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Transcription Factor Dynamics

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To predict transcription, one needs a mechanistic understanding of how the numerous required transcription factors (TFs) explore the nuclear space to find their target genes, assemble, cooperate, and compete with one another. Advances in fluorescence microscopy have made it possible to visualize real-time TF dynamics in living cells, leading to two intriguing observations: first, most TFs contact chromatin only transiently; and second, TFs can assemble into clusters through their intrinsically disordered regions. These findings suggest that highly dynamic events and spatially structured nuclear microenvironments might play key roles in transcription regulation that are not yet fully understood. The emerging model is that while some promoters directly convert TF-binding events into on/off cycles of transcription, many others apply complex regulatory layers that ultimately lead to diverse phenotypic outputs. Cracking this kinetic code is an ongoing and challenging task that is made possible by combining innovative imaging approaches with biophysical models.

Cell-fate control rests with a series of proteins generally termed transcription factors (TFs): cell types are the product of the sequential expression of distinct TFs during development and forced expression of the right TF(s) can reprogram cells into different cell fates (Takahashi and Yamanaka 2006). TFs recognize specific sequences within the promoter or enhancer(s), which regulates a given gene (Suter 2020). Once bound, they recruit coactivators and chromatin remodelers, culminating in the assembly at the gene promoter of the preinitiation complex that loads a functional RNA polymerase II (Pol II). Pol II is then licensed for elongation by regulatory complexes and proceeds to synthesize the nascent transcript (Cramer 2019). For the purpose of this review, we adopt an umbrella definition of TFs that, in addition to sequence-specific TFs, also includes chromatin remodelers, general transcription factors (gTFs), Pol II, coactivators, and repressors.

In vitro biochemical assays and in vivo footprinting assays have provided important insights into the DNA sequences targeted by TFs (Lambert et al. 2018). In higher eukaryotes, sequence-specific TFs typically bind thousands of targets to regulate hundreds of genes, although some TFs display increased specialization (Zolotarev et al. 2017), in extreme cases controlling a single gene such as the TF ZNF410 that uniquely controls γ-globin transcription in erythroid cells (Yang et al. 2017; Lan et al. 2020). Because transcription programs are often long-lived (days), TFs have generally been assumed to bind their targets for long periods (Perlmann et al. 1990), consistent with the complexity of

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the transcription machinery, deemed incompatible with rapid assembly. However, over the last two decades, live imaging studies have demonstrated that most TFs are highly dynamic with residence time of seconds (Hager et al. 2009), that different TF subpopulations exhibit specific mobility dynamics, and that TFs often form non-stoichiometric complexes consisting of many molecules (Liu and Tjian 2018). These complexes have been referred to as clusters, condensates, or hubs across the literature. As condensates are often implied to form through phase separation and hubs hint at a functional role, we restrict ourselves to the term cluster here when we refer to those complexes without assumption of their assembly mechanism or function. In parallel, live-cell mRNA imaging experiments have similarly uncovered complex transcription dynamics. Active genes do not synthesize mRNAs steadily over time; rather, transcription occurs stochastically in bursts that alternate with off periods (Rodriguez and Larson 2020). As a result, expression levels, and thus phenotypes, are probabilistic rather than deterministic (Symmons and Raj 2016). Despite recent progress, we still lack mechanistic models linking stochastic transcription kinetics with upstream TF biophysics. In this article, we review our current understanding of TF mobility and discuss mechanisms linking TF dynamics with stochastic transcription outputs.

TECHNIQUES TO CAPTURE TF DYNAMICS

Understanding TF dynamics requires tools to detect where TFs bind in the genome, at what amount, what percentage of TFs are bound to DNA (% bound), and what their association (k_{on}) and dissociation rates with the DNA (k_{off}, the inverse of the TF residence time) are.

In Vitro Binding Specificity

Systematic evolution of ligands by exponential enrichment (SELEX) probes DNA motifs preferentially bound by TFs in vitro (Jolma et al. 2010). A purified TF is incubated with a large library of DNA molecules, from which TF-bound sequences are identified. SELEX may not accurately reflect in vivo binding, and where TFs compete or cooperate with one another, DNA is folded into chromatin and exposed to a very different ionic milieu than in vitro. Nevertheless, SELEX has been used to determine the binding specificity of hundreds of TFs on free and nucleosome-containing DNA, confirming notable differences (Zhu et al. 2018).

In Vivo Binding Specificity

Chromatin immunoprecipitation (ChIP)-seq is widely used to determine in vivo genome-wide binding profiles (Johnson et al. 2007). Chemical cross-linking of a TF of interest to DNA in cells is followed by ChIP and sequencing of TF-bound DNA fragments. Recent variations of the procedure achieve better spatiotemporal resolution than the original methods; for example, digesting fragmented DNA prior to ChIP enables near base-pair resolution (Rhee and Pugh 2011; He et al. 2015). While ChIP traditionally measures average TF occupancy (Fig. 1), recent modifications of the technique enable measurements of kinetic rates (k_{on}, k_{off}):k_{on} can be determined by varying the cross-linking duration (Poorey et al. 2013), whereas measuring bound TFs at various time points after acute depletion of nuclear TFs provides access to genome-wide k_{off} values (Jonge et al. 2020). ChIP, however, is subject to two technical caveats: first, TFs can artificially dissociate from chromosomes upon cross-linking (Teves et al. 2016; Festuccia et al. 2019), which can be overcome by alternatives bypassing cross-linking (Skene and Henikoff 2017), and second, ChIP accuracy is limited by antibody quality (Shah et al. 2018). ChIP-seq and its derivatives have two further limitations: first, they require tens to millions of cells to produce a robust signal, and therefore only provide binding profiles averaged over many cells, and second, free TFs are lost as only the chromatin-bound fraction is captured. Despite these limitations, ChIP-seq remains the sole approach capable of capturing genome-wide target sites of TFs in cells.

