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REVIEW

# Robust and efficient gene regulation through localized nuclear microenvironments

**Albert Tsai*, Rafael Galupa and Justin Crocker***

## ABSTRACT

Developmental enhancers drive gene expression in specific cell types during animal development. They integrate signals from many different sources mediated through the binding of transcription factors, producing specific responses in gene expression. Transcription factors often bind low-affinity sequences for only short durations. How brief, low-affinity interactions drive efficient transcription and robust gene expression is a central question in developmental biology. Localized high concentrations of transcription factors have been suggested as a possible mechanism by which to use these enhancer sites effectively. Here, we discuss the evidence for such transcriptional microenvironments, mechanisms for their formation and the biological consequences of such sub-nuclear compartmentalization for developmental decisions and evolution.

**KEY WORDS: Gene regulation, Development, Nucleus, Transcription factors, Transcriptional microenvironment**

## Introduction

In 1968, the geneticist David E. Comings discussed 'the rationale behind the possibility that the nucleus is a well-ordered place' (Comings, 1968). He noted that chromatin reliably condenses into highly ordered chromosomes during cell division despite its apparently random distribution outside of mitosis and meiosis. Comings postulated that the space within the nucleus is highly organized and that there is an ordered arrangement of the chromatin even during interphase. Despite the lack of internal membrane-delimited sub-compartments, subsequent investigations showed that the nucleus indeed contained several functional compartments, such as the nucleolus, splicing speckles and replication factories (Cook, 1999; Hozák and Cook, 1994; Spector, 2003). The question of whether gene transcription also occurs within compartmentalized structures, which could provide specific mechanisms to regulate gene expression, has been pursued with great interest for some decades now, using both microscopy and molecular biology approaches.

Perhaps one of the first observations that transcription does not occur homogeneously inside the nucleus was the visualization of discrete foci of nascent RNAs and RNA polymerases, and their overlap, leading to the concept of 'transcription factories' (Glossary, Box 1) (Bregman et al., 1995; Iborra et al., 1996; Jackson et al., 1993, 1998; Wansink et al., 1993). Further experiments suggested that the movement of genes into or out of transcription factories results in an increase or reduction of transcription (Osborne et al., 2004; Rieder et al., 2012). New advances in imaging technologies have taken these models further by allowing for a more dynamic view of transcription (recently reviewed by Furlong and Levine, 2018). As we will discuss here, binding events of transcription factors frequently occur in clusters that are highly dynamic and distributed across the nucleus in a heterogeneous organization. The transient and stochastic nature of these interactions (see Glossary, Box 1) poses challenges in establishing a direct link between transcription factor binding, RNA polymerase recruitment and transcriptional output, especially in the context of development, often regarded as a robust and stereotypical process (Fig. 1A).

Within the dynamically changing environment of a developing embryo, there are likely to be intermediate layers of mechanisms inside the nucleus that coherently coalesce these molecular interactions to make reliable cell-fate decisions (Fig. 1B). Such mechanisms could filter out the stochastic noise inherent in transcription factor binding to allow for consistent regulatory decisions during development (Arias and Hayward, 2006). Mechanisms that buffer against noise in gene expression have been observed in gene-regulatory networks (Lagha et al., 2012; Raj et al., 2010; Stapel et al., 2017). In fact, some of the mechanisms could be integral parts of the regulatory architecture of the genes themselves, where the system integrates inputs from multiple regulatory elements, achieving additive or non-additive effects (Bothma et al., 2015). Genes and transcription factors may also interact in networks tuned for specific types of behaviors (Krotov et al., 2014). It is possible that post-transcriptional steps of gene expression could provide an additional contribution to, or regulation of, the noise in protein levels (Vogel and Marcotte, 2012). Nevertheless, protein levels, in general, show some positive correlation to their mRNA levels (Csárdi et al., 2015; Wilhelm et al., 2014), with the effect possibly stronger in genes that are not constitutively active (i.e. dynamically regulated genes) (Gong et al., 2017). Furthermore, transcription and its regulation have been proposed to dominate the final expression noise unless mRNAs are infrequently translated (Eldar and Elowitz, 2010; Swain et al., 2002), highlighting the need for stringent regulation of transcription to limit downstream propagation of fluctuations in mRNA levels.

Furthermore, these middle-layer transcriptional mechanisms operating at the level of nuclear compartmentalization and genome architecture might provide phenotypic robustness (see Glossary, Box 1) to developmental systems facing environmental and genetic perturbations (Payne and Wagner, 2019). As gene expression in embryos is highly time- and location-dependent, the middle-layer mechanisms, and their relative importance, could differ between developmental and homeostatic systems. As a result, the mechanisms observed in cell culture and adult animals may not, in all cases, be generalizable. Unfortunately, our specific understanding of how embryos coherently organize the possible components of this middle layer and prevent mistimed and misplaced cross-interactions within the physical confines of the nucleus is currently limited. A better understanding of how areas within the nucleus can be dynamically organized to achieve specific

European Molecular Biology Laboratory, 69117 Heidelberg, Germany.

*Authors for correspondence (albert.tsai@embl.de; justin.crocker@embl.de)

A.T., 0000-0002-1643-0780; J.C., 0000-0002-5113-0476

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## Box 1. Glossary

**Active chromatin hub.** This spatial unit (hub) contains active genes interacting with multiple cis-regulatory elements on the chromatin with intervening inactive genes looping out. First observed in β-globin genes (de Laat and Grosveld, 2003; Tolhuis et al., 2002), the genes selectively expressed in this hub would depend on their proximity to promoters and specificity for cis-regulatory elements in the hub. This term highlights the transcriptional state of the chromatin, which could be an integral part of the broader concept of transcriptional hubs.

**Dwell time.** The time that a molecule remains bound, also known as residence time. For transcription factors binding to low-affinity sites and many Pol II-binding events on DNA, this could last on average for less than 1 s (e.g. Chen et al., 2014; Cisse et al., 2013; Mir et al., 2017; 2018; Tsai et al., 2017). In contrast, transcriptional repressors [e.g. Polycomb Group proteins (Fonseca et al., 2012)] and histones (Misteli et al., 2000) could have dwell times on DNA of seconds to minutes.

**Phase separation.** Liquid-liquid phase separation in cells is the spontaneous separation of macromolecules into droplets of different physical states (analogous to that between water and gels) (Alberti, 2017). When the concentrations of molecules having specific physical and chemical properties, such as proteins that have disordered domains favoring hydrophobic interactions, reach a threshold, they condense into droplets where they are highly enriched. The interface between the droplets and the outside only allows for the selective passage of specific molecules based on their physical and chemical properties, leading to a membraneless mechanism of compartmentalization.

**Robustness.** Robustness refers to the ability of a system to function consistently under perturbations and is a fundamental feature of complex and evolvable systems. The general concept of biological robustness encompasses a wide range of concepts and mechanisms (reviewed by Frederik Nijhout et al., 2017; Kitano, 2004; Masel and Siegal, 2009). Transcriptional robustness as applied to development means that the expression of developmental genes remains consistent and functional even in the face of fluctuations in their regulatory signals due to stochastic noise, environmental perturbations (e.g. temperature) and/or genetic mutations.

**Stochastic interactions.** The exact timing and duration of individual binding interactions of transcription factors to the DNA are not precisely predictable. While properties of the binding interactions, such as their frequency, duration and correlation with the presence of other factors and co-factors, should follow statistical distributions, they only become apparent when multiple interactions over long periods are sampled. There could also be multiple binding attempts by a transcription factor before it successfully recruits Pol II, making the exact consequence of each binding difficult to ascertain. Finally, transcription factor binding and subsequent mRNA production can occur at uneven intervals.

**Topologically associating domains.** Topologically associating domains (TADs) represent submegabase domains: (1) in which genomic elements interact preferentially with each other; and (2) that correlate with functional properties of the genome, such as transcriptional co-regulation, enhancer-promoter communication, domains of active histone marks and CTCF clustering at boundaries.

**Transcription factories.** Nascent mRNAs and RNA polymerases tended to form overlapping foci within the nucleus, leading to the proposal that the bulk of transcription selectively occur within these centralized 'factories' (reviewed by Rieder et al., 2012). Anchored to the nuclear matrix, these factories would form a stationary scaffold for the continuous trapping and recycling of polymerases and transcription factors to sustain high levels of transcriptional output. Their proposed stability and persistence led to the early hypothesis that moving genes into and out of these compartments regulates their transcriptional state.

