Accepted Manuscript

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PII: S2589-0042(18)30169-X

DOI: 10.1016/j.isci.2018.10.010

Reference: ISCI 169

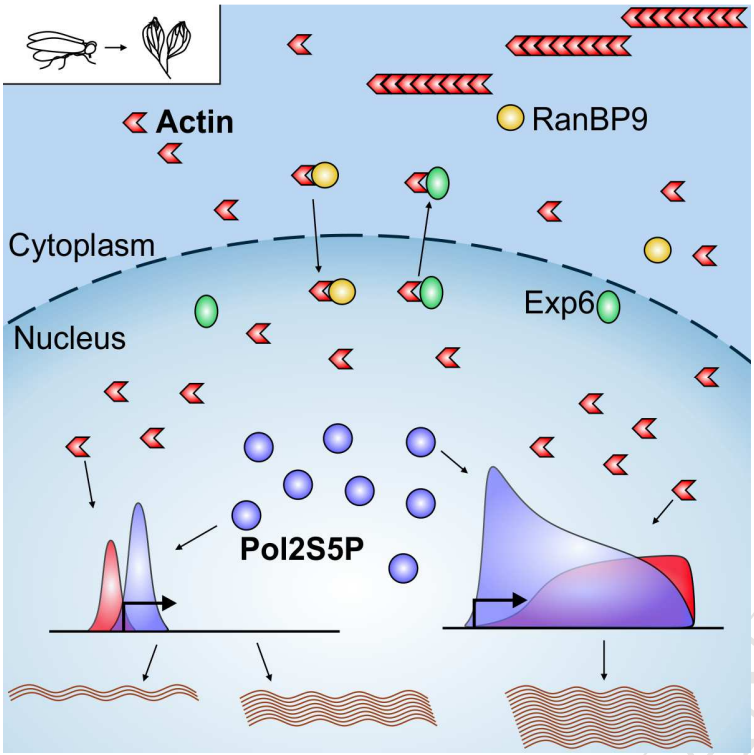
To appear in: *ISCIENCE*

Received Date: 21 August 2018

Revised Date: 28 September 2018

Accepted Date: 10 October 2018

Please cite this article as: Sokolova, M., Moore, H.M., Prajapati, B., Dopie, J., Meriläinen, L., Honkanen, M., Matos, R.C., Poukkula, M., Hietakangas, V., Vartiainen, M.K., Nuclear actin is required for transcription during *Drosophila* oogenesis, *ISCIENCE* (2018), doi: https://doi.org/10.1016/j.isci.2018.10.010.



Nuclear actin is required for transcription during *Drosophila* oogenesis

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Key words: actin, nuclear actin, transcription, *Drosophila* oogenesis, Mrtf

Abstract

Actin has been linked to processes spanning the whole gene expression cascade, from regulating specific transcription factors, such as myocardin-related transcription factor (Mrtf), to chromatin remodelling and RNA polymerase (Pol) function. However, whether actin controls transcription of only specific genes or has a global role in gene expression has remained elusive. Our genome-wide analysis reveals, for the first time, that actin interacts with essentially all transcribed genes in *Drosophila* ovaries. Actin co-occupies majority of gene promoters together with Pol II, and on highly expressed genes these two proteins also associate with gene bodies. Mechanistically, actin is required for Pol II recruitment to gene bodies and manipulation of nuclear transport factors for actin leads to decreased expression of egg shell genes. Collectively, these results uncover a global role for actin in transcription, and demonstrate the *in vivo* importance of balanced nucleo-cytoplasmic shuttling of actin in transcriptional control of a developmental process.

Introduction

In addition to its essential roles as part of the cytoskeleton, actin also regulates gene expression in the nucleus. Actin is a component of many chromatin remodeling complexes [reviewed by (Kapoor and Shen, 2013)] and linked to transcription by all three RNA polymerases (Hofmann et al., 2004; Hu et al., 2004; Philimonenko et al., 2004). Actin seems to have a positive role on general transcription, since reduced availability of nuclear actin, due to either inhibition of the active nuclear import of actin (Dopie et al., 2012), activation of a mechanosensory complex consisting of emerin, non-muscle myosin II and actin (Le et al., 2016) or polymerizing nuclear actin into stable filaments (Serebryannyy et al., 2016), attenuates transcription. Nevertheless, the exact mechanism and the *in vivo* relevance of this process have remained unclear. Actin also negatively regulates the transcription of specific genes. For example, actin regulates both the nuclear localization and activity of myocardin related transcription factor A (MRTF-A; also known as MAL/MKL1), which is cofactor of the essential transcription factor SRF (Miralles et al., 2003; Vartiainen et al., 2007). Actin monomer-binding prevents MRTF-A from activating SRF in the nucleus. This regulation has been postulated to take place at the level of target genes (Vartiainen et al., 2007), but how the opposing effects of actin on transcription are resolved on chromatin is not obvious. Moreover, the genome-wide binding pattern of actin in the context of RNA polymerase II (Pol II) mediated transcription has remained elusive, and previous studies of actin-chromatin interactions are based on few selected genes (Hofmann et al., 2004; Obrdlik et al., 2008). Importantly, actin itself is one of the target genes for SRF (Salvany et al., 2014), generating a feedback loop, where actin levels are controlled by the actin dynamics cycle. Here we show that chromatin-binding of actin is not dependent on Mrtf transcription factors and that, at the genome-wide level, actin interacts with essentially all transcribed genes in *Drosophila*

ovaries, with a pattern depending on the expression level of the gene. Finally, we demonstrate the functional relevance of nuclear actin for gene transcription *in vivo*.

Results and discussion

Actin is involved in transcription of *Act5C* independently of Mrtf

To clarify the role of actin in general vs. gene-specific transcriptional regulation, we examined actin-chromatin interactions in *Drosophila* ovaries, where Mrtf has been shown to regulate *Act5C* transcription (Salvany et al., 2014). We performed chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) of Mrtf-GFP, actin and Pol II phosphorylated at serine 5 (Pol II S5P) in ovaries of wild type (w^{1118}) and *Mrtf* mutant ($mal\text{-}d^{\Delta 7}$) flies, where *Mrtf* expression is abolished (Somogyi and Rorth, 2004), as well as in flies ubiquitously expressing GFP-tagged version of Mrtf (*tub mal-d3xGFP*) (Salvany et al., 2014) (**Figure 1A**). Deletion and overexpression of Mrtf displayed decreased and increased expression of *Act5c*, respectively (**Figure 1B**), and Mrtf bound to promoter and upstream region of the *Act5C* gene (**Figure 1A,C,D**), in agreement with previous studies (Salvany et al., 2014). Pol II S5P bound to transcription start sites of *Act5C* in all three fly strains (**Figure 1A,C,D**). Interestingly, the binding pattern of actin was different than that of Mrtf, and a substantial actin signal was found on the gene body of the *Act5C* gene (**Figure 1A,E**). Importantly, actin signal was not reduced in $mal\text{-}d^{\Delta 7}$ flies (**Figure 1F**), indicating that actin-binding to the *Act5C* gene is not dependent on Mrtf. The functional significance of actin-binding to its own gene remains to be investigated.

Actin interacts with transcribed genes with a pattern depending on their expression level

To obtain a genome-wide view of actin-chromatin interactions, further ChIP-Seq analysis of the w^{1118} fly strain revealed actin on the promoters of essentially all transcribed genes together with Pol II S5P (**Figure 2A**). Peak-calling confirmed the substantial overlap between actin and Pol II S5P binding sites (**Figure 2B**). However, detailed analysis showed that actin binds promoters slightly before the transcription start site (TSS) and Pol II S5P enrichment (**Figure 2C**), indicating that actin could be involved in transcriptional initiation, perhaps via pre-initiation complex formation, as suggested before (Hofmann et al., 2004).

Similarly to the *Act5C* gene (**Figure 1**), actin was also found, together with Pol II S5P, on gene bodies of certain genes (**Figure 2A**, genes at the bottom have highest expression). These included, for example, the highly transcribed *chorion* genes (**Figure 2D**) involved in eggshell formation. On these genes actin is enriched more towards the transcription end site (TES) than the TSS (**Figure 2E**). Notably, both actin antibodies produced a very similar binding pattern on chromatin (**Figure 2A,B,D,E**). This genome-wide analysis shows that actin interacts with most transcribed genes in *Drosophila* ovaries, and that depending on the expression level of the gene, actin can be found both on the promoters and gene bodies. This genome-wide data can thus consolidate previous ChIP studies of actin that have reported variable binding to different genomic sites depending on the specific gene analyzed (Hu et al., 2004; Obrdlik et al., 2008; Philimonenko et al., 2004; Ye et al., 2008). Whether the binding pattern of actin reflects its dual roles in transcription, both during transcription initiation and elongation, or whether the recruitment to gene bodies represents a specific requirement for actin upon high transcriptional activity, awaits further studies. An obvious candidate for recruiting actin to the genes is Pol II,

which based on our ChIP-seq studies co-occupies most actin-binding sites (**Figure 2**), although not with exactly the same pattern. Other candidates include the different chromatin remodeling complexes containing actin (Kapoor and Shen, 2013), as well as the elongation factor P-TEFb (Qi et al., 2011).

