

REVIEW

Of numbers and movement – understanding transcription factor pathogenesis by advanced microscopy

Julia M. T. Auer^{1,§}, Jack J. Stoddart^{1,*}, Ioannis Christodoulou^{1,*}, Ana Lima^{1,*}, Kassiani Skouloudaki¹, Hildegard N. Hall¹, Vladana Vukojević^{2,‡,§} and Dimitrios K. Papadopoulos^{1,‡,§}

ABSTRACT

Transcription factors (TFs) are life-sustaining and, therefore, the subject of intensive research. By regulating gene expression, TFs control a plethora of developmental and physiological processes, and their abnormal function commonly leads to various developmental defects and diseases in humans. Normal TF function often depends on gene dosage, which can be altered by copy-number variation or loss-of-function mutations. This explains why TF haploinsufficiency (HI) can lead to disease. Since aberrant TF numbers frequently result in pathogenic abnormalities of gene expression, quantitative analyses of TFs are a priority in the field. *In vitro* single-molecule methodologies have significantly aided the identification of links between TF gene dosage and transcriptional outcomes. Additionally, advances in quantitative microscopy have contributed mechanistic insights into normal and aberrant TF function. However, to understand TF biology, TF-chromatin interactions must be characterised *in vivo*, in a tissue-specific manner and in the context of both normal and altered TF numbers. Here, we summarise the advanced microscopy methodologies most frequently used to link TF abundance to function and dissect the molecular mechanisms underlying TF HIs. Increased application of advanced single-molecule and super-resolution microscopy modalities will improve our understanding of how TF HIs drive disease.

KEY WORDS: Haploinsufficiency, Quantitative microscopy, Transcription factors, Super-resolution microscopy, Transcriptional regulation

Introduction

Precise tissue- and cell-specific regulation of gene expression is required for development and homeostasis. Transcription factors (TFs) tightly control transcriptional programmes in a cell- and tissue-dependent manner. General TFs and the core transcriptional machinery bind to gene promoters, whereas specific activating and repressing TFs bind to gene regulatory elements – i.e. transcriptional enhancers or silencers – and interact with promoter-bound complexes to control transcription (Horikoshi et al., 1988). This interaction is

thought to occur through the Mediator complex, a multi-subunit protein complex that recognises enhancer-bound TF complexes and signals to RNA polymerase II to transcribe genes. The Mediator complex was first discovered in yeast (Kim et al., 1994); in humans, the basic Mediator complexes generally enhance transcription (Andrey et al., 2013; Bergiers et al., 2018; Ernst et al., 2011; Golan-Lagziel et al., 2018; Heintzman et al., 2009; Noordermeer et al., 2011; van Bömmel et al., 2018). Thus, cell type-specific gene expression is mainly influenced by the ability of TFs to bind their target sites within promoters or enhancers, which, in turn, relies on the chromatin state of these sites (Ernst et al., 2011).

The main TF families are classified according to their DNA-binding domains. These include the helix-turn-helix (HTH), zinc-finger (ZNF), basic helix-loop-helix (bHLH), basic leucine zipper (bZIP) and nuclear hormone receptor binding domains (Table 1), each featuring distinct mechanisms of sequence-specific DNA recognition and binding (Badis et al., 2009; Lambert et al., 2018; Wei et al., 2010). Furthermore, TFs often work in unique combinations, for example during development and differentiation (Bergiers et al., 2018). Thus, different cell types can be distinguished based on their TF repertoires. Thereby, the clustering of TF-binding sites in enhancer sequences favours functional synergies of TFs that are co-expressed in the same tissue (Golan-Lagziel et al., 2018).

The aberrant function of TFs can have profound effects on development and disease. Of particular interest is how reduced levels of some TFs may influence their function and result in haploinsufficient phenotypes. Haploinsufficiency (HI) is the inability of a gene to rely on only one (of its two) alleles to exhibit its normal function. Therefore, HIs generally result in loss-of-function (LOF), often abnormal, phenotypes. It follows that HIs are properties of genes and, although ‘dosage-sensitive genes’ is a broader term than ‘HI genes’, henceforth, for simplicity, we use the two terms interchangeably.

LOF mutations or deletions of HI genes are inherited in a dominant manner, resulting in phenotypic alterations or abnormalities caused by the insufficiency of one intact allele to confer full gene functionality. Computational models aim to predict the tolerance of a system at the cell, tissue or organismal level to a single functional copy of a gene. Such models have mainly focussed on predicting the probability of genes being LOF-intolerant (pLI) (Lek et al., 2016), i.e. rating genes with a score of $n \geq 0.9$ as intolerant and with a score of $n \leq 0.1$ as tolerant to LOF mutations (Lek et al., 2016). Bioinformatics approaches and machine learning have identified 7841 HI genes in the human genome (Shihab et al., 2017), linking them to a number of dominantly inherited HI diseases. Key examples are eye disorders, such as aniridia (Jordan et al., 1992), keratitis (Mirzayans et al., 1995) and ocular colobomas (Sanyanusin et al., 1995; Williamson et al., 2014), as well as multiple cranial, facial and limb diseases – including synpolydactyly (Muragaki et al., 1996), schizencephaly (Brunelli et al., 1996), craniosynostosis of Adelaide type (Hollway et al., 1995;

¹MRC Human Genetics Unit, University of Edinburgh, Edinburgh EH4 1XU, UK.

²Center for Molecular Medicine (CMM), Department of Clinical Neuroscience, Karolinska Institutet, 17176 Stockholm, Sweden.

*These authors contributed equally to this work

†These authors should be considered as joint senior authors

§Authors for correspondence (s1611128@sms.ed.ac.uk; vladana.vukojevic@ki.se; dpapado2@ed.ac.uk)

DOI D.K.P., 0000-0003-0914-3051

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Table 1. Overview of TF families according to their DNA-binding domains and main structural characteristics of binding site recognition

TF families and DNA-binding domains	DNA-binding properties and structural characteristics	TF examples (human or as indicated)	References
Helix-turn-helix (HTH)	These TFs contain four α -helices and an N-terminal flexible arm. The third α -helix forms base-specific contacts with the major DNA groove, the N-terminal flexible arm interacts with the minor DNA groove in a sequence-specific manner. The turn between the first and second helix interacts with the DNA backbone.	PAX6, POU5F1 (OCT4), HOXA1, DUX4, SOX2	(Gruschus et al., 1997; