Supporting Information

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SI Text

Supplement 1: Determination of DNA – Scr-HD Binding Constants in the Polytene Nucleus. ***Nonspecific interactions.*** Nonspecific interactions between DNA and Scr-HD$_{DD}$ can be described as a reversible reaction:

$$\mathrm{DNA} + \mathrm{ScrHD_{DD}} \underset{k_{-ns}}{\overset{k_{ns}}{\Leftrightarrow}} \mathrm{DNA-ScrHD_{DD}}, \qquad \textbf{[S1]}$$

where k_{ns} and k_{-ns} are rate constants for the formation and dissociation of the nonspecific complex between Scr-HD$_{DD}$ and DNA, respectively. At equilibrium the forward and backward reactions are equal:

$$k_{ns} \cdot [\mathrm{DNA}] \cdot [\mathrm{ScrHD_{DD}}] = k_{-ns} \cdot [\mathrm{DNA-ScrHD_{DD}}] \qquad \textbf{[S2]}$$

and the concentration of nonspecifically bound Scr-HD$_{DD}$ is:

$$[\mathrm{DNA-ScrHD_{DD}}] = \frac{k_{ns}}{k_{-ns}} \cdot [\mathrm{DNA}] \cdot [\mathrm{ScrHD_{DD}}]. \qquad \textbf{[S3]}$$

The total concentration of the bound and unbound reactive species is constant and equal to the respective initial concentrations. Therefore, concentrations of the reactive species at any time are:

$$[\mathrm{ScrHD_{DD}}] = [\mathrm{ScrHD_{DD}}]_0 - [\mathrm{DNA-ScrHD_{DD}}] \qquad \textbf{[S4]}$$

$$[\mathrm{DNA}] = [\mathrm{DNA}]_0 - [\mathrm{DNA-ScrHD_{DD}}] \qquad \textbf{[S5]}$$

The total concentration of potential binding sites in a polytene nuclei is noticeably higher than the total concentration of the transcription factor under physiologically relevant conditions. Changes in the DNA concentration due to Scr-HD$_{DD}$ binding can be therefore neglected and the DNA concentration can be regarded as constant and equal to the total DNA concentration:

$$[\mathrm{DNA}] \approx [\mathrm{DNA}]_0. \qquad \textbf{[S6]}$$

Introducing relations **S4** and **S5** in Eq. **S6** gives:

$$[\mathrm{DNA-ScrHD_{DD}}] = \frac{k_{ns}}{k_{-ns}} \cdot [\mathrm{DNA}]_0 \cdot ([\mathrm{ScrHD_{DD}}]_0 - [\mathrm{DNA-ScrHD_{DD}}]) \qquad \textbf{[S7]}$$

$$\left(1 + \frac{k_{ns}}{k_{-ns}} \cdot [\mathrm{DNA}]_0\right)[\mathrm{DNA-ScrHD_{DD}}] = \frac{k_{ns}}{k_{-ns}} \cdot [\mathrm{DNA}]_0 \cdot [\mathrm{ScrHD_{DD}}]_0 \qquad \textbf{[S8]}$$

$$[\mathrm{DNA-ScrHD_{DD}}] = \frac{\frac{k_{ns}}{k_{-ns}}}{\left(1 + \frac{k_{ns}}{k_{-ns}} \cdot [\mathrm{DNA}]_0\right)} \cdot [\mathrm{DNA}]_0 \cdot [\mathrm{ScrHD_{DD}}]_0 \qquad \textbf{[S9]}$$

$$[\mathrm{DNA-ScrHD_{DD}}] = \frac{k_{ns}}{(k_{-ns} + k_{ns} \cdot [\mathrm{DNA}]_0)} \cdot [\mathrm{DNA}]_0 \cdot [\mathrm{ScrHD_{DD}}]_0. \qquad \textbf{[S10]}$$

As expected, concentration of the nonspecific complex [DNA-Scr-HD$_{DD}$] increases linearly with the concentration of the transcription factor.