Active transport of nuclear actin is required for egg shell gene transcription

To study if active maintenance of nuclear actin levels is required for transcription in *Drosophila* ovaries similarly as in mammalian cells (Dopie et al., 2012), we generated a mutant of the nuclear actin import receptor, RanBP9 (*Drosophila* orthologue of Importin-9) (**Figure 3A**; see also Materials and methods). Similarly to Importin-9 knockdown in mammalian cells (Dopie et al., 2012), loss of RanBP9 in *Drosophila* resulted in decreased nuclear actin levels (**Figure 3B,C**), while the total actin levels were not significantly altered (**Figure 3D**). On the same genetic background, the *RanBP9*$^{\Delta 1}$ mutants were viable, but females laid fewer eggs than control flies (**Figure 3E**), and these eggs failed to develop.

In contrast to our previous results from mammalian cells, RNA-seq analysis of the *RanBP9*$^{\Delta 1}$ mutant ovaries did not reveal dramatic transcriptional downregulation upon inhibiting active nuclear import of actin (**Figure 4A and Table S1**). We note that in mammalian cells, Importin-9 depletion led to a greater reduction in nuclear actin levels (Dopie et al., 2012) than the *RanBP9*$^{\Delta 1}$ deletion reported here (**Figure 3C**). Whether the fly utilizes additional nuclear import mechanisms for actin or whether the underlying biological complexity creates differential sensitivity to nuclear actin levels remains to be determined. Nevertheless, several genes encoding for chorion proteins showed reduced expression in the *RanBP9*$^{\Delta 1}$ compared to control (marked

as red in **Figure 4A)**, and RT-qPCR confirmed the significant downregulation for a subset of them (**Figure 4B)**. Importantly, the same transcripts showed reduced expression also when RanBP9 expression was silenced by RNAi specifically in the follicle cells (**Figure 4C**), which are the cells that express the *chorion* genes to deposit the eggshell over the oocyte. Since RanBP9 could also have other import cargoes than actin, and rescue with an NLS-actin construct (Dopie et al., 2012) was not possible due to technical reasons in this experimental system, we used overexpression of Exportin 6, the nuclear export receptor for actin (Stuven et al., 2003), as an alternative method to manipulate nuclear actin in follicle cells. Also this led to reduction in *chorion* gene expression (**Figure 4C**), further supporting the notion that balanced nuclear transport of actin is required for appropriate transcription of egg shell genes. Mechanistically, the *RanBP9*$^{\Delta 1}$ deletion led to decreased binding of both actin and Pol II (**Figure 4D**) to the *chorion genes*. Finally, the eggs laid by the *RanBP9*$^{\Delta 1}$ females displayed morphologically abnormal (**Figure 4E**) and short (**Figure 4F**) dorsal appendages, which are specialized structures of the egg shell used by the embryo for breathing. Deregulated *chorion* gene expression thus has phenotypic consequences and could explain why the eggs laid by the *RanBP9*$^{\Delta 1}$ females do not develop.

Taken together, these results enforce the importance of actin for transcription by showing in a genome-wide format that actin interacts with virtually all genes transcribed by Pol II and that its balanced nuclear transport is required for transcription *in vivo*. Further studies are required to elucidate the molecular machineries that recruit actin both to the promoters and gene bodies.

Limitations of the study

Although this study shows the genome-wide binding pattern of actin on chromatin, which has not been available before, the transcription complexes containing and functionally interacting with actin remain unclear, and will be an important avenue for further studies. This study takes advantage of the nucleo-cytoplasmic shuttling mechanism of actin to decrease the amounts of actin in the nucleus. While targeting both import and export pathways of actin alleviates some specificity issues, development of more previse tools to manipulate actin specifically in the nucleus would benefit the whole research field.

Acknowledgements

We thank Paula Maanselkä for technical assistance and Dr. Joachim Urban for supplying UASp-Exp6 fly strain. Imaging was carried out at the Light Microscopy Unit (LMU) of the Institute of Biotechnology, SEM sample preparation as well as imaging at Electron Microscopy Unit of the Institute of Biotechnology, and next-generation sequencing at Biomedicum Functional Genomics Unit (FuGU) all of the University of Helsinki. This work was supported by the Academy of Finland, ERC, Sigrid Juselius and Jane & Aatos Erkko foundation grants to MKV.

Author contributions

Conceptualization, M.K.V. and M.S.; Methodology, M.K.V., M.S., V.H. and M.P.; Investigation, M.S., H.M.M., B.P., J.D., M.H. and R.C.M; Writing-original draft, M.S, H.M.M. and M.K.V.; Writing –Review and editing, M.S., and M.K.V. Supervision, M.K.V. and V.H; Project administration, M.K.V.; Funding acquisition, M.K.V.

Competing interests: None

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Figure legends

Figure1.

Actin-binding to the *Act5C* gene is not dependent on Mrtf.

(A) ChIP-seq analysis of Mrtf-GFP, actin and Pol II S5P at the *Act5C* gene region on chromosome X. Fly strains and antibodies used are indicated on the left, and signal intensity as number of reads is shown above each track; actin and the control antibody IgG are shown on the same scale.

(B) mRNA levels of Act5C in the indicated fly strain measured by qPCR. Rpl32 was used as internal control, data is normalized to mal-dΔ7/+ (heterozygous Mrtf deletion) and is mean from two independent measurements with standard deviation.

(C,D) Binding profile of Pol II S5P (purple) and Mrtf-GFP (green) on *Act5C* gene in ovaries from *mal-dΔ7* (C) and *tub mal-d3xGFP* (D) fly strains. Read counts are normalized to inputs.

(E) Binding profile of actin (black) and Mrtf-GFP (green) on the Act5C gene in ovaries from *tub mal-d3xGFP* fly strain. Read counts are normalized to inputs.

(F) Binding profile of actin on the *Act5C* gene in ovaries from *tub mal-d3xGFP* (black) and in *mal-dΔ7*(light brown) flies.

Figure 2.

Actin colocalizes with Pol II at TSS and gene bodies of transcribed genes.

(A) Heatmap of the ratio between the sample (histone H3K4met3, Pol II S5P, and actin with two antibodies, AC-74 and AC-15) and input ChIP-Seq signals across gene regions, standardized and segmented into 200 bins. Transcription start sites (TSS) and transcription end sites (TES) are indicated. Genes are sorted according to normalized read count (NRC) of RNA-seq data from w^{1118} fly ovaries (right panel).

(B) Venn diagram showing overlap of actin (AC-74) and Pol II S5P peaks from ChIP-seq.

(C) Average signal of read counts normalized to the input from −500 bp to +500 bp from the TSS of gene loci (n=10843).

(D) Binding profile of actin and Pol II on *chorion* genes at 66D locus of chromosome 3L. Antibodies used in ChIP-Seq are indicated on the left, and signal intensity as number of reads is shown in parentheses above each track. Results from two experiment replicates (rep) are shown.
(E) ChIP-seq with the indicated antibodies with average signal of read counts normalized to input shown across the gene body of known eggshell protein encoding genes (Tootle et al., 2011).

Fig. 3. Generation of RanBP9 mutant fly with decreased nuclear actin

(A) Schematic of the RanBP9 locus. The region of deletion (light yellow) generated by imprecise excision of P{GSV6}GS13460.

(B) Confocal microscopy images of nurse cell nuclei of ovarian egg chambers stained with actin antibodies and DAPI. Scale bar 10 μm.

(C) Quantitation of nucleus to cytoplasm ratio of actin staining intensities in nurse cells. Data is from three independent experiments with N=32 (wt/def) and N=29 (*RanBP9*$^{\Delta 1}$/def). Mann-Whitney Test, $p<0.05$. Boxes represent 25-75% and the error bars range within 1.5IQR. The line in the middle is median and the open square is mean.

(D) Western blots from the whole fly lysates probed with anti-actin antibody. Quantitation of actin amount (below the blots) is from three independent experiments with wt/def normalized to 1 and ± representing SD. No significance by Student's T test.

(E) Numbers of eggs laid by the indicated flies. N=289 (wt/def) and N=214 (*RanBP9*$^{\Delta 1}$/def) from six independent experiments. Student's t-test, $p<0.001$. Data shown as in C. Black diamonds are outliers.

Fig. 4. RanBP9 mutants display decreased expression of chorion protein genes and defective egg shell formation.

(A) MA-plot of RNA-Seq data. The transcripts of known eggshell proteins are indicated in red.

(B) Relative expression of 4 chorion protein transcripts in wt/def and *RanBP9*$^{\Delta 1}$/def fly ovaries from five independent experiments. Data is normalized to wt/def. Statistics with student's t-test. Error bars represent +/- SD.

(C) Relative expression of 4 chorion protein transcripts in the indicated fly strains from two independent experiments. Data is normalized to c204>RNAi-GFP and error bars represent +/- SD. Asterisk indicates $p<0.05$ with Students t-test.

(D) ChIP-seq with actin (right) and Pol II ser5 (left) with average signal of read counts normalized to input shown across the gene body of *chorion* protein genes.

(E) Scanning electron micrographs of fly eggs with dorsal appendages. Representative images of control (wt/def) and *RanBP9*$^{\Delta 1}$/def eggs are shown. Magnification 450x. Scale bar 200 µm.

(F) Relative lengths of dorsal appendages from eggs of indicated fly strains. Data is normalized to wt/def. N=91 (wt/def) and N=120 (*RanBP9*$^{\Delta 1}$/def) from three independent experiments. Student's t-test, $p<0.001$. Data shown as in 3C.

Table S1. RNA-seq, related to Figure 4A.

Data from RNA-seq analysis.