Live-Cell Kinetics

Because transcription bursts are heterogeneous among cell populations (Chubb et al. 2006; Raj

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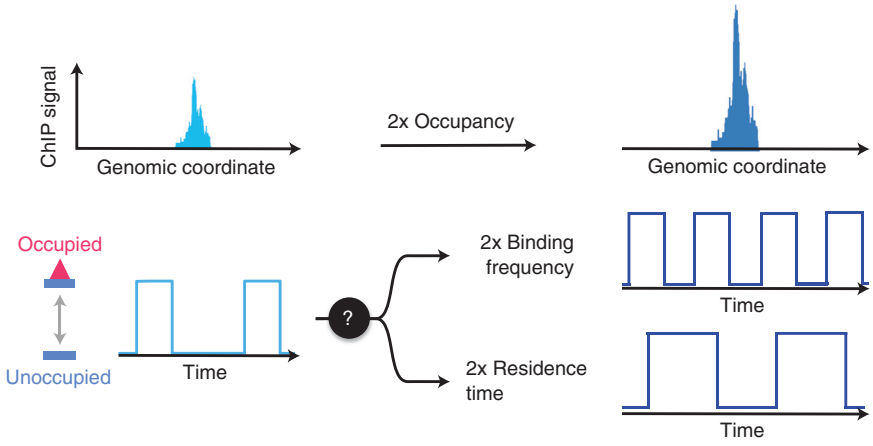

Figure 1. Occupancy versus kinetics. ChIP-seq (*top*) measures the average occupancy of a given transcription factor (TF) at its binding site. Increased occupancy may result from higher TF-binding frequency (higher k_{on}), or longer residence times ($1/k_{off}$, *bottom*). Kinetic profiles can be decoded into distinct transcriptional outputs by promoters.

et al. 2006; Suter et al. 2011), resolving transcription kinetics requires single-cell sensitivity. Given that TFs turn over within seconds, temporal resolution is key for any method to measure in vivo TF dynamics. Furthermore, in situ studies are necessary to recapitulate physiological chromatin states and cofactors. Live imaging satisfies all these conditions, and various methods discussed below have emerged as tools of choice to probe TF dynamics (Mueller et al. 2013).

Fluorescence recovery after photobleaching (FRAP) involves photobleaching fluorescently tagged TFs in a nuclear region of interest, and subsequently measuring fluorescence recovery, typically over seconds to hours, as photobleached TFs exchange with fluorescent TFs freely diffusing into the focal volume from the rest of the nucleus (Phair and Misteli 2000). Fitting recovery curves to reaction-diffusion models provides estimates of average diffusion coefficients of free TFs, residence times of bound TFs, and the relative proportions of the two states (Darzacq et al. 2007; Maiuri et al. 2011).

Fluorescence correlation spectroscopy (FCS) measures the passage of individual molecules through a focused beam (Elf et al. 2007). The number of molecules crossing the beam per unit time provides access to the absolute TF concentration in the chosen region, while the duration each molecule dwells in the focal volume provides similar observables as FRAP, albeit in a different time regime (ms-sec).

Single-molecule tracking (SMT) captures the dynamics of TFs over an entire nuclear plane (Elf and Barkefors 2019). TFs labeled with a photoactivatable fluor are initially dark but upon a brief pulse of blue light, a few TF molecules turn on and are tracked until they either photobleach or diffuse out of the focal volume. Many cycles of activation followed by tracking generate hundreds to thousands of individual trajectories per cell. SMT is usually performed in one of two imaging modes: the fast-tracking mode (exposure times of 1–50 msec per frame) captures fast diffusing TFs in the nucleoplasm, from which one can determine the relative amounts of free versus chromatin-bound TFs and their diffusion coefficients. The slow-tracking mode (exposure times of 100–500 msec per frame) motion-blurs free molecules to calculate the residence times ($1/k_{off}$) of TFs bound to chromatin.

A SHORT STAY AFTER A LONG SEARCH

TF Search Dynamics

Live imaging studies have helped shape a clearer picture of TF behavior. TFs reside on DNA only

for short intervals (1–100 sec) and at any given time, less than half the TF population is bound to chromatin (Figs. 2 and 3; Tables 1 and 2). According to the facilitated diffusion model (von Hippel and Berg 1989), while searching for its target(s), a TF molecule diffuses through the nuclear space in 3D, occasionally binding and briefly sliding along accessible DNA regions (<1 sec) (Chen et al. 2014; Marklund et al. 2020). After several attempts, the TF molecule eventually lands on a cognate binding site, where it resides longer (~10 sec). The average time it takes for a TF to travel between two specific binding sites is defined as its search time,

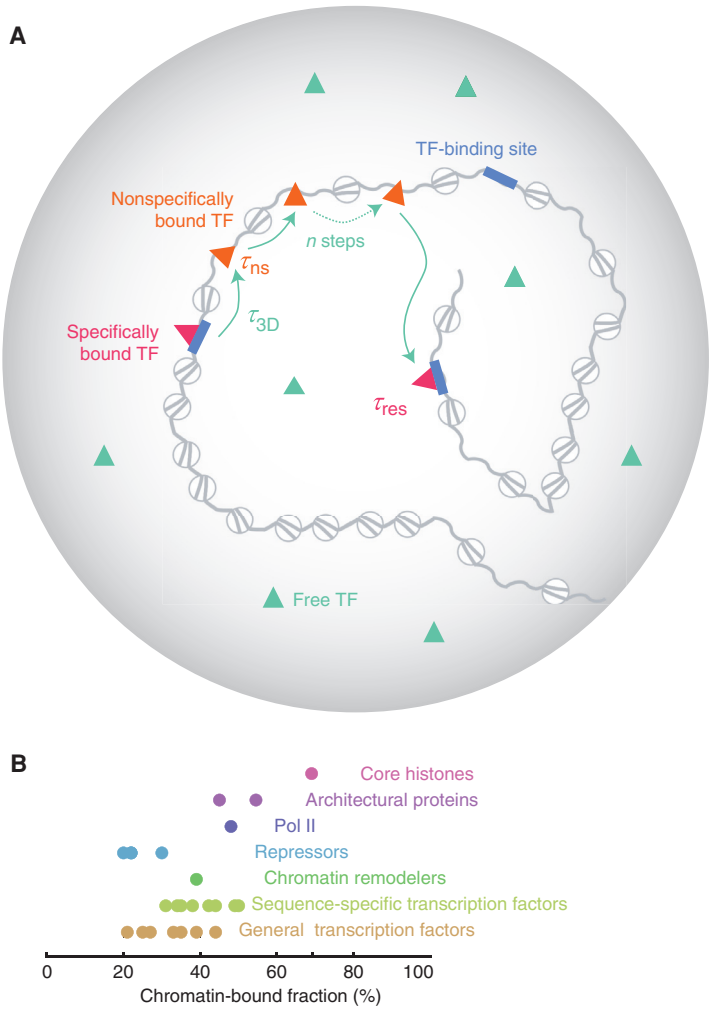

Figure 2. Transcription factor (TF) mobility in the nucleus. (*A*) Facilitated 3D diffusion model. While searching for its target sites (blue), a TF makes multiple, brief, and nonspecific contacts ($\tau_{ns} < 1$ sec; orange) with open chromatin before landing on its cognate site where it dwells longer ($\tau_{res} \sim 1$–100 sec; magenta). The average time between two specific binding events is defined as the search time $\tau_{search} = (n-1)^*(\tau_{3D} + \tau_{ns}) + \tau_{3D}$, where $n \sim 10$–100 is the number of trials and τ_{3D} is the averaged diffusion time between two trials. (Panel *A* is based on data in Chen et al. 2014.) (*B*) Experimentally measured chromatin-bound fraction (circles) for various TFs compiled from the community resource developed by Mir and colleagues (www.mir-lab.com/dynamics-database) and the recent literature. Only factors expressed as knockins or rescuing a knockout background are featured here.

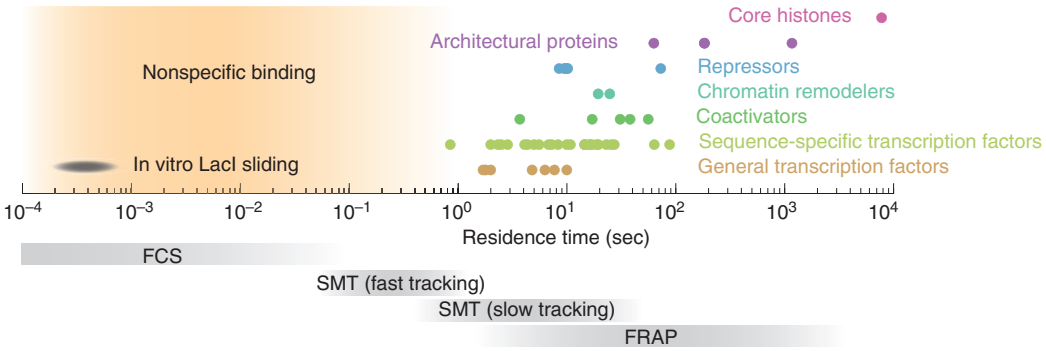

Figure 3. Chromatin association timescales in vivo. Experimentally measured residence times (circles) compiled from the community resource developed by Mir and colleagues (www.mir-lab.com/dynamics-database) and the recent literature, relative to the temporal resolution of different imaging techniques (*bottom*, gray). (FCS) Fluorescence correlation spectroscopy, (SMT) single-molecule tracking, (FRAP) fluorescence recovery after photobleaching.

which is inversely proportional to k_{on}, and sets a limit to how fast a gene can be activated (Elf and Barkefors 2019). As a result of the dozens of nonproductive interactions with chromatin, the search time is often orders of magnitude longer than the TF residence time (Fig. 2). The chromatin-bound fraction consists of both specifically and nonspecifically bound TF molecules and the search time is impacted by the concentration of target sites (Reisser et al. 2018), DNA folding (Cortini and Filion 2018), the chromatin states surrounding the target sites (Mehta et al. 2018), the uneven spatial distribution of protein/DNA barriers in the nucleoplasm (Izeddin et al. 2014; Li et al. 2016), and the presence of coregulators at the target site (Mir et al. 2017). The effective k_{on} is also directly proportional to the nuclear concentration of TFs, enabling dynamical regulation of target occupancy across a large dynamic range (Di Ventura and Kuhlman 2016).

Repressive chromatin complexes are generally smaller than activating ones (Miron et al. 2020), but TF mobility and accessibility to their target sites are largely independent of molecular weights, ruling out steric hindrance as a main partition mechanism (Grünwald et al. 2008; Bancaud et al. 2009; Liu et al. 2014). Lower TF mobility and higher trapping frequencies in heterochromatin appear more likely to explain the observed enrichment of repressive complexes in chromatin-dense regions. Additionally, while 3D diffusing, some TFs move isotropically as expected for free diffusion, while others exhibit anisotropy, making more U-turns than expected by chance, which affects their search times (Izeddin et al. 2014). Anisotropic exploration results in the TF oversampling its nuclear neighborhood. Conversely, isotropic diffusion enables global exploration where any target is equally likely to be reached, regardless of distance. Nuclear exploration is therefore likely more complex than the three discrete states envisioned in the facilitated diffusion model (3D diffusion, 1D DNA sliding, stable binding to a target): interactions with nucleosomes (Lerner et al. 2020) or association into local clusters (Hansen et al. 2020) impact TF mobility and could introduce apparent bound states guiding search in preferred compartments. Single-molecule trajectories constitute rich data sets, and mobility metrics extending beyond the diffusion coefficient bring important insights (Shukron et al. 2019).

TFs Interact Briefly with Chromatin

Unlike core histones that stably bind chromatin for tens of minutes to hours (Kimura and Cook 2001; Dion et al. 2007; Deal et al. 2010), the residence times of sequence-specific TFs, gTFs, and repressive complexes tend to be short, in the range of seconds (Fig. 3; Choi et al. 2017;

Table 1. Experimentally measured residence times for various transcription factors (TFs) related to Figure 3

Protein name	Protein type	Residence time (seconds)	References
Histone H1	Architectural protein	183	Phair et al. 2004
CTCF	Architectural protein	63	Hansen et al. 2017
CTCF	Architectural protein	184.3	Hansen et al. 2017
RAD21	Architectural protein	1170.6	Hansen et al. 2017
HMGN1	Chromatin remodeler	24.8	Phair et al. 2004
BRG1	Chromatin remodeler	19.4	Phair et al. 2004
PCAF	Coactivator	17.1	Phair et al. 2004
CYCT1	Coactivator	56	Lu et al. 2018
BRD4	Coactivator	38.1	Phair et al. 2004
ARNT	Coactivator	30.9	Phair et al. 2004
Mediator	Coactivator	3.7	Nguyen et al. 2020
H2B	Core histone	7800	Kimura and Cook 2001
TFIIA	General TF	6.3	Nguyen et al. 2020
TFIIB	General TF	1.8	Nguyen et al. 2020
TFIID	General TF	4.8	Nguyen et al. 2020
TFIIE	General TF	2	Nguyen et al. 2020
TFIIF	General TF	1.7	Nguyen et al. 2020
TFIIH	General TF	10	Nguyen et al. 2020
TFIIK	General TF	7.7	Nguyen et al. 2020
Cbx7	Repressor	8.5	Tatavosian et al. 2018
HT-Cbx7/F-H3.3	Repressor	9.7	Tatavosian et al. 2018
Eed	Repressor	9.5	Tatavosian et al. 2018
EZH2	Repressor	10	Tatavosian et al. 2018
HT-EZH2/F-H3.3	Repressor	10.2	Tatavosian et al. 2018
HP1β	Repressor	73	Phair et al. 2004
ESRRB	Sequence-specific TF	10	Xie et al. 2017
STAT3	Sequence-specific TF	8.3	Xie et al. 2017
TBP	Sequence-specific TF	88	Teves et al. 2018
Sox2	Sequence-specific TF	14.6	Teves et al. 2016
Estrogen receptor	Sequence-specific TF	4.36	Swinstead et al. 2016
FoxA1	Sequence-specific TF	10.8	Swinstead et al. 2016
CREB1	Sequence-specific TF	2.86	Sugo et al. 2015
Glucocorticoid receptor	Sequence-specific TF	0.85	Stasevich et al. 2010
Sox19b	Sequence-specific TF	2	Reisser et al. 2018
TBP	Sequence-specific TF	6.8	Reisser et al. 2018
Glucocorticoid receptor	Sequence-specific TF	7.25	Presman et al. 2016
AhR	Sequence-specific TF	25.6	Phair et al. 2004
C/EBP	Sequence-specific TF	18.8	Phair et al. 2004
FBP	Sequence-specific TF	63.6	Phair et al. 2004
Fos	Sequence-specific TF	14.6	Phair et al. 2004
Jun	Sequence-specific TF	27.3	Phair et al. 2004
Mad	Sequence-specific TF	19.5	Phair et al. 2004
Myc	Sequence-specific TF	16.3	Phair et al. 2004
NF1	Sequence-specific TF	16.2	Phair et al. 2004
XBP	Sequence-specific TF	23.1	Phair et al. 2004
TetR	Sequence-specific TF	5	Normanno et al. 2015
Bicoid	Sequence-specific TF	2.33	Mir et al. 2018
Zelda	Sequence-specific TF	5.56	Mir et al. 2018
p53	Sequence-specific TF	2.5	Hinow et al. 2006

Continued

Table 1. *Continued*

Protein name	Protein type	Residence time (seconds)	References
Gal4	Sequence-specific TF	17	Donovan et al. 2019
OCT-4	Sequence-specific TF	14.6	Chen et al. 2014
P65 (NF-κB)	Sequence-specific TF	4.1	Callegari et al. 2019

Youmans et al. 2018; Suter 2020). The linker histone H1 exchanges from chromatin with similarly fast kinetics (~20 sec–3 min) (Lever et al. 2000; Misteli et al. 2000), while the architectural proteins cohesin and CTCF fall in between core histones and TFs (min) (Hansen et al. 2017). The residence time depends on the affinity between a TF and its target (Clauß et al. 2017; Callegari et al. 2019; Donovan et al. 2019; Popp et al. 2020), but can be modulated across loci, cell types, and cell states. For example, the residence time of the gTF TBP (TATA-binding protein) ranges from seconds in the developing zebrafish embryo (Reisser et al. 2018), interphase U2OS cells, and in vitro assays (Zhang et al. 2016), to hours at the histone locus in *Drosophila* cells (Guglielmi et al. 2013). TBP resides longer at active genes on mitotic chromosomes than during interphase in mouse embryonic stem (ES) cells, enabling rapid transcriptional reactivation upon mitotic exit (Teves et al. 2018). SRF (serum response factor) (Hipp et al. 2019), the glucocorticoid receptor (Stavreva et al. 2019), and GAL4 (Donovan et al. 2019) all exhibit increased residence times upon activation of their upstream pathways. In the case of GAL4, the promoter nucleosome is a key modulator of TF residence time (Donovan et al. 2019) but it is not clear whether this is a general mechanism. In most cases studied so far, the changes in TF residence times are small compared to the changes in the transcription output of their downstream genes, suggesting additional regulation of k_{on} and/or downstream amplifying mechanisms (Fig. 1). Short TF residence times mirror the observations that target sites favor low-affinity TF motifs, a feature that confers extended sensitivity to TF concentration (Kribelbauer et al. 2019).

The biological interpretation of residence times measured by SMT faces two challenges: first, photobleaching limits the direct observation of very long events, even though photobleaching contributions can be corrected from measurements (Gebhardt et al. 2013; Chen et al. 2014; Hansen et al. 2017; Reisser et al. 2020; Garcia et al. 2021). Second, in each experiment, binding events are measured across the nucleus without knowledge of the locus bound by each TF. The original separation of binding events into two discrete populations, assumed to represent nonspecific versus specific events, based on the observation of short (<1 sec) and long (~10 sec) subpopulations in residence time distributions, and supported by DNA-binding domain (DBD) deletion experiments (Chen et al. 2014), might not be valid in all cases. Some TFs exhibit broad residence time distributions, consistent with a continuum of affinities across diverse genomic targets (Normanno et al. 2015; Stavreva et al. 2019; Garcia et al. 2021). A novel kinetic analysis suggests on the other hand the existence of 5–6 discrete dissociation rates ranging from subseconds to minutes (Popp et al. 2020; Reisser et al. 2020). Despite their differences, the different SMT analyses converge on the fact that only a minority of TF-binding events extend beyond the seconds regime, consistent with dozens of earlier studies by FRAP (Hemmerich et al. 2011). In contrast, an indirect approach suggests exceptionally long-lived TF binding in the *Xenopus* oocyte (hours to days) (Gurdon et al. 2020) but it remains unclear whether the chromatin environment of the oocyte fosters this unusual behavior. Albeit chromatin motion and microscopy constrain the live-cell-imaging resolution to ~10 kbp (Li et al. 2019), new microscopes able to measure TF binding at a specific locus have validated that seconds-long TF interactions do occur at relevant targets, and that they correlate with productive transcription (Donovan et al. 2019; Li et al. 2019; Stavreva et al. 2019). Combined with biological perturbations, these tools hold great potential to decipher the TF kinetic code.

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Table 2. Experimentally measured chromatin-bound fraction for various transcription factor (TFs), related to Figure 2

Protein name	Protein type	Bound (%)	References
RAD21	Architectural protein	45	Hansen et al. 2017
CTCF	Architectural protein	54.5	Hansen et al. 2017
Mediator	Coactivator	39	Nguyen et al. 2020
H2B	Core histone	69	Nguyen et al. 2020
TFIID	General TF	39	Nguyen et al. 2020
TFIIA	General TF	35	Nguyen et al. 2020
TFIIB	General TF	21	Nguyen et al. 2020
TFIIF	General TF	25	Nguyen et al. 2020
TFIIE	General TF	33	Nguyen et al. 2020
TFIIH	General TF	27	Nguyen et al. 2020
TFIIK	General TF	44	Nguyen et al. 2020
TBP	General TF	34	Nguyen et al. 2020
TBP	General TF	31	Teves et al. 2018
Eed	Repressor	22	Tatavosian et al. 2018
Ring1B	Repressor	20	Huseyin and Klose 2021
Cbx7	Repressor	30	Tatavosian et al. 2018
EZH2	Repressor	22	Tatavosian et al. 2018
Zelda	Sequence-specific TF	49	Mir et al. 2018
Bicoid	Sequence-specific TF	50	Mir et al. 2018
STAT3	Sequence-specific TF	35	Xie et al. 2017
Sox2	Sequence-specific TF	38	Liu et al. 2014
OCT-4	Sequence-specific TF	42.2	Chen et al. 2014
ESRRB	Sequence-specific TF	44	Xie et al. 2017
RPB1	Subunit of Pol II	48	Nguyen et al. 2020

From TF Binding to Transcripts

The short TF residence times echo the short bursts that constitute the basic unit of transcription (Rodriguez and Larson 2020). It is thus tempting to propose that TF-binding events coincide with bursts (Fig. 4A). This simple "one-to-one" model predicts that the burst frequency should equal the product of k_{on} and TF concentration and the burst duration should equal the TF residence time. Several observations are consistent with the one-to-one model: (1) the transcription machinery assembles within seconds, well within typical TF residence times (Zhang et al. 2016; Nguyen et al. 2020); (2) increasing TF concentration increases burst frequency (Senecal et al. 2014; Stavreva et al. 2019); (3) search time estimates are inversely proportional to burst frequencies observed in yeast (Larson et al. 2011); (4) decreasing k_{off} increases the number of transcripts per burst (Senecal et al. 2014); (5) enhancers regulate burst frequency (Walters et al. 1995; Bartman et al. 2016; Fukaya et al. 2016; Chen et al. 2018); and (6) elegant experiments directly observe that single TF-binding events correlate temporally with burst firing (Donovan et al. 2019; Stavreva et al. 2019). While the simple two-state model might be valid in simple systems, it often breaks down in higher eukaryotes (Bartman et al. 2016; Corrigan et al. 2016; Rodriguez et al. 2019; Lammers et al. 2020; Popp et al. 2020). In a striking example, pluripotency genes cease to transcribe early in differentiation, long before their enhancers lose TF occupancy (Hamilton et al. 2019). gTFs constitute obvious kinetic intermediates that could mediate this decoupling: TBP binding regulates permissive periods over 5–20 min timescales, while Mediator ensures rapid back-to-back Pol II initiations (seconds) (Tantale et al. 2016), and pausing regulates the number of transcripts per burst (Bartman et al. 2016). Besides gTFs, supercoiling and chromatin also shape burst timing (Muramoto et al. 2010; Chong et al. 2014; Teves and Henikoff 2014). Thus, dis-

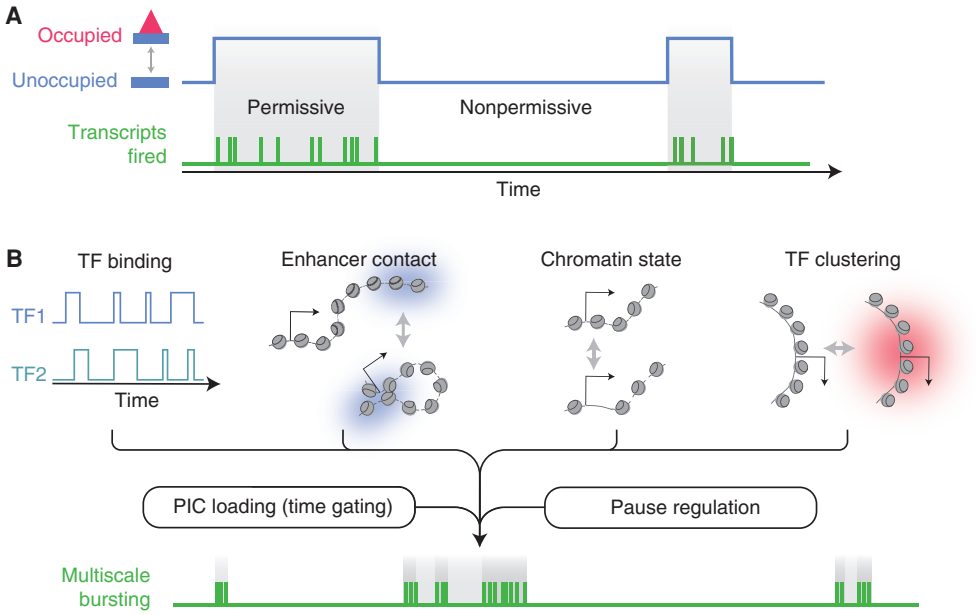

Figure 4. Decoding of transcription factor (TF) kinetics by promoters. (*A*) In simple systems, TF binding directly leads to permissive periods (gray) during which many Pol II are rapidly fired. In this one-to-one model, the TF residence time equals the burst duration. (*B*) Promoters often integrate complex regulation from multiple TFs and enhancers, as well as the state of chromatin at the promoter, and the kinetics of cluster formation. These interdependent inputs are processed by the transcription machinery, which applies further control layers, leading to multistate bursting dynamics. (PIC) Preinitiation complex.

tinct steps of the transcription cycle are controlled by separate TFs (Stasevich et al. 2014), leading to multiscale bursting kinetics (Corrigan et al. 2016) and complex regulatory logic (Fig. 4B; Scholes et al. 2017). These features likely explain why predicting enhancer combinations remains challenging (Vincent et al. 2016). As the enhancer–promoter looping paradigm has recently been called into question (Alexander et al. 2019; Benabdallah et al. 2019), biophysical models of enhancer function remain sorely needed (Bothma et al. 2015). Ideally such descriptions will integrate binding of the various players, DNA organization, and local TF clustering (see last section) to predict bursting kinetics.

Intrinsically Disordered Regions Provide a Flexible Platform for TF Dynamics

Regulatory sequences favor low-affinity TF-binding sites that ensure specificity and sensitivity to TF concentrations over a wide range (Kribelbauer et al. 2019). Could the same principles hold for interactions between TFs and their protein partners? Indeed, it has long been appreciated that the activating domains of TFs contain intrinsically disordered regions (IDRs), which are unstructured peptides that are essential sites for TF–TF interactions and retain function upon extensive mutations (Sigler 1988; van der Lee et al. 2014; Wright and Dyson 2015). Over 80% of all eukaryotic TFs contain one or more IDRs (Liu et al. 2006), also called low complexity regions because of their limited repertoire of amino acids. While protein–protein interactions are classically thought of as stoichiometric complexes forming via a lock–key mechanism relying on complementary structures, IDRs by definition cannot fit this model. In addition, IDR sequences are poorly conserved, challenging our understanding of how TFs associate with specific partner(s) to regulate transcription. The possible role of IDRs in TF clustering (see below) has fueled renewed interest in these elusive do-

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mains, with the hope to better detect and interpret pathological mutations, many of which fall within IDRs (Uyar et al. 2014).

Stoichiometric IDR Complexes

While IDRs explore a large ensemble of conformations in solution, they can exhibit a more ordered conformation when bound to a cofactor, enabling the appearance of a traditional protein–protein interface (Fig. 5; Schuler et al. 2020). This process, termed binding-coupled folding, can occur in two ways: (1) the ordered bound state pre-exists in the conformational ensemble of the free IDR and is required for binding to the cofactor ("conformational selection"); or (2) the TF can recognize its partner in its disordered state and fold while binding ("induced fit") (Staby et al. 2017). Binding-coupled folding offers flexibility; for instance, the IDR of the coactivator CBP adopts different structures when bound to different TFs (Demarest et al. 2002; Qin et al. 2005; Waters et al. 2006). IDRs can in some cases form "fuzzy complexes" that do not exhibit a fixed conformation but gain stability through multiple weak and dynamic contacts between the IDR and its partner (Tompa and Fuxreiter 2008; Henley et al. 2020). Thanks to their dynamic nature, fuzzy complexes can be easily remodeled via competitive substitution, enabling rapid gear switching of the transcription machinery (Schuler et al. 2020).

IDR-Driven Phase Separation

Beyond fuzzy complexes, IDRs can mediate the formation of nonstoichiometric clusters (Fig. 6). Pioneering work showing that IDRs can form or associate with hydrogels in vitro suggested that IDRs of TFs may drive clustering via phase separation (Frey et al. 2006; Kwon et al. 2013).

IDRs are often repetitive in sequence, and thus intrinsically multivalent, a prerequisite of phase separation (Choi et al. 2020). For instance, the carboxy-terminal domain (CTD) of the largest Pol II subunit, Rpb1, is a well-studied IDR that consists of repeats of the tyrosine-serine-proline-threonine-serine-proline-serine (YSPTSPS) motif (Corden 2013; Eick and Geyer 2013; Zaborowska et al. 2016; Gibbs et al. 2017; Portz et al. 2017). Pol II and many purified TFs or their IDRs indeed self-organize into phase-separated droplets in vitro (Larson et al. 2017; Boehning et al. 2018; Boija et al. 2018; Lu et al. 2018; Sabari et al. 2018; Guo et al. 2019; Plys et al. 2019; Zamudio et al. 2019; Daneshvar et al. 2020; Li et al. 2020a). In vivo, some features of TF clusters are consistent with liquid–liquid phase separation. TFs exchange dynamically within clusters as revealed by FRAP, and TF clusters undergo fusion and fission and are sensitive to treatment with 1,6-hexanediol, which disrupts some of the weak interactions that can contribute to phase separation (Cho et al. 2018; Chong et al. 2018; Sabari et al. 2018). However, these features alone do not rule out nonphase separation

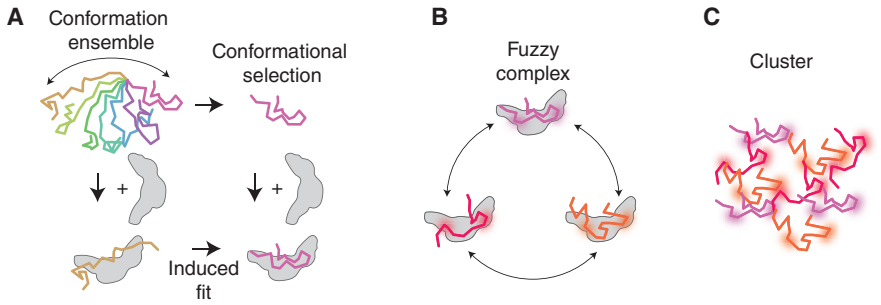

Figure 5. Intrinsically disordered regions (IDRs) mediate different types of complexes. (*A*) Binding-coupled folding: free IDRs explore a vast conformation space (colors), but some IDRs adopt a fixed conformation when bound to a partner (gray). (*B*) In a fuzzy complex, the IDR is dynamic yet remains bound to its partner. (*C*) Nonstoichiometric complexes (clusters) can form via networked interactions between multivalent IDRs.

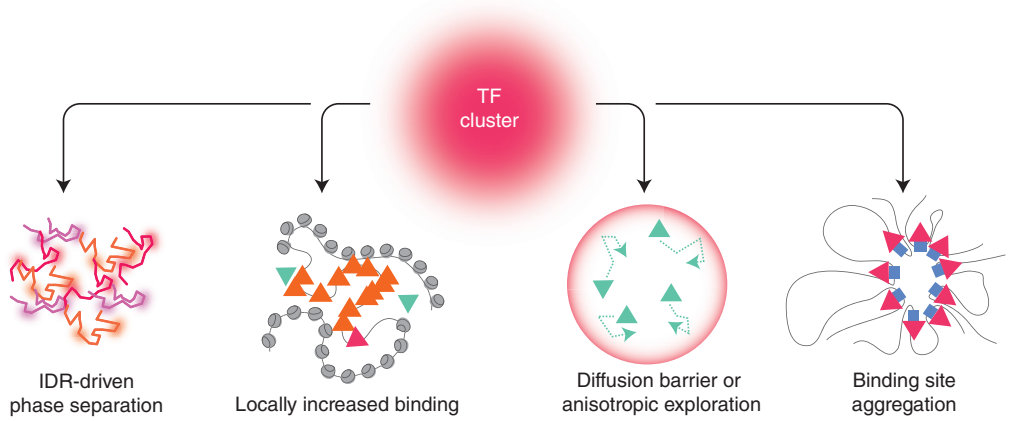

Figure 6. Clustering mechanisms. Transcription factor (TF) clustering can occur via phase separation, thanks to multivalent interactions between intrinsically disordered regions (IDRs) (*left*). Other mechanisms also exist; locally enhanced TF binding on highly accessible chromatin, locally anisotropic diffusion, and collapse of a TF-binding site. Triangles represent TFs; green, orange, and magenta denote, respectively, free, nonspecifically bound, and specifically bound species.

mechanisms (McSwiggen et al. 2019b) (see last section). A substantial caveat is that in vitro assays often use crowding agents and are done at TF concentrations much higher than physiological conditions and thus favor phase separation. For this reason, while these assays provide useful insights into the relative role of different parameters in clustering, they likely offer only a limited representation of in vivo clusters, and do for example not take into account the role of RNA and other TFs in clustering in vivo (Wei et al. 2020; Henninger et al. 2021).

