hypo-phosphorylated RNA Pol II at Pp4-19c
Relative enrichment/ Input
***
**
N/S
Control
Mtor KD
Control IgG
Mtor KD IgG
G.
Serine 5-phosphorylated RNA Pol II at Pp4-19c
Relative enrichment/ Input
0.8
0.6
0.4
0.2
0.0
N/S
*
**
Promoter/5' UTR
5' end
Mid gene body
3' UTR

Figure S7. Mtor restrains dosage compensation by affecting nascent transcription uniformly. Related to Figure 7.

(A) Intron/Exon ratios obtained in the TT-Seq experiment in control, Mtor and MOF depleted conditions. 0.6 is known to be the standard ratio value in TT-Seq in which sufficient detection of nascent transcripts can be confirmed. P-values not shown are all N/S (P>0.05). P-values were obtained with a one-way ANOVA followed by the Holm-Sidak test for multiple comparisons. (B) Venn diagrams showing the overlap of the TT-Seq DEGs that are down in MOF KD conditions and up in Mtor KD conditions on all chromosomes on the left and on the X chromosome on the right. (C) Boxplot showing the correlation of the TT-Seq DEGs that are down in MOF KD and up in Mtor KD conditions with MSL-2 ChIP-Seq signal (dataset from Straub et al., 2013). Categories include overlapping DEGs that are both downregulated upon MOF KD and upregulated upon Mtor KD (Down in MOF and Up in Mtor, these are the shared genes in B) and DEGs that are downregulated upon Mtor KD (Down in Mtor), either genome wide or only on the X chromosome. Random set nonDEGs represents 49 randomly selected genes that are not affected by MOF or Mtor KD amongst all chromosomes. P-values were obtained from a one-sample t-test to determine if mean MSL-2 ChIP signal is greater than 0. From left to right, X-axis categories, N= 49, 133, 28, 10, 49. Error bars represent SEM. * is P<0.05, N/S is P>0.05. (D) Boxplots showing the log2 ratio of pausing and elongation indexes for genes, downregulated in MOF KD conditions, on the X chromosome vs. autosomes (Aut) (left), and for genes, upregulated in Mtor KD conditions, on X chromosome vs. autosomes (right). Values are represented as the log2 ratio of the index values from the respective KD over the control, obtained from metagene analysis described in text, using TT-Seq data. See methods for additional details on index calculations. (E) Boxplots showing pausing and elongation index for X chromosome genes, upregulated in Mtor KD conditions, in control and Mtor KD treatments. Values are represented as index values, obtained from metagene analysis described in text, using TT-Seq data. See methods for additional details on index calculations. (F) ChIP-qPCR for hypo-phosphorylated RNAP II (as recognized by the 8WG16 antibody) at Pp4-19c in control and Mtor depleted conditions at 4 different gene positions in S2 cells. IgG controls are included for each primer set. N= 4 biological replicates, error bars represent SEM. *** is P<0.001, ** is P<0.01, N/S is P>0.05. P-values obtained with unpaired t-tests. (G) ChIP-qPCR for Serine-5 phosphorylated RNAP II (as recognized by the CTD4H8 antibody) at Pp4-19c in control and Mtor depleted conditions at 4 different gene positions in S2 cells. IgG controls are included for each primer set. N= 2 biological replicates, error bars represent SEM. ** is P<0.01, * is P<0.05, N/S is P>0.05. P-values obtained with unpaired t-tests.