Figure 1

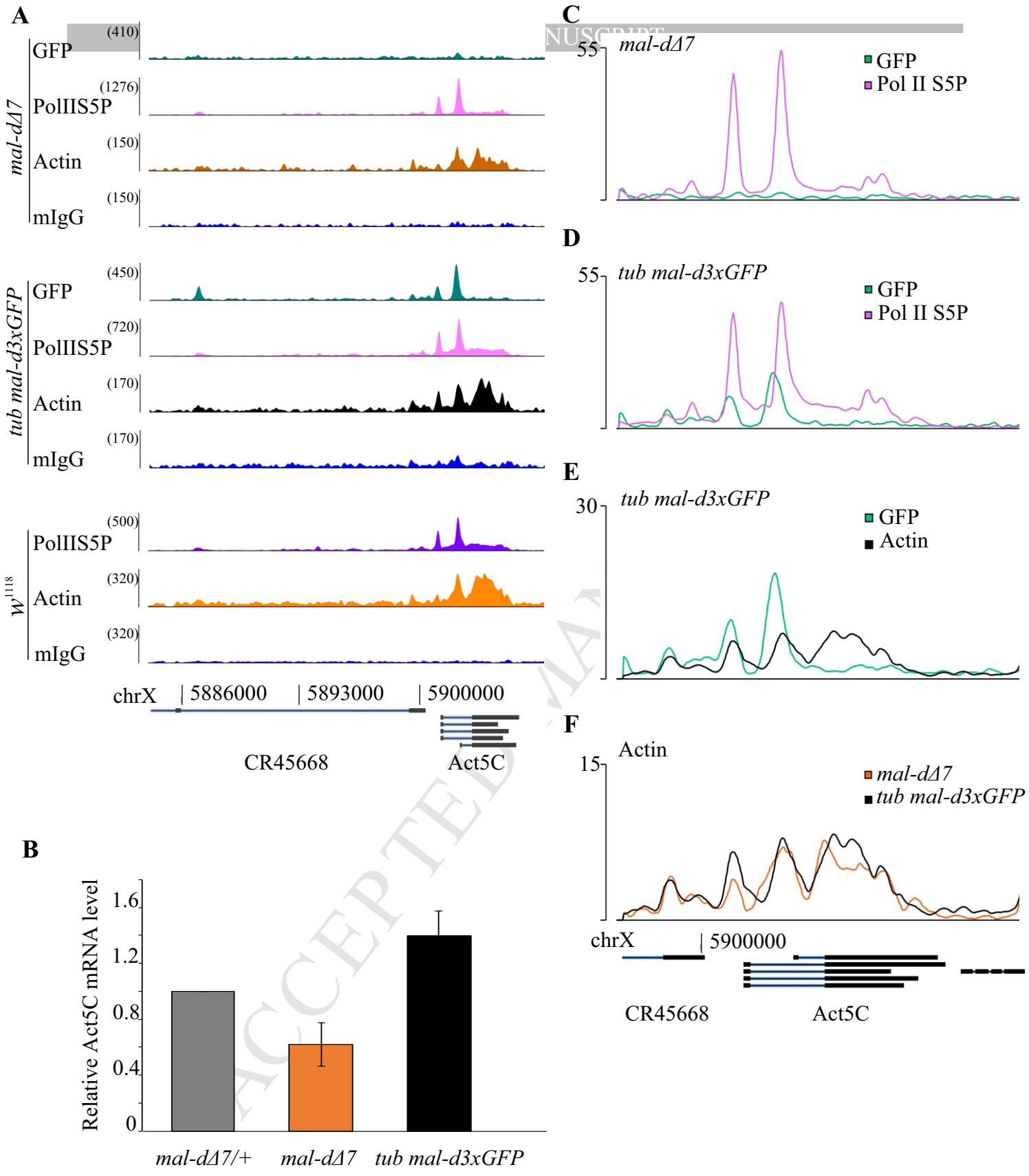


Figure 2

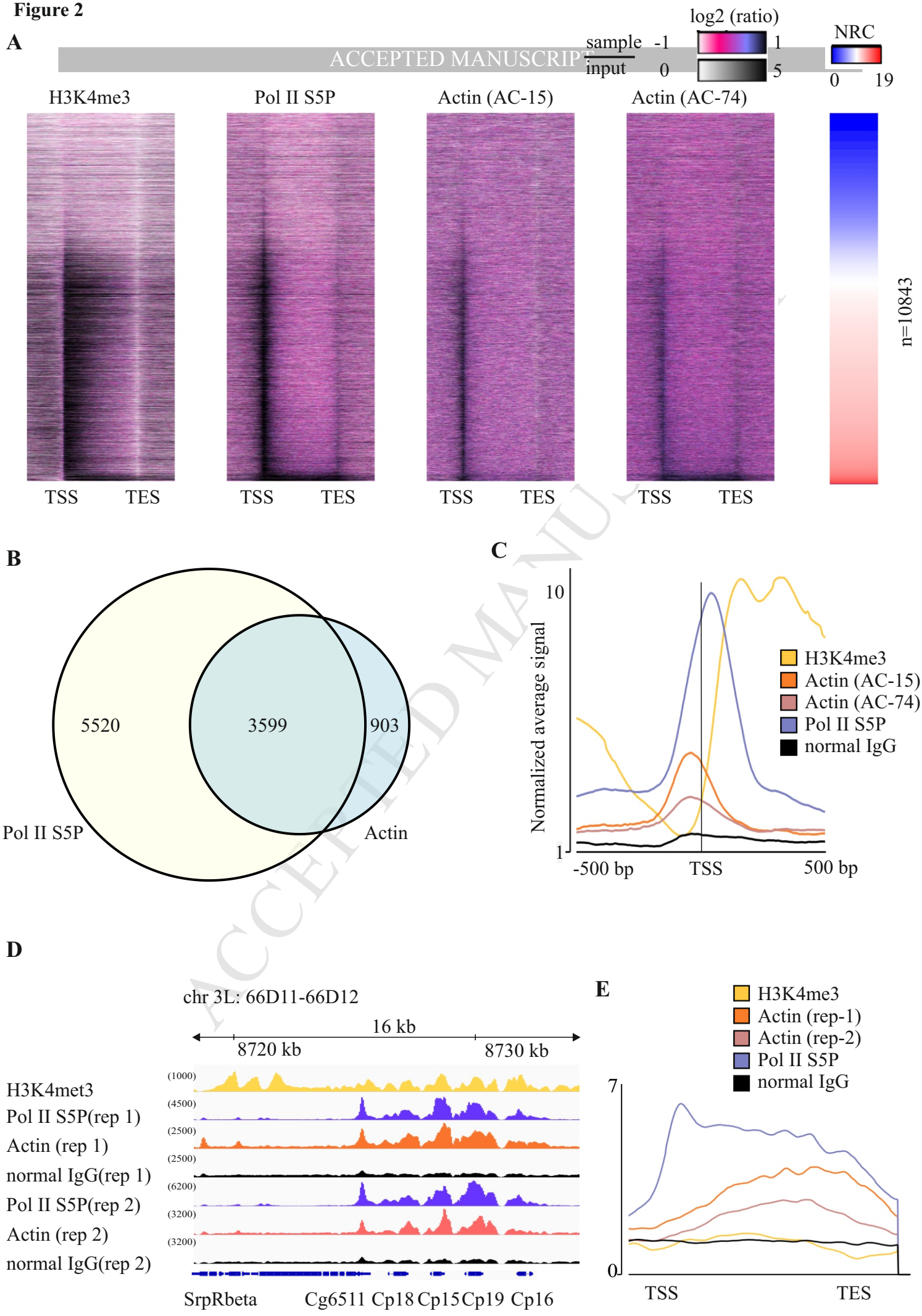