Beyond Complexes: IDRs Facilitate Target Search

IDRs might not just mediate the affinity of TFs to each other, but may also influence search kinetics. The IDRs of sequence-specific TFs Msn2 and Yap1 are both necessary and sufficient for promoter specificity, but their DBDs are not (Brodsky et al. 2020), suggesting a two-step target search. TFs first localize to an open promoter by promiscuously scanning it with their IDR, and later stably bind to target DNA motifs via their DBDs. The separation of tasks between DBD and activation domains might therefore not be binary (Liu et al. 2008). IDRs could boost search efficiency in several ways including (1) the large interaction surface of IDRs may increase TF affinity for DNA, enhancing its sliding propensity; (2) IDRs may facilitate TF translocation to a neighboring DNA segment (Vuzman and Levy 2012); or (3) IDRs could guide TFs by facilitating clustering or anisotropic diffusion in specialized compartments (Izeddin et al. 2014; Hansen et al. 2020; Nguyen et al. 2020).

Sequence Determinants of IDR Interactions

In contrast to lock–key interactions driven by unique motifs, fuzzy complexes and phase separation involve individually weak but multivalent interactions between protein partners and/or nucleic acids, which could explain the low sequence conservation of IDRs. Indeed, mutation scans suggest that IDRs interact through a sum of weak interactions distributed along their entire domain (Wang et al. 2018; Brodsky et al. 2020). Furthermore, the *Drosophila* Pol II CTD, which consists of heptapeptide repeats diverged from the canonical YSPTSPS sequence, can be replaced with a shorter canonical CTD, but not with a canonical CTD at wild-type length (Lu et al. 2019). These findings suggest a model in which the sum interaction strength required for proper biological function can be achieved either through many low affinity sites (noncanon-

ical repeats), or fewer high affinity ones (canonical repeats). Consistent with this picture, truncated CTDs decrease the frequency and size of transcription bursts in yeast (Quintero-Cadena et al. 2020), likely through reduced Pol II clustering (Boehning et al. 2018), while longer CTDs exhibit aberrant clustering (Lu et al. 2019). *Drosophila* Pol II mutants containing exceedingly long or short CTDs are inviable, confirming that sum interaction strength constitutes a key selection pressure. The fact that a specific sum interaction strength can be achieved in many ways (e.g., using distinct residues with similar physicochemical properties, and/or distributing these residues differently along the IDR sequence, accounts for the low sequence conservation of IDRs.

The prevalence of IDRs in TFs presents a conundrum. TFs need to selectively associate with specific sequence(s) or cofactors, yet IDRs mediate promiscuous interactions. How is specificity encoded into the underlying sequence of IDRs? It appears that small motifs or even a single residue within the IDR can convey specificity for a partner for instance by biasing a fuzzy complex toward a subset of its binding modes (Sims et al. 2011; Warfield et al. 2014; Desai et al. 2015; Zhao et al. 2016; Henley et al. 2020). Non-stoichiometric IDR clustering also exhibits specificity (Chong et al. 2018), although it remains unclear what mechanisms apply here. The numbers and positions of acidic, hydrophobic, and aromatic residues all modulate the affinity of IDR interactions (Staller et al. 2018; Erijman et al. 2020), as do posttranslational modifications (Guo et al. 2019). These observations suggest a departure from the dogma of sequence-defining function via a deterministic 3D protein structure. Rather, IDR sequences specify the physicochemical properties of residues such as hydrophobicity, charge distribution, and flexibility, in turn constraining both the conformation space explored by the protein chain as well as its potential for interactions through the number and strength of sticky residues, thus encoding specific TF functions including clustering propensity, exploration mode, and target selectivity (Vernon et al. 2018; Wang et al. 2018; Martin and Holehouse 2020).

Evolution of IDRs

Eukaryotic proteomes contain more disordered segments than those of simpler organisms (Ward et al. 2004; Tompa et al. 2006; Peng et al. 2015) and individual transcription regulators, such as the Pol II CTD (Quintero-Cadena et al. 2020) or Mediator subunits (Tóth-Petróczy et al. 2008) exhibit increasing disordered content over evolutionary timescales. An interesting idea is that regulatory innovation is often gained by adding new components to existing complexes. It is likely faster in evolutionary terms to achieve binding to an existing complex through a flexible domain, rather than creating a de novo specialized 3D structure matching the complex interface. Consistent with this idea, proteins that participate in large complexes are more disordered (Hegyi et al. 2007). Altogether, IDR size selection likely results from a series of tradeoffs between regulatory potential (longer IDRs enabling binding to more targets), nuclear exploration mode (longer positive tails increase affinity for DNA, enhancing a TF sliding propensity at the expense of its ability to hop to a locus in *trans* [Vuzman and Levy 2012]), and clustering potential (IDRs with higher valency or interaction strength generate static aggregates unable to respond to dynamic signals).

TFs ASSEMBLE IN CLUSTERS

Transcription has long been proposed to occur in stable, self-assembled "hubs" that could outlive the binding of individual components (Cook 1999; Edelman and Fraser 2012), similar to larger subnuclear structures such as the nucleolus (Phair and Misteli 2000). Consistent with the hub model, a variety of factors form clusters in cells: sequence-specific TFs (Liu et al. 2014; Mir et al. 2017, 2018; Chong et al. 2018; Basu et al. 2020; Li et al. 2020b), coactivators (Cho et al. 2018; Sabari et al. 2018; Guo et al. 2019; Li et al. 2019, 2020b; Zamudio et al. 2019), Pol II (see below), splicing factors (Guo et al. 2019), corepressors (Treen et al. 2020), repressive complexes (Wollman et al. 2017; Plys et al. 2019; Ruault et al. 2020), chromatin modifiers (Tatavosian et al. 2019), and HP1 (heterochromatin pro-

tein 1) (Strom et al. 2017; Erdel et al. 2020; Li et al. 2020a). Contrasting with the model of a stable factory, cluster lifetimes are generally short (Cisse et al. 2013).

Pol II Clustering

Single-molecule imaging has revealed that RNA polymerase II (Pol II) forms clusters in various mammalian cultured cell models (Cisse et al. 2013; Cho et al. 2016, 2016; Boehning et al. 2018; Li et al. 2019). Pol II clusters are short-lived, generally on the order of seconds, but some last minutes, and vary in size (Cisse et al. 2013; Cho et al. 2016, 2018; Boehning et al. 2018), from diffraction-limited foci all the way to micron-sized accumulations at the histone locus body (Guglielmi et al. 2013) and viral replication compartments (McSwiggen et al. 2019a). The number, size, and lifetime of clusters change upon induction (Cisse et al. 2013; Cho et al. 2016; Li et al. 2019) or inhibition of transcription (Cho et al. 2018; Li et al. 2019) and during differentiation (Cho et al. 2018), suggesting that clustering may contribute to transcription control.