Using the data from FCS measurements (Fig. 5, *Black Line*), we estimate the dissociation constant for the nonspecific DNA – Scr-HD DD complex to be:

$$\frac{k_{ns} \cdot [\mathrm{DNA}]_0}{(k_{-ns} + k_{ns} \cdot [\mathrm{DNA}]_0)} = (0.35 \pm 0.15) \qquad \textbf{[S11]}$$

$$k_{ns} \cdot [\mathrm{DNA}]_0 = 0.35 \cdot (k_{-ns} + k_{ns} \cdot [\mathrm{DNA}]_0) \qquad \textbf{[S12]}$$

$$(1 - 0.3) k_{ns} \cdot [\mathrm{DNA}]_0 = 0.35 \cdot k_{-ns} \qquad \textbf{[S13]}$$

$$k_{ns} \cdot [\mathrm{DNA}]_0 = \frac{0.35}{(1 - 0.35)} \cdot k_{-ns} \qquad \textbf{[S14]}$$

$$k_{ns} \cdot [\mathrm{DNA}]_0 = 0.54 \cdot k_{-ns} \qquad \textbf{[S15]}$$

$$\frac{k_{-ns}}{k_{ns}} = K_d^{\mathrm{ScrHD_{DD},ns}} = \frac{[\mathrm{DNA}]_0}{0.54} \qquad \textbf{[S16]}$$

Nonspecific and Specific Interactions. The transcriptionally active variants of Scr-HD$_{wt}$ and Scr-HD$_{AA}$, undergo both specific and nonspecific interactions with DNA. Assuming that nonspecific interactions precede the specific ones, a two-step process of consecutive reactions was anticipated:

$$\mathrm{DNA_{ns}} + \mathrm{ScrHD_{wt}} \underset{k_{-ns}}{\overset{k_{ns}}{\Leftrightarrow}} (\mathrm{DNA-ScrHD_{wt}})_{ns}$$

$$(\mathrm{DNA-ScrHD_{wt}})_{ns} + \mathrm{DNA}_s \underset{k_{-s}}{\overset{k_s}{\Leftrightarrow}} (\mathrm{DNA-ScrHD_{wt}})_s \qquad \textbf{[S17]}$$

The turnover rate for the nonspecific complex is:

$$\frac{d[(\mathrm{DNA-ScrHD_{wt}})_{ns}]}{dt} = k_{ns} \cdot [\mathrm{DNA_{ns}}] \cdot [\mathrm{ScrHD_{wt}}] - (k_{-ns} + k_s \cdot [\mathrm{DNA}_s]) \cdot [(\mathrm{DNA-ScrHD_{wt}})_{ns}] + k_{-s} \cdot [(\mathrm{DNA-ScrHD_{wt}})_s] \qquad \textbf{[S18]}$$

Assuming a quasi-steady state approximation:

$$\frac{d[(\mathrm{DNA-ScrHD_{wt}})_{ns}]}{dt} = 0 \qquad \textbf{[S19]}$$

$$(k_{-ns} + k_s \cdot [\mathrm{DNA}_s]) \cdot [(\mathrm{DNA-ScrHD_{wt}})_{ns}] = k_{ns} \cdot [\mathrm{DNA_{ns}}] \cdot [\mathrm{ScrHD_{wt}}] + k_{-s} \cdot [(\mathrm{DNA-ScrHD_{wt}})_s]. \qquad \textbf{[S20]}$$

Using the mass balance equation to express the concentration of the free transcription factor:

$$[\mathrm{ScrHD_{wt}}] = [\mathrm{ScrHD_{wt}}]_0 - [(\mathrm{DNA-ScrHD_{wt}})_{ns}] - [(\mathrm{DNA-ScrHD_{wt}})_s] \qquad \textbf{[S21]}$$

and assuming as previously that:

$$[\mathrm{DNA}]_{ns} \approx [\mathrm{DNA}]_0, \qquad \textbf{[S22]}$$

Eq. **S23** becomes:

$$\begin{aligned}&(k_{-\mathrm{ns}}+k_s\cdot[\mathrm{DNA}_s])\cdot[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_{\mathrm{ns}}]\\&=k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0\cdot([\mathrm{ScrHD}_{\mathrm{wt}}]_0-[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_{\mathrm{ns}}]\\&-[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_s])+k_{-s}\cdot[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_s]\end{aligned}\quad\textbf{[S23]}$$