**Transcriptional hubs.** Transcriptional hubs are localized nuclear compartments that can sustain high levels of transcriptional output through high local concentrations of polymerases and transcription factors. Hubs could have specific compositions of transcription factors and co-factors, depending on the regulatory elements present. As individual transcription factor and polymerase-binding events to DNA are transient, hubs likely depend on cooperative interactions between transcription factors and polymerases to sustain themselves. In contrast to stable and persistent transcription factories, they would be highly dynamic, allowing for specific transcriptional regulation with high spatial and temporal precision.

**Transcriptional microenvironments.** These are regions within the nucleus that are locally enriched for specific transcription factors and co-factors. Depending on the compositions of microenvironments, they can be transcriptionally activating or repressing, perhaps even leading to localized changes in the chromatin that alter their transcriptional state over the long term. Transcriptional hubs are a specific example of an activating transcriptional microenvironment. A nucleus containing many transcriptional microenvironments would be heterogeneous – genes would experience very different conditions, depending on their exact location.

and efficient gene regulation will lead to better quantitative models of transcriptional regulation, allowing us to unravel fundamental principles in signal processing in developmental systems.

In this Review, we will cover what could function as the centerpiece of this middle layer that can physically bring together many of the proposed buffering mechanisms: localized transcriptional environments that form membraneless subnuclear-compartments across developmental systems (reviewed by Kribelbauer et al., 2019). Using a variety of imaging approaches, these compartments have been shown to contain highly enriched levels of transcription factors and polymerases, forming local 'hubs' whose specialized environment differs from other areas of the nucleus (Fig. 1C). We will discuss how these sub-nuclear structures can form dynamically during embryogenesis, and provide examples of their functional importance in preserving phenotypic robustness in the face of environmental perturbations (Fig. 1D). Finally, we will discuss the implications of these hubs as a general principle of gene regulation during development in multicellular organisms, and how these environments can be a source and target of evolutionary changes.

## The heterogeneous and organized nuclear transcriptional space: concepts from microscopy and molecular biology

New imaging technologies promise to advance our understanding of transcriptional processes during development. Already in stem cells, microscopy has revealed that the subnuclear distributions of transcription factors and polymerases are heterogeneous (Cho et al., 2016; Hipp et al., 2019; Liu et al., 2014; Verneri et al., 2018).Similar observations in embryos are still limited, but a few examples also show localized distributions of transcription factors and Pol II in *Drosophila* (Mir et al., 2018; Tsai et al., 2017) and zebrafish (Sato et al., 2019). Specifically, Tsai et al. (2017) have shown that, in *Drosophila melanogaster* embryos, the Hox factor Ultrabithorax (Ubx) and its co-factor Homothorax (Hth) are heterogeneously distributed in the nucleus, and their patterns do not, in general, overlap. However, transcription sites driven by *shavenbaby* (*svb*) enhancers specifically reside in locations enriched for both Ubx and Hth (Fig. 2A). These data suggest that regions of Ubx and Hth overlap could be specialized environments driving *svb* expression. In general, there could be many more of such subnuclear compartments containing combinations of different transcription factors and co-factors used for the regulation of specific genes.

From a dynamic standpoint, molecular interactions within these transcriptional microenvironments operate over short timescales – in the order of milliseconds to seconds. Recent data from super-resolution and live-imaging microscopy have shown that, for example, RNA polymerase II (Pol II) molecules in live cells have dwell times (see Glossary, Box 1) in the order of seconds in localized clusters (Cisse et al., 2013). Furthermore, single-molecule

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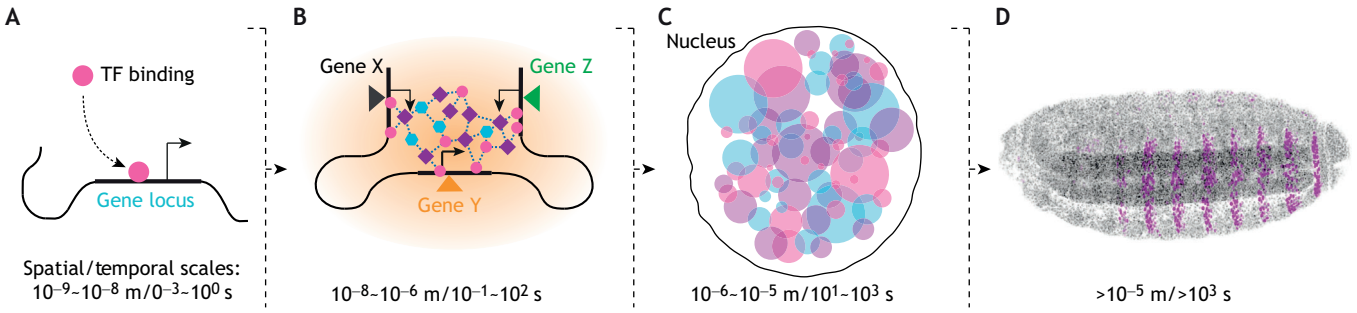


**Fig. 1. Localized transcriptional environments bridge molecular interactions and robust embryo development.** Transcriptional regulation during embryo development involves many processes across a variety of spatial and temporal scales, ranging from molecular interactions to development of body segments. (A) Molecular interactions of transcription factors (TFs). At one end of this range (between a few to tens of nanometers), transcription factor molecules bind stochastically to regulatory elements in the gene locus. The dwell time of transcription factors on the DNA is short in many eukaryotic systems, often lasting less than a few seconds. (B) Middle layer of mechanisms in transcriptional microenvironments. For consistent and robust gene regulation to occur during embryo development, there should be a middle layer of mechanisms that use multiple regulatory components to buffer the short-term noise of the molecular interactions, providing stability over minutes. We propose that this middle layer of mechanisms could physically reside in the same transcriptional microenvironment (approximately hundreds of nanometers across) containing cooperatively interacting transcription factors, *cis*-regulatory regions and even multiple genes in a regulatory network. (C) Integrating the middle layers: compartmentalized nucleus. During development, the nucleus may be organized into many specialized transcriptional compartments, contributing to efficient and specific gene expression. This allows the precise temporal and spatial control of transcription depending on the dynamics and sizes of the transcriptional environments. (D) Cell fate specification and body plan development. The consistency and reliability in these transcriptional microenvironments would lead to robust cell-fate specification and the formation of the correct animal body plan (shown here, the *Drosophila* embryo) even under stressful conditions, potentially also contributing to the evolvability of the system.

imaging experiments in embryos [e.g. Ultrabithorax (Tsai et al., 2017), Zelda (Dufourt et al., 2018; Mir et al., 2018) and Bicoid (Mir et al., 2017)] and in embryonic stem cell culture [e.g. Sox2 (Chen et al., 2014)] have shown that binding events within the nucleus for transcription factors are short, often lasting less than a few seconds (Fig. 2B-D). In addition to the stochasticity on the side of transcription factor binding, transcriptionally active genes also produce mRNAs in short stochastic bursts in between periods of no transcriptional activity (Bothma et al., 2014; Corrigan et al., 2016; Fukaya et al., 2016; Lammers et al., 2020). In summary, the cases examined above support the view that transcription factor and Pol II-binding events are short-lived and occur in localized clusters, forming binding hotspots that are highly dynamic and distributed across the nucleus in a heterogeneous organization (Fig. 2E). These hotspots could then nucleate the formation of localized transcriptional environments through high levels of transcription factor-DNA and transcription factor-transcription factor interactions.