The CTD is a key regulator of Pol II clustering. On its own, it forms liquid condensates in vitro and its length regulates Pol II clustering in vivo (Boehning et al. 2018). Phosphorylation of the CTD during early stages of transcription could control CTD clustering via charge modulation (Harlen and Churchman 2017). Indeed, inhibitors against P-TEFb, which triggers Pol II pause release via CTD phosphorylation, stabilize Pol II clusters (Cisse et al. 2013; Cho et al. 2016) while CTD phosphorylation disperses clusters in vitro (Boehning et al. 2018; Lu et al. 2018). These observations place Pol II clustering at the transcription preinitiation or initiation stage. CTD phosphorylation also biases Pol II association with splicing factor condensates versus those containing Mediator (Guo et al. 2019), while electrostatic repulsion by charged nascent RNAs dismantles Pol II clusters (Henninger et al. 2021). Together, these observations suggest a model wherein RNA accumulation and/or CTD phosphorylation force cluster turnover once a Pol II convoy has initiated on a transcribed gene (Quintero-Cadena et al. 2020).

Clustering without Phase Separation

Besides IDR-driven phase separation, other clustering mechanisms exist (Fig. 6). In cells infected with herpes simplex virus (HSV), viral replication compartments form micron-sized Pol II clusters due to locally enhanced Pol II binding to nucleosome-free DNA (McSwiggen et al. 2019a). Strikingly, these Pol II clusters are insensitive to CTD length in the HSV context, confirming their distinctive mechanism. TF clustering can also emerge from locally hindered diffusion as indicated by the observation that CTCF molecules are partially retained in specific nuclear zones, likely via interactions with RNA (Hansen et al. 2020). Similarly, upon heat shock, various factors are retained at induced loci, in a poly(ADP-ribose) polymerase (PARP) activity-dependent manner. This led to the speculation that PAR polymerization could create a diffusion barrier around the locus, favoring local recycling of TF molecules once they finish a round of transcription (Yao et al. 2007; Zobeck et al. 2010). Alternatively, PARP-induced chromatin decondensation could enhance TF binding, and/or PARylation could increase TF mutual affinity (Benabdallah et al. 2019). Finally, TFs may cluster due to the collapsing of their DNA targets, as suggested for HP1 in mouse embryonic fibroblasts, where the formation of chromocenters occurs independently of HP1 (Erdel et al. 2020). Interestingly, HP1 clusters reminiscent of phase separation are observed during *Drosophila* embryogenesis (Strom et al. 2017). The Pol II and HP1 examples suggest that a given factor can evolve distinct biophysical mechanisms to cluster in different contexts.

Functions of Clustering in Transcription

Clusters generate high local TF concentrations, which could ensure robust TF recruitment via mass action law even if TFs exchange from the cluster faster than the cluster lifetime (Dufourt et al. 2018). Indeed, clustering ensures high target site occupancy at low TF concentrations (Mir et al. 2017, 2018), and artificially induced clustering of TFs or IDRs in vivo is sufficient to

recruit higher levels of transcription components and increase levels of transcription locally (Wei et al. 2020; Schneider et al. 2021). Increased local concentration of TFs is also expected to favor efficient transcription reinitiation. This prediction is consistent with the observation that increased Pol II clustering leads to increased burst size (Cho et al. 2016; Quintero-Cadena et al. 2020), and that rapid reinitiation is regulated by Mediator, a factor prone to clustering (Cho et al. 2018; Nguyen et al. 2020). TF cluster lifetimes are in the range of seconds, and thus are unlikely to constitute the molecular substrate of long-term transcription memory (Cisse et al. 2013; Cho et al. 2016, 2018; Mir et al. 2018). Clustering may also form a molecular bridge between distant loci (Tsai et al. 2019), which could explain why enhancers do not always directly contact promoters upon activation (Alexander et al. 2019; Benabdallah et al. 2019). Instead TF clusters may generate a regulatory environment shared by *cis*-regulatory elements without the need for molecular contact. Disruption of clustering does not abolish existing enhancer–promoter contacts, suggesting that clusters are not needed to maintain long-range interactions (Crump et al. 2021). Clustered enhancers confer robustness (Tsai et al. 2019) and constitute a flexible platform able to encode a variety of regulatory responses (Ezer et al. 2014). Similar to activators, repressors may also bring together distant loci (Ruault et al. 2020). Finally, clusters could facilitate nuclear exploration by guiding TFs to specialized compartments (Hansen et al. 2020; Nguyen et al. 2020).

CONCLUDING REMARKS

Advances in live imaging have uncovered novel modes of TF exploration and transient assemblies whose function and regulation are just beginning to be understood. Since TF dynamics parameters such as search time, residence time, concentration, fraction bound, etc. all impact transcription levels, experimental separation of individual factors is a challenge that will need to be overcome to build mechanistic models (Popp et al. 2020).

Clustering is emerging as a ubiquitous feature of transcription regulation that locally boosts transcription via mass action, while ensuring a nimble architecture able to rapidly respond to changing cues. One could envision other functions, for instance that TF clustering away from active sites could also titrate out TFs when transcription needs to be globally turned down. The next challenge is to better understand the mechanisms of cluster formation, and how clustering dynamics are decoded by promoters into transcription outputs. Biophysical regulators of clustering identified so far include phase separation, locally enhanced DNA binding, and local diffusion barriers. One difficulty is that these mechanisms are likely intertwined; for instance, transcription-coupled clustering could enhance DNA binding and/or generate local diffusion barriers. Since the respective roles of these mechanisms likely depend on the biological context, a key question is how biochemical pathways interface with TF biophysics. So far, posttranslational modifications, particularly of the Pol II CTD, have been demonstrated to control clustering by rapidly modulating IDR affinities. The role of other pathways or regulators remains to be fully explored.

Overall, the dominating feature of TF dynamics is that they follow a distributed interaction principle, apparent at many scales. First, in stoichiometric fuzzy complexes, multiple weak interaction sites between two partners rapidly exchange without complex dissociation. Second, multivalent interactions distributed across IDRs ensure the formation of nonstoichiometric clusters. Finally, enhancers favor multiple weak TF-binding motifs over high affinity ones to ensure expression specificity (Frankel et al. 2010; Crocker et al. 2015, 2016; Farley et al. 2015). This unifying principle offers many advantages needed for regulatory function, particularly robustness, tunability, and responsiveness.

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