$$\begin{aligned}&(k_{-\mathrm{ns}}+k_s\cdot[\mathrm{DNA}_s]+k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0)\cdot[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_{\mathrm{ns}}]\\&=k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0\cdot([\mathrm{ScrHD}_{\mathrm{wt}}]_0-[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_s])\\&+k_{-s}\cdot[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_s]\end{aligned}\quad\textbf{[S24]}$$

$$\begin{aligned}&(k_{-\mathrm{ns}}+k_s\cdot[\mathrm{DNA}_s]+k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0)\cdot[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_{\mathrm{ns}}]\\&=k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0\cdot[\mathrm{ScrHD}_{\mathrm{wt}}]_0\\&-(k_{\mathrm{ns}}\cdot[\mathrm{DNA}]-k_{-s})\cdot[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_s]\end{aligned}\quad\textbf{[S25]}$$

$$\begin{aligned}[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_{\mathrm{ns}}]=&\frac{k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0}{k_{-\mathrm{ns}}+k_s\cdot[\mathrm{DNA}_s]+k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0}\\&\cdot[\mathrm{ScrHD}_{\mathrm{wt}}]_0\\&-\frac{k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0-k_{-s}}{k_{-\mathrm{ns}}+k_s\cdot[\mathrm{DNA}_s]+k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0}\\&\cdot[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_s].\end{aligned}\quad\textbf{[S26]}$$

According to Eq. **S26** and the FCS data presented in Fig. 5 (*Red Stars*), the slope of the linear dependence gives:

$$\frac{k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0}{k_{-\mathrm{ns}}+k_s\cdot[\mathrm{DNA}_s]+k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0}=(0.60\pm0.05)\quad\textbf{[S27]}$$

and the intercept:

$$\begin{aligned}&\frac{k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0-k_{-s}}{k_{-\mathrm{ns}}+k_s\cdot[\mathrm{DNA}_s]+k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0}\cdot[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_s]\\&=(50\pm30)\ \mathrm{nM}.\end{aligned}\quad\textbf{[S28]}$$

If k_{-s} is small compared to $k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0$ and can therefore be neglected, then:

$$0.6\cdot[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_s]=50\ \mathrm{nM}.\quad\textbf{[S29]}$$

Thus, the concentration of specific complex between *Scr*-HD wild type and DNA in the polytene nucleus can be estimated to be:

$$[(\mathrm{DNA}-\mathrm{ScrHD}_{\mathrm{wt}})_s]=(80\pm50)\ \mathrm{nM}.\quad\textbf{[S30]}$$

FCS data enabled us also to estimate the dissociation constant for the nonspecific DNA – Scr-HD wt complex (Fig. 5, *Dotted Red Line*):

$$\frac{k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0}{(k_{-\mathrm{ns}}+k_{\mathrm{ns}}\cdot[\mathrm{DNA}]_0)}=(0.65\pm0.05)\quad\textbf{[S31]}$$

$$\frac{k_{-\mathrm{ns}}}{k_{\mathrm{ns}}}=K_d^{\mathrm{ScrHD}_{\mathrm{wt,ns}}}=\frac{[\mathrm{DNA}]_0}{1.9}.\quad\textbf{[S32]}$$

Supplement 2: Calculation of the Concentration of DNA Sites for Specific and Nonspecific Binding of the Scr-HD Within a Polytene Nucleus. The Drosophila haploid genome size is 1.76×10^8 bp long; this means that the diploid genome size is $2\times1.76\times10^8=3.52\times10^8$ bp long.

Polytene cells normally undergo 10 DNA-replication cycles without cell division, thus $2^{10}=1024$ chromosomal copies are produced.

This results in $1024\times3.52\times10^8=3.6\times10^{11}$ bp within a polytene nucleus.

However, a protein molecule can interact (bind specifically or nonspecifically) only with a few base-pairs of the DNA.