## The dynamic and multifaceted interactions that form transcriptional hubs

What are the mechanisms that drive the heterogeneous distributions of transcription factors? As internal membranes do not subdivide the nucleus, non-interacting molecules tend to distribute themselves independently of each other (Fig. 3A), forming regions of overlap by random chance. For transcription factors to colocalize to specific areas to perform specialized regulatory functions (e.g. Ubx and Hth), they must specifically interact, either directly or through co-factors. Alternatively, transcription factors could be distributed apart from each other in a mutually exclusive manner (Fig. 3B) if co-factors with different localization preferences guide them or if they compete for the same binding sites. A frequent strategy in nature to enrich substrates without membranes is ‘local trapping’, whereby increasing the overall dwell time of molecules at a location greatly increases their local concentrations (Battich et al., 2015; Saunders et al., 2012; Slepchenko and Terasaki, 2003). Recent works have shown that nuclear regions of frequent transcription factor binding are likely co-enriched for other transcription factors, co-factors and polymerases (Chen et al., 2014; Liu et al., 2014; Tsai et al., 2017). These locations may contain enhancers with clusters of binding sites for multiple transcription factors and even multiple enhancers from different genes, sometimes even located on different chromosomes (Allahyar et al., 2018; Tsai et al., 2019; De Wit et al., 2013). Although further investigation is needed to establish this as a general occurrence in embryos, this hypothesis is in line with the proposal of transcriptional ‘hubs’ (Glossary, Box 1) (Fukaya et al., 2016; Lim et al., 2018; Tsai et al., 2019). Coupled with promoter regions pre-loaded with polymerases (Ghavi-Helm et al., 2014; Lagha et al., 2013; Saunders et al., 2013), these hubs can produce large numbers of mRNAs with minimal delays as soon as a gene becomes transcriptionally active (Fig. 3C) (Boettiger and Levine, 2009; Gaertner and Zeitlinger, 2014).

In order to concentrate transcription factors and polymerases into localized hubs over specific regions of the genome, there must be features at these locations that promote frequent interactions of transcription factors and polymerases with the chromatin. Importantly, the subnuclear location of transcription factor binding hotspots do not move perceptibly over timescales of minutes, as observed for Bicoid and Ubx (Mir et al., 2017; Tsai et al., 2017). Ubx microenvironments were not observed with a mutant Ubx that cannot recognize its binding sites but that can still interact with DNA non-specifically (Tsai et al., 2017). This argues for specific interactions between proteins and DNA as the cornerstone for hub formation and is consistent with several studies (Denholtz et al., 2013; Monahan et al., 2019; De Wit et al., 2013). For example, Nanog binding can directly induce spatial clustering of target genes to form consolidated hubs (De Wit et al., 2013). In line with the observed locational stability of transcription factor binding hotspots, the movement of chromatin fibers within the nucleus is slow due to their size (Dion and Gasser, 2013). Therefore, DNA can be sufficiently stable over timescales of minutes to anchor transcriptional hubs to specific locations within the nucleus. However, the contents within these hubs are likely dynamic over timescales of minutes because the individual binding interactions with the DNA are transient. Transcription hubs would dissolve once the underlying DNA-binding sites are no longer accessible, e.g. due to heterochromatinization (Allshire and Madhani, 2018; van Steensel

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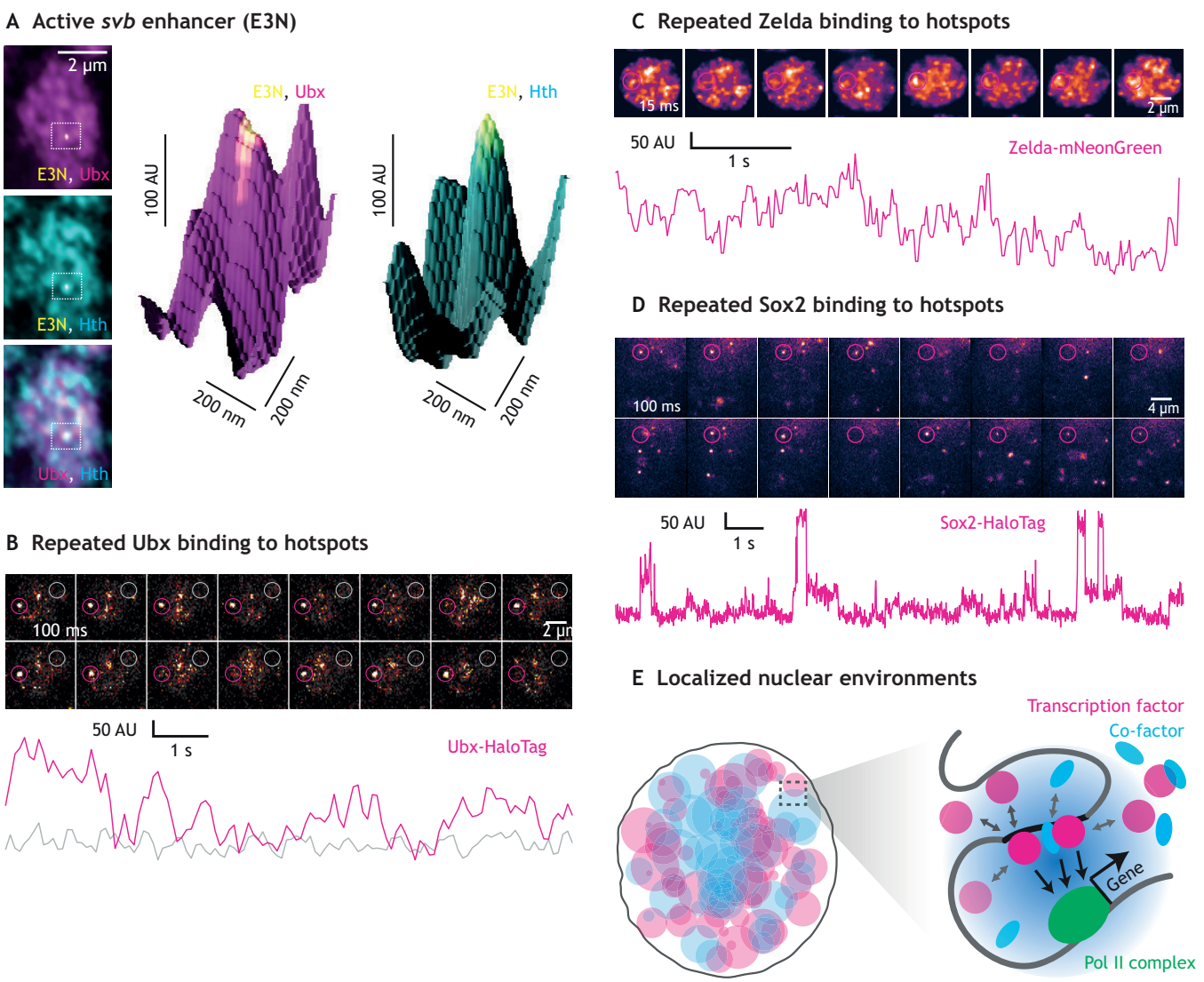


**Fig. 2. Localized transcription environments with frequent and dynamic transcription factor binding.** (A) The distributions of Ultrabithorax (Ubx) and the co-factor Homothorax (Hth) within *Drosophila melanogaster* embryos are heterogeneous and do not, in general, overlap. The E3N enhancer of *svb* is regulated by Ubx and specifically requires Hth for expression. Transcription sites driven by E3N colocalize with concentration maxima of both Ubx and Hth, suggesting that they reside in transcriptional hubs with specific compositions of transcription factors and co-factors. The surface plots on the right show zoomed in views of the region outlined by the white box. The height of these surface plots indicate Ubx (left) or Hth (right) intensity. (B) Binding of individual Ubx molecules on the chromatin is short lived, lasting on average less than a few seconds. However, there are hotspots within the nucleus that show repeated Ubx-binding events (pink circle, in contrast to the grey circle that is not a hotspot). (C) The distribution of Zelda in cells of *D. melanogaster* embryos is also heterogeneous and Zelda dynamically binds to specific locations within the nucleus (circled). (D) Sox2 binding in mouse embryonic stem cells is also short lived but repeatedly occurs at specific hotspots in the nucleus (circled). (E) These locations inside the nucleus with frequent transcription factor binding may contain a multiple binding sites for specific transcription factors, therefore trapping them. Interactions of transcription factors with the binding sites and with each other could then nucleate the formation of localized transcriptional environments, subdividing the nucleus into many different enriched and specialized regions for transcriptional regulation. (A,B) Adapted, with permission, from Tsai et al. (2017). (C) Adapted, with permission from Mir et al. (2018). (D) Adapted, with permission, from Liu et al. (2014).

and Furlong, 2019). Owing to this potential volatility, reliably forming transcriptional hubs containing genes that are not close to each other on the linear DNA may require specific distributions of the chromatin that promote interactions between relatively distal parts of the genome.