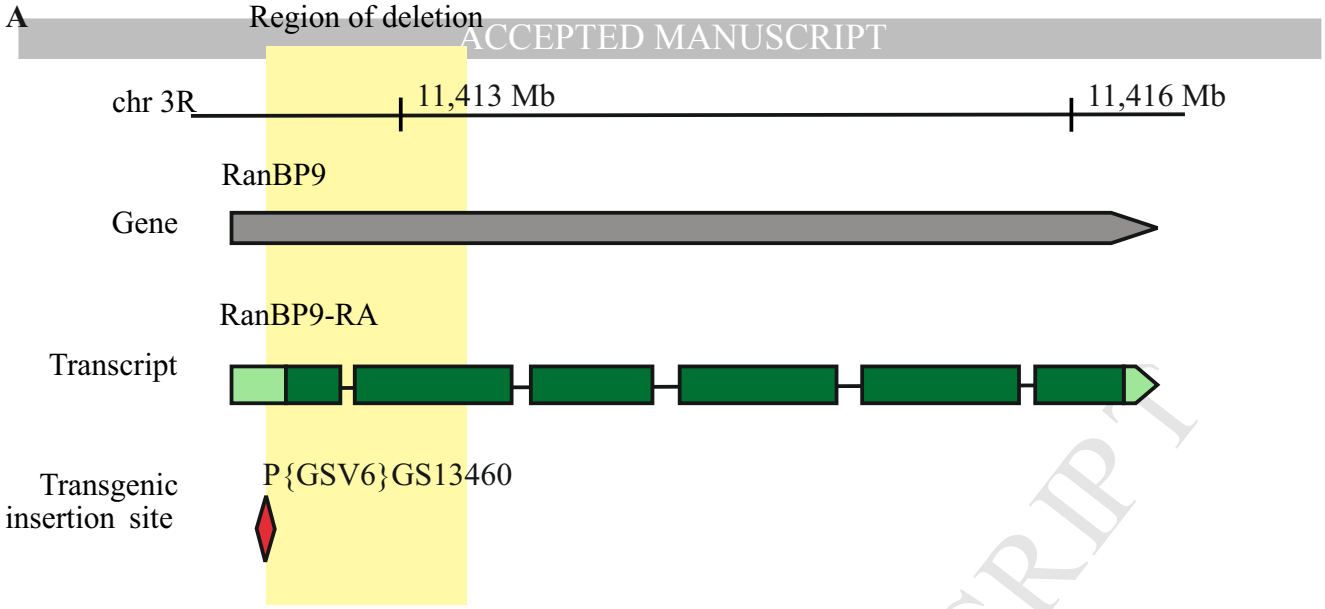
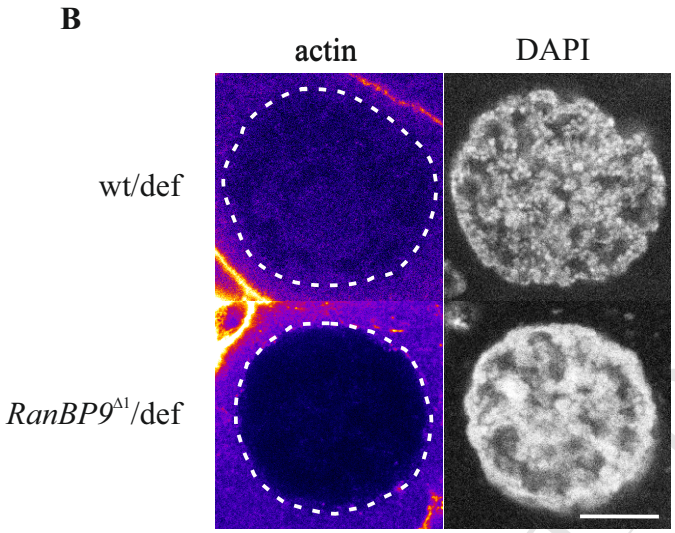