A) For nonspecific binding (i.e., binding to DNA sequences that do not belong to the putative *cis*-regulatory elements bound by Scr) we assume on the average a 6 bp DNA interaction domain with the consensus sequence TAATCG or TAATGG found for Scr, Unx, and Antp (1–4). Taking into consideration that this consensus occurs every 2048 bp in the genome, this results in $\frac{3.6\times10^{11}}{2048}=1.758\times10^8$ sites within a polytene nucleus. 1 mol of potential sites contains 6.023×10^{23} sites.

n mol of potential sites contain 1.758×10^8 sites, thus $n=\frac{1.758\times10^8}{6.023\times10^{23}}=0.292\times10^{-15}$ mol of nonspecific sites within the polytene nucleus.

However, the Drosophila euchromatin comprises 117 Mbp (5), which means that only 66.5% of the total DNA length can be considered as candidate sequences for transcription factor–DNA interactions. This results in $0.292\times10^{-15}\times0.665=0.194\times10^{-15}$ mol of binding sites.

Experimentally we have determined the diameter of a polytene nucleus as 20 μm, thus the radius is 10 μm. The volume of the nucleus will be

$$\begin{aligned}V_{\mathrm{nucleus}}=&\frac{4}{3}\pi r^3=\frac{4}{3}\times3.14\times(10\times10^{-6})^3\ \mathrm{m}^3=4.187\times10^{-15}\ \mathrm{m}^3\\&=4.187\times10^{-15}\times10^3\ (\mathrm{dm})^3=4.187\times10^{-12}\ \mathrm{L}.\end{aligned}\quad\textbf{[S33]}$$

The concentration of the potential DNA-transcription factor binding sites will be thus

$$C_{\mathrm{sites}}^{\mathrm{non-specific}}=\frac{n}{V_{\mathrm{nucleus}}}=\frac{1.94\times10^{-16}}{4.187\times10^{-12}}=46\ \mu\mathrm{M}.\quad\textbf{[S34]}$$

B) For specific binding to occur, usually a larger sequence is necessary. One example are the *fkh250* and *fkh*250$^{\mathrm{con}}$ elements, which consist of 10 bp (AGATTAATCG) and can bind either an Scr-Exd or a Ubx-Exd complex, respectively (6). Assuming thus a minimum requirement of 10 bp to confer Scr binding specificity, this sequence may occur once every $4^{10}=1048576\cong1.048\times10^6$ bp in the genome.

This results in $\frac{3.65\times10^{11}}{1.048\times10^6}=3.48\times10^5$ sites within a polytene nucleus.

1 mol of potential sites contains 6.023×10^{23} sites.

n mol of potential sites contain 3.48×10^5 sites thus $n=\frac{3.48\times10^5}{6.023\times10^{23}}=0.578\times10^{-18}$ mol.

Thus the concentration of specific binding sites within the polytene nucleus will be

$$C_{\mathrm{sites}}^{\mathrm{specific}}=\frac{n}{V_{\mathrm{nucleus}}}=\frac{0.578\times10^{-18}\times0.665}{4.187\times10^{-12}}=92\ \mathrm{nM}.\quad\textbf{[S35]}$$

Supplement 3: Isoelectric Points of *Scr*-HD Variants. Isoelectric points for *Scr*-HD variants fused with mCitrine were estimated using the Protein Calculator v3.3 that is available on-line (http://www.scripps.edu/cgi-bin/cdputnam/protcalc3). The primary sequences given below were submitted and the charge at different pH was estimated by the program (Table S1).

mCitrine-*Scr*-HD$_{\mathrm{wt}}$

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEG-DATYGKLTLKFICTTGKLPVPWPTLVTTFGYGLMCFARY-PDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAE-VKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHN-

VYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNT-PIGDGPVLLPDNHYLSYQSALSKDPNEKRDHMVLLEFVT-AAGITLGMDELYKLEPPQIYPWMKRVHLGTSTVNANGE-TKRQRTSYTRYQTLELEKEFHFNRYLTRRRRIEIAHALC-LTERQIKIWFQNRRMKWKKEHKMASMNIVPYHMGPYG-HPYHQFDIHPSQFAHLSA