### Chromatin as the foundation for local transcriptional environments

Chromosomes and contiguous regions of a chromosome tend to occupy relatively globular and defined territories in space, instead of spreading out evenly throughout the nucleus (Cremer et al., 2006). This behavior conforms to the prediction of polymer physics where long polymer chains mixed in a restricted space tend to distribute themselves such that each chain occupies a defined territory (Chiariello et al., 2016; Sazer and Schiessel, 2018). The result of a relatively compact globular distribution would be that the physical distances between distal parts of the same chromosome are shorter than if the same chromosome were spread across the nucleus, increasing the chances of interactions. Given that the borders between different chromosome territories tend to contain gene-rich regions, this distribution of chromosomes could additionally promote inter-chromosome interactions between genomic regions with similar properties (Zheng and Xie, 2019).

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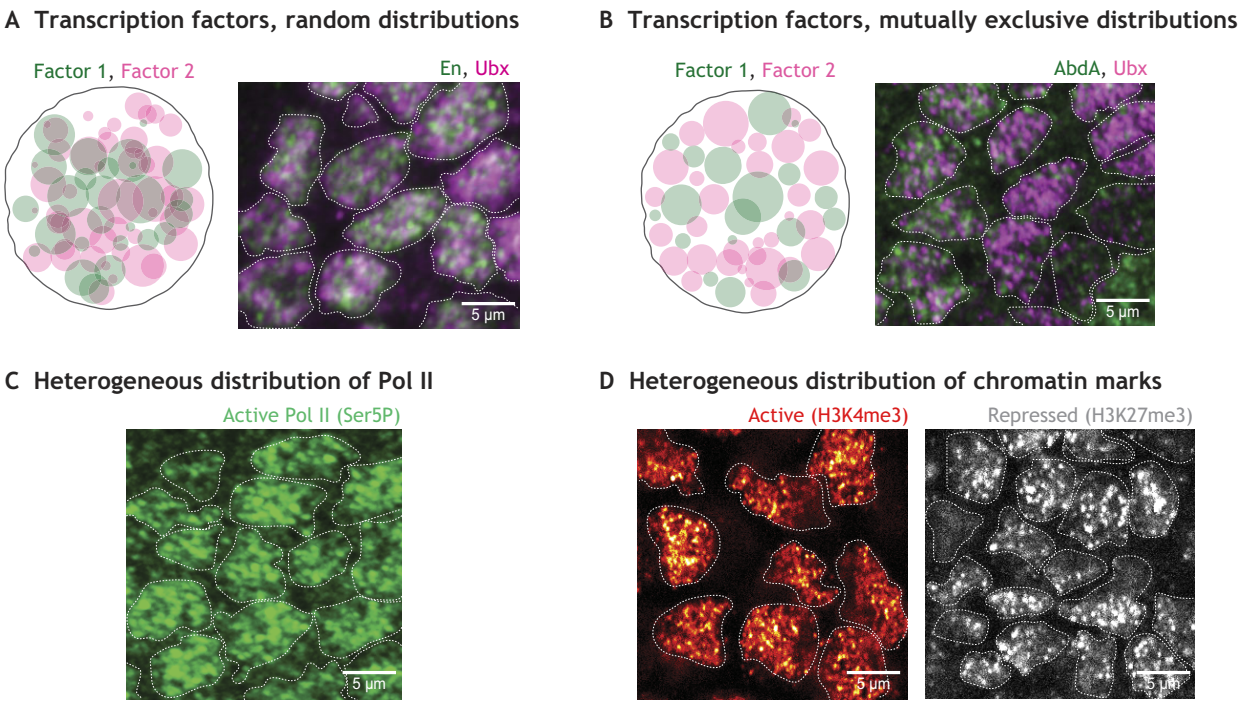


**Fig. 3. The nucleus is a heterogeneous environment for transcriptional regulation.** (A) Without specific protein-to-protein interactions or binding sites in proximity, transcription factors should distribute randomly relative to each other, as appears to be the case for Ubx and Engrailed (En). (B) Distribution of transcription factors that show mutual exclusion, e.g. Ubx and Abdominal A (AbdA), may result from mechanisms such as competition for similar binding sites and/or differential interactions with co-factors localized to different parts of the nucleus. (C) Active Pol II distribution within the nucleus is heterogeneous within embryos, with concentrated regions possibly denoting highly active transcriptional compartments. (D) The distributions of active and repressed chromatin, as marked by histone modifications (H3K4me3 and H3K27me3, respectively), may also be heterogeneous in embryos. This difference in chromatin state and, accordingly, its accessibility could guide the formation of transcriptional microenvironments over specific locations inside the nucleus. All cells shown are from the ectoderm in the first abdominal (A1) segment of stage 15 *Drosophila melanogaster* embryos. (A-C) Adapted, with permission, from Tsai et al. (2017) (D) A.T. and J.C., unpublished.

Therefore, one can imagine a model whereby the conformation and positioning of chromosomes help to bring distal enhancers and genes sharing common regulatory logic together, where high availability of binding sites for particular transcription factors would create regions with high local concentrations of the transcription factors and their co-factors through a positive-feedback loop. These regions could be highly effective and specific transcriptional microenvironments (Glossary, Box 1) that activate or repress transcription. Alternatively, they could perform other roles beyond directly modulating transcription (e.g. promoting changes in the chromatin state). According to this model, changing the spatial distances between parts of the genome would either promote or suppress interactions between genes and enhancers, perhaps resulting in hubs with different contents and/or hubs forming in different locations. It is therefore of great interest to track how multiple points on the genome are distributed in the nucleus and to follow them through different phases of embryogenesis to see whether stretches of chromatin are consistently brought together or kept apart.

In addition to genome organization within the nucleus, direct control of DNA accessibility is likely a crucial component for efficiently funneling transcription factors and polymerases to hubs (Kaplan et al., 2011; Porcelli et al., 2019). As an extreme example, large regions of open chromatin, such as those induced through herpes simplex virus infection (McSwiggen et al., 2019), are sufficient for creating sub-nuclear compartments enriched for DNA-binding proteins and polymerases. As local variations in the accessibility of the DNA can profoundly alter where the transcriptional machinery is recruited, it is important to observe which chromatin states (Heintzman et al., 2009) can form, sustain and dissolve transcriptional environments containing specific genes (Fig. 3D). From the other direction, transcriptional hubs could also change the state of the chromatin to sustain their activities. Applying specific and local methods to modify histone marks in developmental systems would be a crucial tool for understanding how the chromatin and transcriptional hubs interact with and influence each other (Crocker and Stern, 2013; Mendenhall et al., 2013).

Genomics has provided us with panoramic views of enhancer activity across eukaryotic genomes and suggests a high degree of organization at the level of the chromatin (Gibcus and Dekker, 2013). In the case of humans, estimates for the total number of enhancers are in the order of hundreds to thousands of elements (ENCODE Project Consortium, 2012). Many enhancers can regulate promoters over long distances, sometimes spanning over one million base pairs (Lettice et al., 2003). In some loci, long-range interactions are formed *de novo* and are associated with gene activation (Dixon et al., 2015; Mifsud et al., 2015; Rao et al., 2014; Schoenfelder et al., 2015; Suter et al., 2011). Others are pre-formed and invariant across tissues, and are independent of gene activation (Galupa and Heard, 2017; Ghavi-Helm et al., 2014), suggesting interactions with longer timescales. In these two scenarios, different mechanisms must presumably operate to induce active transcription. Furthermore, one gene is often regulated by multiple enhancers, which can drive partially overlapping patterns of gene expression. How are such long-range, multi-loci interactions choreographed across development to provide precise patterns of gene expression?

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How do transcriptional microenvironments relate to recently described domains of chromatin organization?

Chromosome conformation capture (3C)-based techniques have shown that there are sub-megabase stretches of chromosomes that show increased interactions (including between promoters and enhancers) internally but decreased interactions with outside elements (Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012). These topologically associating domains (TADs; Glossary Box 1) represent higher-order chromatin structures that span multiple genes and regulatory regions (Fig. 4A). TAD boundaries delineate these regions and could constrain the search space for distal regulatory elements to find their promoters and reduce the chances that they interact with off-target promoters. Weakened and misplaced TAD boundaries can lead to changes in gene expression profiles (Lupiáñez et al., 2015) and defects in development (Narendra et al., 2016; Schwarzer et al., 2017; Soshnikova et al., 2010), although the overall impact of TAD organization on dynamic gene expression is still not entirely clear. It also remains unclear whether TADs are functionally equivalent structures across the phylogenetic tree (reviewed by Dekker and Heard, 2015), as different molecular players underlie the physical nature of TADs in various taxa (reviewed by Szabo et al., 2019). Genes in the same TAD tended to follow the same expression kinetics (up- or downregulation) upon embryonic stem cell differentiation in mammals (Nora et al., 2012; Zhan et al., 2017). Additionally, in breast cancer cells undergoing hormone treatment, the majority of genes in 20% of the TADs were so tightly coordinated that each TAD functioned as a discrete regulatory unit (Le Dily et al., 2014). This type of coordination suggests that genes within the same TAD share and are responsive to an environment with similar transcription factors, polymerases and histone modifications. TADs and transcriptional microenvironments may feedback upon one another, reinforcing underlying interactions (e.g. topological interactions, *cis*-regulatory interactions such as enhancer-promoter) across development and creating tightly regulated conditions encompassing several genes (Fig. 4A).

