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A

В

Figure S1. Sequence alignments of the CTDs of Drosophila, related to Figure 1B.

(A) Nucleotide sequence alignment of the CTDs from 12 *Drosophila* species. The colors identify nucleotides that are identical (green) or different (magenta) compared with *D. melanogaster*.

(B) Amino acid sequence alignment of the CTDs from 12 *Drosophila* species. The colors identify amino acids that are identical (green), similar (yellow) or dissimilar (magenta) compared with *D. melanogaster*. Pink boxes highlight the only two consensus heptads in the CTD of *D. melanogaster*.









Figure S2. PCR validation of fly lines and additional physiological parameters of selected Rpb1 mutants, related to Figure 3.

(A) PCR validation of various fly lines carrying *UAS-Rpb1* transgenes. One primer was specific to *UAS-Rpb1* insertion and the other hybridized to the body of *Rpb1*.

(B to F) Replacing the endogenous Rpb1 with either Rpb1²⁰⁰⁰ or Rpb1²⁴⁰⁰ using CRISPR-Cas9 produces healthy homozygous flies. (B) Hatch rates of embryos at 24°C, measured 36h after egg deposition, n>350 for each genotype. (C) Percentages of hatched embryos that developed into adults when raised at 24°C, n>300 for each genotype. (D) Hatch rates of embryos at 30°C, measured 36h after egg deposition, n>350 for each genotype. (E) Percentages of hatched embryos that developed into adults when raised at 24°C, n>300 for each genotype. (E) Percentages of hatched embryos that developed into pupae when raised at 30°C, n>400 for each genotype. (F) Quantification of recovery time after 14h of cold shock on ice, n=64 for *Rpb1*_{FLAG}; n=64 for *Rpb1*²⁴⁰⁰ (equal numbers of male and female adults were assayed). Dots represent individual adults. Red bars show the medians and interquartile ranges.

(G) PCR validation of hemizygotic males with the modified endogenous Rpb1 locus using primers flanking the CTD. A male fly with unmodified Rpb1 locus [w(control)] provides the PCR product of the unmodified locus. Changes in the sizes of the PCR products reflect modification of the endogenous Rpb1 locus.

Two-sided chi-squared tests were used for (B) to (E). Mann–Whitney *U* tests were used for (F). ns: not significant (p>0.05), *p < 0.05, **p < 0.01, ***p < 0.001 or ****p < 0.0001 values were considered statistically significant from *Rpb1*_{FLAG}.





Figure S3. Fluorescence detection of NLS-GFP derivatives in the nuclei of salivary glands, related to Figure 4 and Figure 5. Expression was driven by *Sgs3-GAL4*. Scale bar represents 50 μ m.





Figure S4. GFP-DmCTD forms static and dynamic foci, part of which is sensitive to 1,6-hexanediol, related to Figure 4.

(A) FRAP analysis reveals that GFP-DmCTD containing foci can be static (upper) or dynamic (lower).

(B) Treatment with 1% 1,6-hexanediol dissolved a subset of GFP-DmCTD foci. Maximum intensity projections of entire Z stacks. The diffuse fluorescence detected after 1,6-hexanediol treatment is due to the release of GFP-DmCTD from the chromosomes.

Both scale bars represent 5 µm.

Single-stack images В GFP-20con ← Early prepupal Stationary larval stage stage С D GFP-24con Stationary Early prepupal \leftrightarrow larval stage stage F ← Early prepupal Stationary larval stage stage G Н

GFP-52con (Maximum Intensity Projections of E, G, H and I respectively)





GFP predominantly enriched in a few large clusters of static compartments (as in panels I & M)

GFP predominantly enriched in numerous small clusters of static compartments (as in panels H & L)

GFP significantly enriched in static compartments yet GFP bands remain distinctive (as in panels G & K)

GFP much more enriched on chromosomes than in static compartments (as in panels E & J)

Figure S5. Varying the CTD sequence composition alters its distribution in the nucleus, related to Figure 5.

(A to I) Single-stack confocal images of nuclei in live salivary glands expressing NLS-GFP-20con (A and B), NLS-GFP-24con (C and D) and NLS-GFP-52con (E to I). A, C and E were collected from the stationary larval stage whereas B, D and F were collected from the early prepupal stage. Examples depicted in G, H and I can be observed in both stages. Scale bar represents 5 μ m. Red arrows demarcate examples of puffs that show enrichment over the nucleoplasm.

(J to M) Maximum intensity projections of the same nuclei shown in E, G, H and I, respectively.

(N) GFP-52con cells are more prone to partition in static compartments. Sample sizes are indicated in parentheses. Darker shades of gray indicate that more CTD associates with static compartments than with chromosomes. p<0.0001, two-sided chi-squared tests.