mCitrine-Scr-HD$_{AA}$

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEG-DATYGKLTLKFICTTGKLPVPWPTLVTTFGYGLMCFARY-PDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAE-VKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNV-YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPI-GDGPVLLPDNHYLSYQSALSKDPNEKRDHMVLLEFVTA-AGITLGMDELYKLEPPQIYPWMKRVHLGTSTVNANGET-KRQRAAYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCL-TERQIKIWFQNRRMKWKKEHKMASMNIVPYHMGPYG-HPYHQFDIHPSQFAHLSA

mCitrine-Scr-HD$_{DD}$

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEG-DATYGKLTLKFICTTGKLPVPWPTLVTTFGYGLMCFAR-YPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRA-EVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSH-NVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQN-TPIGDGPVLLPDNHYLSYQSALSKDPNEKRDHMVLLEF-VTAAGITLGMDELYKLEPPQIYPWMKRVHLGTSTVNA-NGETKRQRDDYTRYQTLELEKEFHFNRYLTRRRRIEI-AHALCLTERQIKIWFQNRRMKWKKEHKMASMNIVPY-HMGPYGHPYHQFDIHPSQFAHLSA

mCitrine-Scr-HD$_{DD}^{Q50AN51A}$

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEG-DATYGKLTLKFICTTGKLPVPWPTLVTTFGYGLMCFAR-YPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTR-AEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNS-HNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQ-NTPIGDGPVLLPDNHYLSYQSALSKDPNEKRDHMVLLE-FVTAAGITLGMDELYKLEPPQIYPWMKRVHLGTSTVNA-NGETKRQRDDYTRYQTLELEKEFHFNRYLTRRRRIEIA-HALCLTERQIKIWFAARRMKWKKEHKMASMNIVPYHM-GPYGHPYHQFDIHPSQFAHLSA

Supplement 4: Materials and Methods. ***Immunohistochemistry.*** Salivary glands from third instar wandering larvae were dissected at room temperature (RT) by pulling the mouth-hooks from the larval body in Grace's medium (Gibco) and and the whole tissue was fixed for 10 min in Grace's medium containing 5.3% EM-grade paraformaldehyde (Electron Microscopy Science, PA) freshly used every 10 days. After twice washed for 15 min in PBT (PBS containing 0.1% TritonX-100) and blocked for at least 30 min in PBT containing 5% normal goat serum (NGS) and 20 μg/mL NaN_3, samples were incubated with primary antibodies in blocking solution overnight. Subsequently, samples were washed 3 times for 20 min in PBT and secondary antibodies were applied in PBS for 4 h at RT. After 3 washes for 20 min with PBT and equilibration in PBS, the salivary glands were separated from the mouth-hooks and mounted in Vectashield (Vector Laboratories). Images were obtained within 24 h using a Leica SP5 Confocal setup.

Purification of mCitrine-Scr-HD$_{wt}$. mCitrine-Scr-HD$_{wt}$ was cloned as an *Eco*RI-*Not*I fragment into the pET-21b(+) vector (Novagen), and the 6× histidine-tagged peptide produced in Rosetta™2 (DE3)pLysS cells (Novagen). Purification of a small amount of recombinant protein was performed using the ProBond™ Purification System (Invitrogen). Expression of the peptide was confirmed by SDS-PAGE and Western blotting using a mouse anti-GFP antibody (Molecular Probes). Protein was concentrated and used for FCS analysis in solution.

Preparation of salivary glands for FCS and heat shock procedures. For FCS measurements 3–4 pairs of salivary glands were dissected from third instar wandering larvae in Grace's medium and transferred to chambered slides (Nalge Nunc International) containing PBS. For titrating the concentration of Scr-HD in the nucleus, variable (from 10 min up to 2 h) heat-shocks were applied to the larvae prior to dissection. Then, larvae were dissected within short time and used in the measurements.

APD imaging. An individually modified instrument (Zeiss, LSM 510, ConfoCor 3) with fully integrated FCS/CLSM optical pathways was used for imaging. The detection efficiency of CLSM imaging was significantly improved by the introduction of APD detectors. As compared to PMTs, which are normally used as detectors in conventional CLSM, the APDs are characterized by higher quantum yield and collection efficiency—about 70% in APDs as compared to 15–25% in PMTs, higher gain, negligible dark current and better efficiency in the red part of the spectrum. Enhanced fluorescence detection efficiency enabled image collection using fast scanning (1–5 μs/pixel). This enhances further the signal-to-noise-ratio by avoiding fluorescence loss due to triplet state formation, enabling fluorescence imaging with single-molecule sensitivity. In addition, low laser intensities (150–750 μW) could be applied for imaging, significantly reducing the photo-toxicity. Advantages of APD imaging for nondestructive observation of molecular interactions in real time in live cells are described in detail in reference 23, listed in the main text.