Previous studies using earlier versions of 3C techniques have shown that, in tissues where the β-globin locus is active, it interacts more frequently with its *cis*-regulatory elements, while intervening sequences loop out – this spatial conformation was named the 'active chromatin hub' (Glossary, Box 1) (de Laat and Grosveld, 2003; Tolhuis et al., 2002). Several other lines of research have shown that concurrent chromatin interactions do indeed occur simultaneously between multiple enhancers and promoters, as opposed to separately (Jiang et al., 2016; Markenscoff-Papadimitriou et al., 2014; Oudelaar et al., 2018; Patrinos et al., 2004). Importantly, these higher-order chromatin conformations, including chromatin loops within topological domains, have been proposed as a mechanism to guide transcription factors such that they can efficiently traverse the genome to find their targets (Mirny et al., 2009). Imaging experiments have demonstrated that enhancers from the *svb* locus tended to be close to each other when their targets are active, even when they are copied onto a different chromosome (Tsai et al., 2019). Thus, these local regions may provide localized and specialized environments for the regulation of specific genes, creating feedback loops in space built upon multiple cooperative interactions. These hubs can position an enriched but localized environment to a group of genes in proximity, such as those within a TAD. Changes in the hub would co-regulate genes under its influence, which could lead to strong correlations between their activities, as seen in the context of TADs.

It will be essential to understand how topological structures, identified with molecular biology techniques, overlap with the transcriptional microenvironments, defined based on microscopy approaches (Fig. 4B,C). For example, will all sequences within the same TAD reside in the same microenvironment? Do

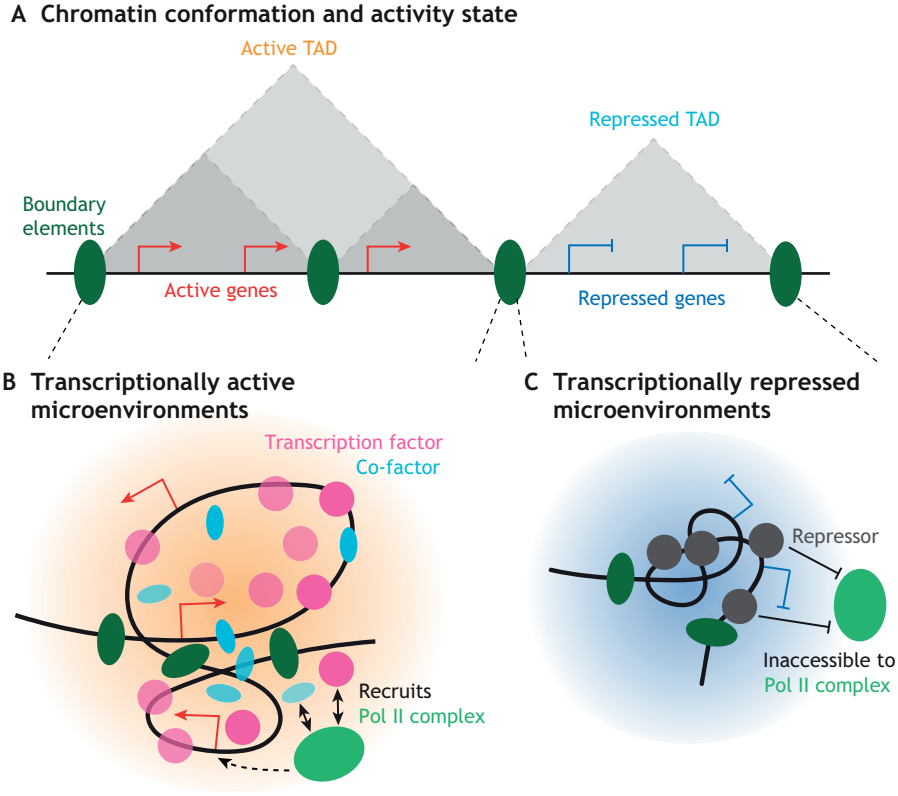


**Fig. 4. Transcriptional microenvironments may underlie topologically associating domains (TADs).** (A) Genomic regions are organized into transcriptionally active and repressed TADs, as detected by 3C-derived techniques and RNA-seq. These regions are bordered by boundary elements form higher-ordered 3D structures, as visualized by tracing the conformation of the chromatin in TADs using super-resolution techniques. (B,C) Transcriptionally active TADs (B) form open and expansive loops, while transcriptionally repressed TADs (C) are condensed into compact globular structures. Different transcriptional microenvironments could form the basis for these TADs with different physical and transcriptional properties, allowing separated and distant genetic loci to interact within the same physical space, which are then detected as TADs.

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microenvironments include sequences from different TADs? As mentioned above, microenvironments can aggregate sequences located on different chromosomes, while probably being confined by the topological organization. Recent imaging methods developed to trace chromatin, combining super-resolution microscopy with multiplexed fluorescence *in situ* hybridization, have revealed TAD-like structures with globular conformation in cultured insect cells and *Drosophila* embryos (Bintu et al., 2018; Boettiger et al., 2016; Cardozo Gizzi et al., 2019; Mateo et al., 2019; Szabo et al., 2018; Wang et al., 2016). In these studies, the authors either measured transcription levels of one (Cardozo Gizzi et al., 2019) to multiple genes (Mateo et al., 2019) or did not measure RNA directly using imaging. Yet the boundaries of globular domains align with known TADs that are either transcriptionally active or inactive. However, the authors also observed significant cell-to-cell variations not captured by 3C experiments. Additionally, the physical size of the associated regions appears to depend on the transcriptional state of the TAD, with active regions being more expansive than inactive and specifically repressed regions (Fig. 4B,C). Further development of such technology would allow us to visualize clusters of DNA and clusters of transcription factors simultaneously.

## The roles of protein-protein interactions in tuning transcriptional hubs

Although transcription factor binding sites and chromatin conformation could provide the foundation for trapping transcription factors and forming the core of transcriptional hubs, additional mechanisms likely exist to tune their properties further. For example, Ubx and a closely related Hox factor Abdominal A (AbdA) are partitioned into different areas of the nucleus in cells expressing both factors, even though they share nearly identical DNA binding preferences (Tsai et al., 2017) (Fig. 3B). As the Ubx and AbdA have different protein-interaction domains, they could interact with different sets of co-factors and other proteins. Although co-factors do not directly activate transcription, enhancers frequently contain specific binding sites for them (Choo et al., 2011; Crocker et al., 2015a), allowing different co-factors to guide transcription factors to different parts of the genome. Furthermore, co-factors pairing with transcription factors could also fine-tune their DNA-binding sequence preference (Rastogi et al., 2018; Slattery et al., 2011), adding means to localize them preferentially. Accordingly, recent work on how enhancers distinguish between Hox transcription factors with close DNA consensus sequences has highlighted the importance of co-factors and other collaborator proteins in achieving specificity and sufficient transcriptional output (Carnesecchi et al., 2020; Sánchez-Higueras et al., 2019).

Currently, the contributions of interactions other than DNA binding to transcription factor function are not well understood, partly due to their less well-defined surfaces of interaction and the potential for multiple interaction partners with similar strengths of interaction. Despite their generally weaker strengths of interaction compared with sequence-specific DNA binding (Shin and Brangwynne, 2017), tuning the compositions of transcriptional hubs through multiple protein-protein interactions could provide a crucial way to increase the fidelity of transcriptional regulation. For example, in the development of mouse olfactory neurons, long-range intergenic transcriptional enhancers converge into interchromosomal hubs through cooperative protein-protein interactions between the transcription factors Lhx2 and Ebf (Monahan et al., 2017).