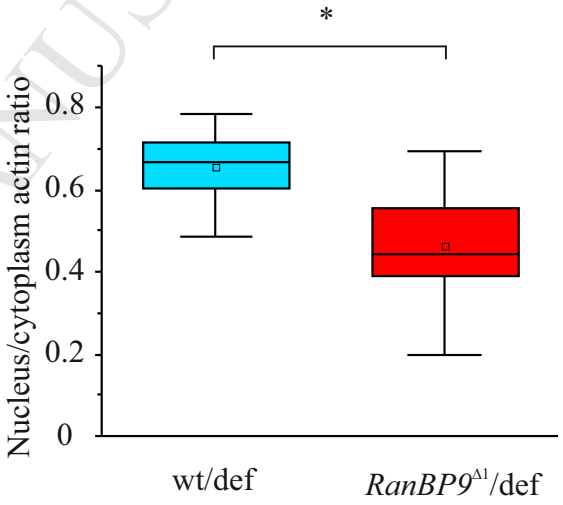
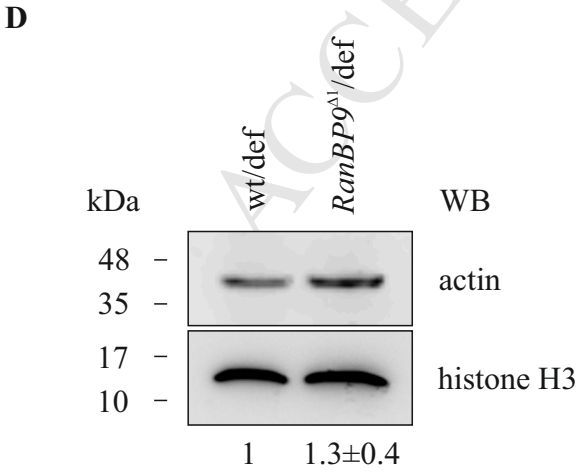