Short background on fluorescence correlation spectroscopy (FCS). FCS measurements are performed by recording fluorescence intensity fluctuations in a very small, approximately ellipsoidal volume element (about 400 nm wide and 1.5 μm long) that is generated in the salivary gland nuclei by focusing the laser light through the objective of the microscope. The fluorescence intensity fluctuations, caused by fluorescently labeled molecules passing through the volume element, are analyzed using statistical methods. Temporal autocorrelation analysis was used in this study, but other methods can be also applied. For recent reviews on FCS see references 27–29 in the main text.

In temporal autocorrelation analysis we first derive the autocorrelation function $G(t)$:

$$G(\tau) = 1 + \frac{\langle \delta I(t) \delta I(t+\tau) \rangle}{\langle I \rangle^2}, \qquad \textbf{[S36]}$$

that gives the correlation between the intensity of light, $I(t)$, measured at a certain time, t, and its intensity, $I(t+\tau)$, measured at a later time $t+\tau$. For further analysis, an autocorrelation curve is derived by plotting $G(\tau)$ as a function of different lags, i.e. different autocorrelation times τ. See for example Figs 2–4 in the main text. The amplitude of the autocorrelation function is reciprocally proportional to the average number of molecules in the observation volume. Thus, the amplitude of the autocorrelation curve decreases for increasing number of molecules (Fig. 2*D*, Fig. 4*A* and *B*). The inflection points on the autocorrelation curves reflect the characteristic times for the investigated molecular processes.

The experimentally obtained autocorrelation curves are compared to autocorrelation functions derived for different model systems. A model describing free diffusion of two components and triplet formation was used in this study:

$$G(\tau) = 1 + \frac{1}{N} \cdot \left(\frac{1-y}{(1+\frac{\tau}{\tau_{D1}})\sqrt{1+\frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_{D1}}}} + \frac{y}{(1+\frac{\tau}{\tau_{D2}})\sqrt{1+\frac{w_{xy}^2}{w_z^2}\frac{\tau}{\tau_{D2}}}} \right) \cdot \left[1 + \frac{T}{1-T} \exp\left(-\frac{\tau}{\tau_T} \right) \right] \qquad \textbf{[S37]}$$

In the above equation, N is the average number of molecules in the observation volume element, y is the fraction of the slowly moving Scr-HD molecules, τ_{D1} is the diffusion time of the free Scr-HD molecules, τ_{D2} is the diffusion time of Scr-HD molecules undergoing interactions with the DNA, w_{xy} and w_z are radial and axial parameters, respectively, related to spatial properties of the detection volume element, T is the average equilibrium fraction of molecules in triplet state and τ_T the triplet correlation time, related to rate constants for intersystem crossing and the triplet decay. Spatial properties of the detection volume, represented by the square of the ratio of the radial and axial parameters ($(w_{xy}/w_z)^2$), are determined in calibration measurements performed by using a solution of a fluorescent dye Rhodamine 6G for which the diffusion coefficient (D) is known. The diffusion time, τ_D, measured by FCS, is related to the translation diffusion coefficient D by:

$$\tau_D = \frac{w_{xy}^2}{4D}. \qquad \text{[S38]}$$

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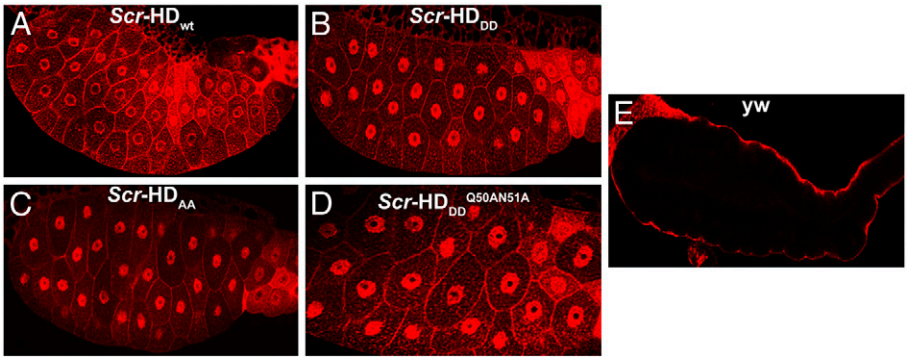