Phase separation (see Glossary, Box 1) has been proposed as a mechanism to bring together proteins and chromatin in membraneless subnuclear domains (Alberti et al., 2019). Proteins with disordered domains that provide large surfaces for hydrophobic interactions have been observed to form phase-separated condensates when their nuclear concentration reaches a critical threshold (Boija et al., 2018; Cho et al., 2018; Feric et al., 2016; Lu et al., 2018; Sabari et al., 2018). Within these droplets, constant interactions between proteins could form scaffolds that trap proteins, preventing them from readily moving through and leaving the droplet. Although subnuclear compartmentalization formed through phase separation has been extensively reported in cells and could provide interesting avenues of localized transcriptional regulations in subnuclear compartments (Alberti, 2017), the functions of these droplets in developing embryos remain relatively unexplored (Mir et al., 2019).

Phase-separated droplets appear to be a phenomenon stemming from extensive protein-protein and protein-nucleic acid interactions (Alberti, 2017), and it is unclear how they are related to the transcriptional hubs, in which dynamic but specific protein-DNA interactions appear to play foundational roles. These two phenomena may overlap, where protein-DNA interactions temporarily creates a localized nucleating hub of transcription factors and polymerases, the protein-interaction domains of which could then recruit other proteins, locally increasing their concentrations above the threshold needed for droplets to form. The extensive protein-protein interactions within a phase-separated droplet could, in turn, influence, tune and stabilize the composition of transcriptional hubs forming around it. Tracking protein movements around transcriptional hubs and phase-separated droplets, especially when they are nearby, to see how droplets modify the movements of transcription factors in transcriptional hubs would clarify how the two phenomena may overlap and influence each other. To achieve this, fluorescently labeled factors compatible with imaging in live embryos must be constructed and validated.

## The significance of microenvironments in development and evolution

In developing embryos, gene regulation must coordinate lineage specification based on hundreds to thousands of intracellular and extracellular signals, while compensating for stresses stemming from the environment outside of the embryo. Ensuring regulatory resiliency and efficiency in the face of all these complexities may be a major reason that multicellular organisms invested heavily into structuring the nucleus (Heger et al., 2012). A heterogeneous nuclear environment opens the door to mechanisms of transcriptional regulation beyond transcription factors finding specific binding sites on the DNA.

## Providing a kinetically efficient mechanism to use low-affinity enhancers

Many essential genes for developmental regulation rely on the use of enhancers harboring low-affinity binding sites for the relevant transcription factors (Crocker et al., 2015a; Gaudet and Mango, 2002; Scardigli et al., 2003). How a low-affinity regime depending on transient interactions translates into consistent and specific gene expression has been a paradox in developmental biology (Crocker et al., 2015b; Kribelbauer et al., 2019). Recent works have discovered that low-affinity sites are developmentally important and can confer specificity in distinguishing between closely related transcription factors (Antosova et al., 2016; Crocker et al., 2015b; Farley et al., 2015, 2016; Gaudet and Mango, 2002; Lebrecht et al., 2005; Lorberbaum et al., 2016; Rister et al., 2015; Rowan et al.,

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2010; Tanay, 2006). For transcription factor families that share similar DNA-binding consensus sequences, such as the Hox family, high-affinity binding sites can recruit many members of the same family with sufficient stability to activate downstream transcription, which results in broad and sometimes ectopic expression (Crocker et al., 2015a). Transient low-affinity interactions may, therefore, be necessary to reduce incorrect regulation through crosstalk within a family of transcription factors.

Owing to the shorter dwell times of transcription factors at low-affinity binding sites, higher factor concentrations would be needed to ensure their functionality (Kribelbauer et al., 2019). However, because the nucleus is a crowded space packed with nucleic acids and other macromolecules (Hancock, 2014a,b), there could be insufficient physical space to significantly increase the concentrations of multiple transcription factors to accommodate all genes relying on low-affinity binding sites. Additionally, with higher affinity binding sites for the same transcription factors likely also operating in the same cell, adjusting the concentrations of transcription factors globally in the nucleus can lead to undesirable consequences. As a result, the straightforward strategy of increasing the total nuclear level of all relevant transcription factors would be untenable. The concept of transcriptional microenvironments, whereby molecules are concentrated to local regions where the relevant genes are located, could circumvent this constraint (Dufourt et al., 2018; Mir et al., 2017; Tsai et al., 2017). We hypothesize that this implies, on the one hand, mechanisms to sustain this increased concentration and, on the other hand, mechanisms to cluster genes with similar regulators. These mechanisms might be intertwined, if, for example, regions binding the same transcription factors tend to cluster together using multiple attractive interactions between transcription factors and co-factors as a scaffold. These hubs can additionally deplete other areas of the nucleus of those transcription factors, reducing the chances of off-target regulation.

As described by Tsai et al. (2017), localized transcription hubs with high local concentrations of transcription factors could effectively use these low-affinity binding sites, overcoming their low intrinsic affinity by promoting constant binding attempts from transcription factors trapped nearby. These low-affinity enhancers may also depend on specific co-factors, as seems to be the case for *svb*. This dual requirement further improves the specificity of these enhancers as environments with the correct combination of factors and co-factors would be required. Transcription dependent on low-affinity interactions would also stop quickly as soon as the local concentration of factors is no longer present, enabling high temporal precision in gene regulation. The activity of genes within heterogeneous nuclei would also depend on their position relative to regions with or without transcription hubs, as well as on the exact composition of the hubs, allowing for differential gene regulation within different areas of the nucleus. In this case, cells that supposedly have the same average nuclear concentrations of transcription factors, co-factors and polymerases could have different regulatory behaviors. Thus, low-affinity binding sites coupled with localized transcriptional hubs make nuanced and complex transcriptional regulation possible with high temporal and spatial precision.

### Preserving phenotypic robustness through ensuring consistent conditions at the gene locus

Beyond providing a means to build complex regulatory networks, localized transcriptional environments could also contribute to buffer the noise and variance stemming from the stochastic nature of molecular interactions between chromatin, transcription factors and polymerases. Recent imaging experiments with sub-second temporal resolution have shown that mRNA transcription occurs in bursts (Bothma et al., 2014; Chubb et al., 2006; Tunnacliffe et al., 2018). The total transcriptional output is likely regulated by tuning the frequency and length of bursting, assuming that maximum polymerase loading rate and, accordingly, maximum mRNA syntheses rate are achieved during a burst (Cai et al., 2008). The inherently stochastic nature of bursts leads to variations in the number of mRNA produced at any given moment, even among similar neighboring cells (Eldar and Elowitz, 2010; Raj and van Oudenaarden, 2008). Although post-transcriptional controls can counteract this variance, this variability imposes fundamental limits on the consistency of the downstream regulatory networks that must sense the results of these transcription events. For gene expression linked to permanent cell fate decisions, the noisy transcriptional output could adversely impact the consistency of embryogenesis without mechanisms to control, compensate or account for these variations. Interestingly, recent studies have suggested that the properties of transcriptional noise are gene dependent (Battich et al., 2015; Blake et al., 2006; Fraser et al., 2004; Metzger et al., 2015; Yan et al., 2017), indicating that the regulatory elements controlling a gene play a substantial role in defining its noise properties. As a result, it would be essential to understand the strategies that specific genes use to constrain noise in their transcriptional output.

One possible way to reduce the sensitivity to noise in expression would be to saturate the system (compare Fig. 5A with A′). Inside transcriptional hubs that can continuously recruit more than sufficient transcription factors to sustain active transcription, short-term fluctuations in the production of upstream factors would have a limited impact on the downstream decisions (Fig. 5B,B′). Over more extended periods, changing the accessibility of the underlying chromatin (e.g. through histone methylation) and the interaction between transcription factors and co-factors (e.g. through post-translational modifications) could alter the transcription factor concentrations and compositions of these hubs or even lead to their disassembly. Additionally, such transcriptional hubs would buffer variations in the regulatory regions that recruit transcription factors. For example, it has recently been shown that the number of trichomes in *Drosophila* larvae that develop as a result of *svb* expression is resistant to temperature stress (Crocker et al., 2015a; Frankel et al., 2010). However, removing some of the *svb* enhancers that provide overlapping expression patterns led to defects in trichome numbers in larvae under temperature stress; this correlated with lower Ubx concentrations and lower transcriptional output (Tsai et al., 2019). Interestingly, such a phenotypic deficiency in trichome numbers could be rescued through introducing the *svb cis*-regulatory region (without the *svb* gene itself) on a different chromosome from the endogenous locus. Concomitantly, when the introduced *cis*-regulatory region colocalized with the transcriptionally active endogenous *svb* locus, the local Ubx concentration around the transcription sites and their transcriptional output were increased. Thus, the wild-type *svb* locus with multiple redundant enhancers may be under-saturating transcription factor concentrations in localized transcriptional environments that use multiple enhancers to trap transcription factors. Creating saturating concentrations of transcription factors over many enhancers and genes would be difficult without the positive feedback of multiple attractive interactions found in transcriptional hubs.