Figure 3









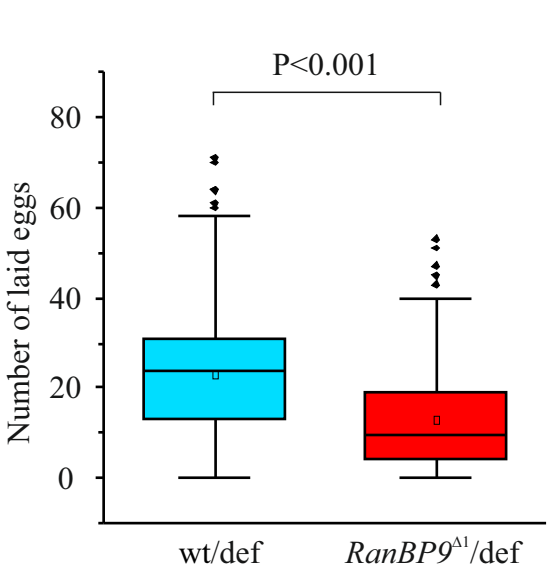
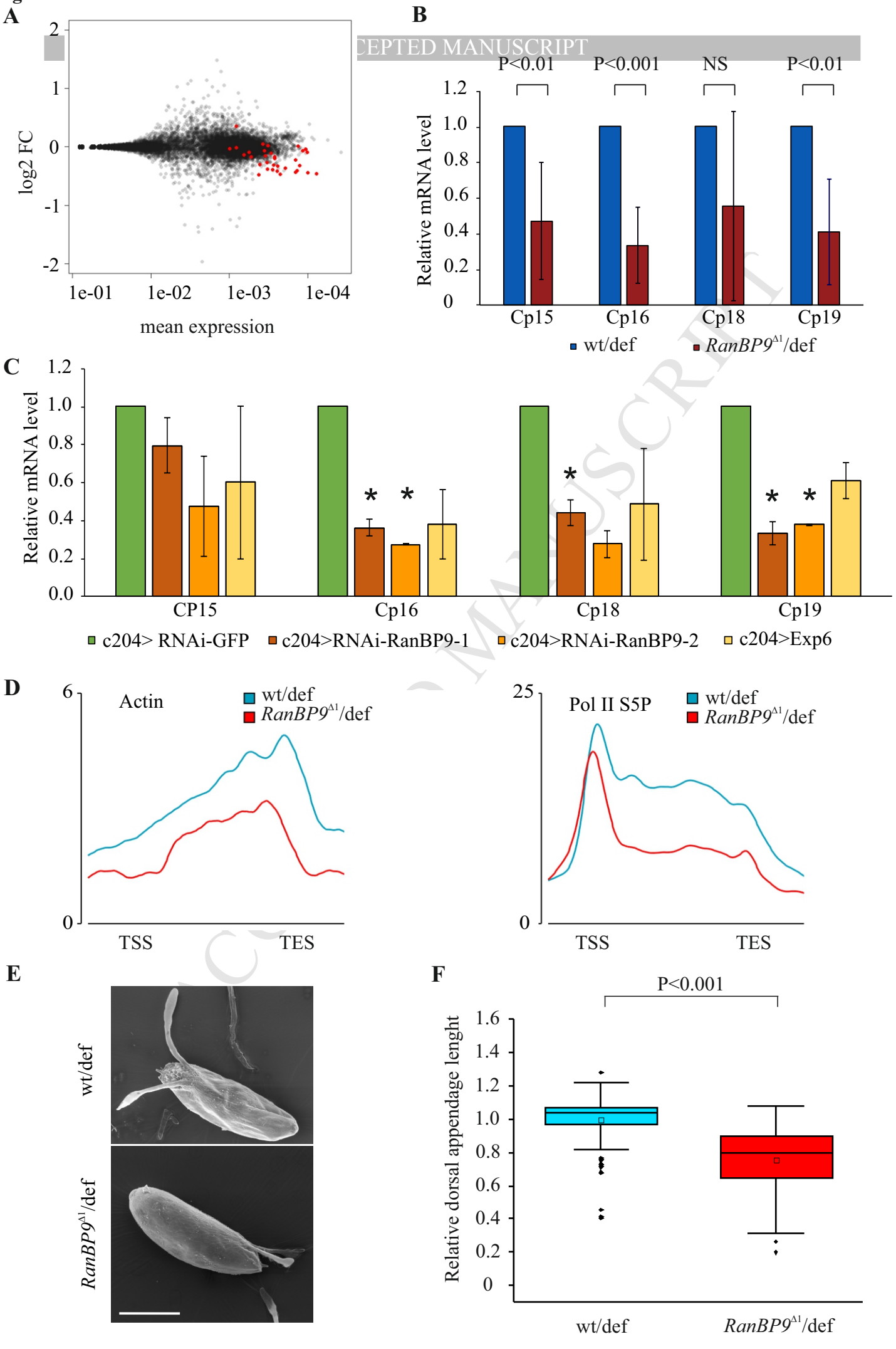


Figure 4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1e-04
B
P<0.01
P<0.001
NS
P<0.01
Relative mRNA level
Cp15
Cp16
Cp18
Cp19
wt/def
RanBP9Δ1/def
C
Relative mRNA level
CP15
Cp16
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Cp19
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c204>RNAi-RanBP9-1
c204>RNAi-RanBP9-2
c204>Exp6
D
Actin
wt/def
RanBP9Δ1/def
TSS
TES
Pol II S5P
E
wt/def
RanBP9Δ1/def
F
P<0.001
Relative dorsal appendage lenght
wt/def
RanBP9Δ1/def

Highlights

- Genome-wide analysis shows actin on all transcribed genes
- Actin binds with RNA polymerase II near transcription start sites of most genes
- On highly expressed genes, actin is also found on the gene bodies
- Nuclear transport of actin is required for transcription during fly development