Fig. S1. Flies carrying UAS constructs but no Gal4 driver constructs express the transgenes in small amounts at normal conditions. (*A–E*) Immunostaining of salivary glands of third instar wandering larvae raised at 25 °C, carrying UAS–mCitrine–*Scr*-HD constructs (without any Gal4 driver). All glands exhibit nuclear localization of the synthetic transcription factor (*A–D*), as compared to the yw^{1118} control (injection background) (*E*). The transcription factor seemingly follows patterned nuclear distribution among all variants, due to paraformaldehyde fixation and cross-linking with the DNA. The tight association with nuclear DNA persists for Scr-HD_{wt} and Scr-HD_{AA}, but not for Scr-HD_{DD} or Scr-$HD_{DD}^{Q50AN51A}$, when visualized in live cells by APD imaging.

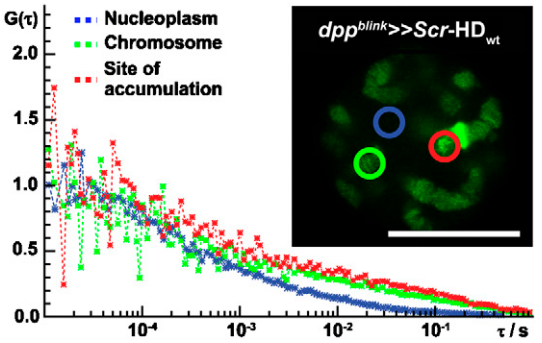

Fig. S2. DNA – Scr-HD_{wt} interactions in different regions of the polytene nucleus. APD imaging of nuclei overexpressing *Scr*-HD_{wt} showed differential nuclear distribution of the transcription factor in the nucleoplasm (*Blue Circle*); on the chromosomes (*Green Circle*) and in sites of accumulation (*Red Circle*). The dynamics of Scr-HD_{wt} is different in the corresponding regions, reflecting differences in its interactions with DNA. Scale bar is 20 μm.

Table S1. Isoelectric points for Scr-HD variants at different pH

	Charge			
pH	mCitrine-Scr-HD_{wt}	mCitrine-Scr-HD_{AA}	mCitrine-Scr-HD_{DD}	mCitrine-Scr-$HD_{DD}^{Q50AN51A}$
7.00	7.2	7.2	5.2	5.2
7.10	6.4	6.4	4.4	4.4
7.20*	**5.7**	**5.7**	**3.7**	**3.7**
7.30*	**5.1**	**5.1**	**3.1**	**3.1**
7.40*	**4.5**	**4.5**	**2.5**	**2.5**
7.50*	**4.0**	**4.0**	**2.0**	**2.0**
7.60*	**3.5**	**3.5**	**1.5**	**1.5**
7.70*	**3.1**	**3.1**	**1.1**	**1.1**
7.80*	**2.7**	**2.7**	**0.7**	**0.7**
7.90*	**2.3**	**2.3**	**0.3**	**0.3**
8.00	1.9	1.9	-0.1	-0.1
8.10	1.5	1.5	-0.5	-0.5
8.20	1.0	1.0	-1.0	-1.0
8.30	0.5	0.5	-1.5	-1.5
8.40	0.0	0.0	-2.0	-2.0
8.50	-0.5	-0.5	-2.5	-2.5
8.60	-1.1	-1.1	-3.1	-3.1
8.70	-1.8	-1.8	-3.8	-3.8
8.80	-2.6	-2.6	-4.6	-4.6
8.90	-3.4	-3.4	-5.4	-5.4

*Indicated values correspond to experimentally measured pH in salivary gland cells isolated from third instar larvae (7).