Of note, the *svb* gene is active late during embryo development and sits at the crux of a regulatory network that resembles an hourglass (Stern and Orgogozo, 2009), where multiple signaling

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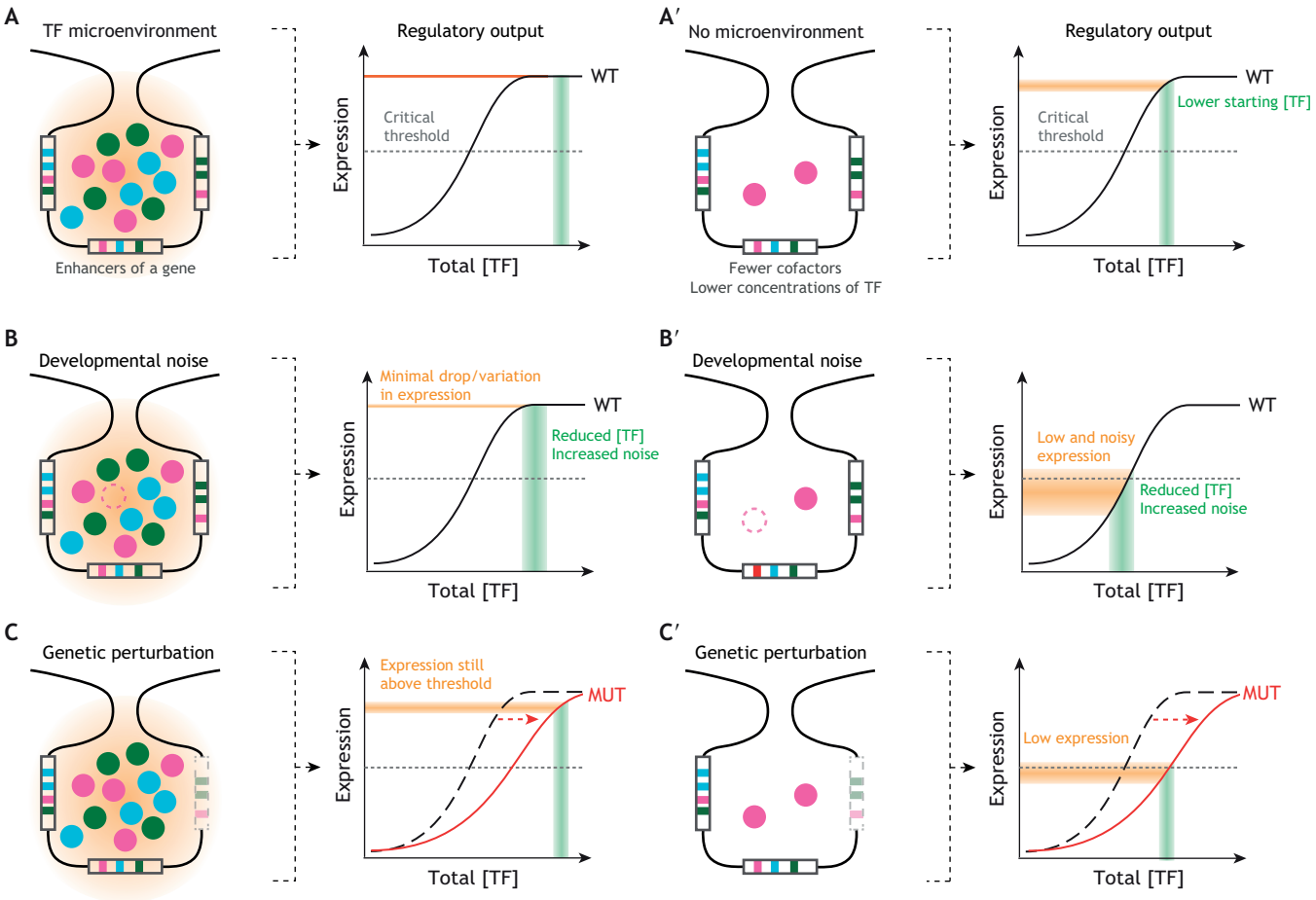


**Fig. 5. Transcriptional microenvironments facilitate robust gene regulation during development.** (A) Highly enriched transcriptional environments around developmental genes ensure stable and consistent regulatory output. Three enhancers for a single gene are shown. Under normal conditions, the regulatory inputs are saturating, with only small amounts of variation. (A′) In contrast, genes functioning outside microenvironments would have lower transcription factor concentrations [TF] and fewer co-factors at their disposal. Under ideal conditions, both A and A′ allow for sufficient gene expression above a critical threshold needed to drive cell fate decisions. (B) In the event that the level of a transcription factor drops (missing pink circle on the schematic) and/or fluctuates (widened green shade on the graph), the buffering capacity of the microenvironment keeps the reduction and/or noise in expression levels to a minimum. The output remains solidly above the critical level and the gene locus is able to specify cell fate unambiguously. (B′) Without the benefit of a microenvironment, any changes in transcription factor concentrations are propagated to the output, and could make cell fate specification ambiguous. (C) Genetic perturbations, such as the deletion of an enhancer, can alter the regulatory response of the system (red line on the graph), even if the concentration of transcription factors around the gene does not change. The mutated locus (MUT) now requires a higher transcription factor concentration to achieve the same level of gene expression. The high concentrations of transcription factors in microenvironments can maintain expression levels above the critical threshold, preserving function. (C′) On the other hand, the effect of the deletion is more severe for genes outside the microenvironments, with lower transcription factor concentrations, which risk a drop in expression levels below the critical threshold. WT, wild type.

pathways converge on *svb*, to then regulate multiple downstream genes that remodel the cell terminally (Chanut-Delalande et al., 2006). Owing to the irreversible nature of the effect of *svb*, the expression level of genes like *svb* may behave like a binary on/off switch. Being in a saturating environment when active should lead to a robust switching of cell fate that does not fluctuate with environmental perturbations. It remains to be seen whether other genes, especially those that require a switch-like behavior to function properly, also adopt this strategy. This setup is likely not ideal for genes that must have graded expression levels depending on their regulatory inputs and thus operate as proportional sensors. Thus, it may not be desirable for such genes to be in microenvironments that are strongly activating or repressive. Considering that many embryo-patterning genes operate based on precisely sensing the levels of morphogen gradients (e.g. Dubuis et al., 2013; Petkova et al., 2019), transcriptional hubs that saturate low-affinity enhancers may play a less important role during earlier stages of embryo development. As transcription factors selectively binding to specific regions of DNA appears to be important in forming transcriptional hubs, the relatively unmarked chromatin conformation that lacks accessible nucleosome-free regions during early embryogenesis (Li et al., 2014) could also mean that transcriptional hubs are not always present during development. Tracking the heterogeneity of transcription factors in the nucleus during different stages of embryogenesis could yield insights into their formation and function.

### Tweaking individual binding sites and enhancers without breaking the regulatory output

Using transcriptional hubs to ensure stable conditions around multiple enhancers from different genes would additionally confer protection if genetic mutations compromised one or more of the regulatory elements (Fig. 5C,C′). As a result, this could reduce the tight pressure on individual enhancers to conserve particular

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binding sites, providing a means for the system to undergo moderate levels of evolutionary changes without compromising the overall functionality of the system. Gradually added and modified low-affinity binding sites would more likely function if they can be pulled into pre-existing transcriptional hubs, because they no longer need to recruit their own transcriptional machinery from scratch (Fig. 6A,B). Moreover, in contrast to evolving new transcription factors with completely different DNA sequence preferences, low affinity sites provide a mechanism to distinguish between transcription factors with similar DNA-binding preferences (Crocker et al., 2015a; Kribelbauer et al., 2019). This type of regulatory mechanism would be more lenient toward a gradual process of building complexity using gene duplication, followed by modifications to generate additional regulatory elements and transcription factors from pre-existing ones (Fig. 6C). The increasing expansion of eukaryotic families of transcription factors congruent with the complexity of the organism (Shiu et al., 2005) suggests that this is likely the case (Wunderlich and Mirny, 2009).

As low-affinity binding sites in isolation are not kinetically efficient, regulatory networks using them as the primary regulatory elements would use a larger number of sites to achieve sufficient transcriptional output. The contribution of each element in such systems would be relatively modest. Thus, changing these elements would be less likely to cause widespread changes throughout the regulatory network, making them amenable to harboring genetic diversity. This could explain why low-affinity binding sites have potentially higher evolvability (Crocker et al., 2015a,b), as intermediate steps of the evolutionary process are less likely to encounter negative selection due to phenotypic deficiencies. The reduced likelihood that mutations are lethal would permit their accumulation in a population, giving rise to a population that contains a diversity genotypes than can turn into beneficial phenotypes under selective pressure – leading to adaptability (Draghi et al., 2010; Paaby and Rockman, 2014). In contrast, high-affinity binding sites could single-handedly effect clear regulatory changes even within a sizeable regulatory network by stably recruiting transcription factors (Preger-Ben Noon et al., 2016), especially repressors, making changes that modify them evolutionarily riskier and leading to reduced phenotypic fitness The ability to create sub-partitions within the nucleus with their own distinct transcriptional environments could, therefore, greatly aid the ability of multicellular eukaryotes to evolve highly complex gene regulation. Exploring when membraneless subnuclear compartments with specialized roles for transcriptional regulation first appeared in the evolutionary tree of life and how their functions have evolved would clarify the molecular basis of complexity in multicellular eukaryotes.

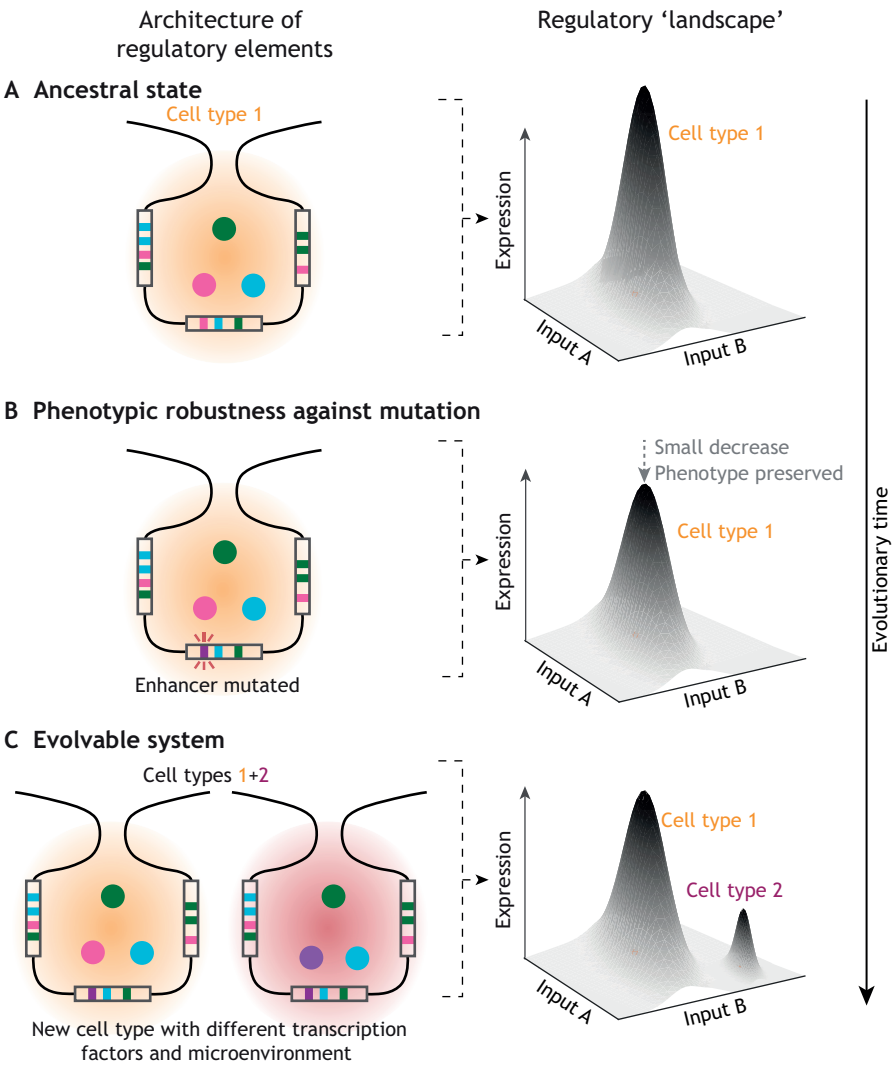


**Fig. 6. Transcriptional hubs support phenotypic robustness and improve evolvability.** (A) A gene with multiple regulatory elements resides in a transcriptional hub and controls the development of a cell fate (cell type 1). The expression of the gene is strong given a set of regulatory inputs (here abstracted as inputs A and B in the right panel), leading to robust cell fate determination. (B) Mutation of one regulatory element (depicted by a pink binding site turning purple) leads to reduced binding of the pink transcription factor, but the presence of other binding sites maintains a sufficient level of transcriptional output to determine cell fate. The phenotype that this gene controls is therefore robust against genetic perturbations. With limited loss of fitness, the genetic variation generated by the mutation is preserved in the population. (C) The ability to retain genetic mutations without reducing fitness increases the likelihood of further evolution through increasing the diversity and, subsequently, adaptability of a population. This permits a gradual process where a transcription factor 'preferring' the mutated regulatory element evolves from duplication and minor modification of a pre-existing transcription factor (the purple transcription factor from the pink). This new transcription factor and its corresponding binding sites could then lead to the development of a different cell type (cell type 2) at a different location on the regulatory landscape (different levels of inputs A and B in the right panel).

## Conclusions

After extensive efforts in dissecting the molecular players of transcriptional regulation and in mapping out the extensive interactions between transcription factors and enhancers, we have now come to a point where we need to integrate the individual components in a coherent whole. Developments in genomics that have found higher-order organizations of the chromatin and in advanced microscopy approaches showing heterogeneous distributions of the transcriptional machinery in cells and animals have borne out Comings' hypothesis that the nucleus is 'a well-ordered place' (Comings, 1968). For regulating gene expression, the order within the nucleus is likely built upon relatively low-affinity interactions between chromatin, transcription factors and other members of the transcriptional machinery, which allows for high degrees of tunability and specialization. The field is now ripe to extend our focus beyond individual binding sites, enhancers and genes in isolation, and to start considering how multiple regulatory regions create, interact with and function within higher-order regulatory networks and structures. These structures are inherently multifactorial, with many different interactions all contributing to their formation, maintenance and dissolution. The structures occupying the 'middle layer' between the molecular interactions of the underlying machinery and the complex patterning of the embryo body plan are currently a missing link between integrating stochastic molecular signals into consistent regulatory decisions during embryogenesis.

Localized transcriptional environments are likely a centerpiece in this middle layer because they provide a physical means in the nucleus to integrate multiple genes and transcription factors in the same space, using localized feedback loops to filter out noises and to preserve adequate gene expression in the face of mutations and environmental challenges. Currently, comprehensive information about the physical properties of these transcriptional microenvironments is lacking. Parameters such as their size distribution, stability over time, total number within the nucleus and location relative to different parts of the genome would have direct effects on the mechanisms governing their formation and strategies to use them effectively. Identifying and understanding these processes would be necessary to form a coherent picture of how robust developmental decisions could emerge out of myriads of stochastic building blocks. Emergent properties are difficult to deduce if only the atomistic elements of the system can be manipulated and observed; thus, developing methods to observe and control the system as coherent units will be crucial to further our understanding of the principles of gene regulation during development. We anticipate rapid developments will soon enable imaging of different elements within the nucleus in living embryos at high resolution. Complementing genomics techniques with increasingly high cell-type specificity and potentially single-cell resolution will also become progressively mature and widely accessible. Together, they will reveal mechanisms during development that can counteract, control and even take advantage of the inherent noise and variability in transcription. Dynamic and multifactorial transcriptional hubs could be merely the first step in understanding how the physical organization within the nucleus shapes and controls gene expression in eukaryotes.

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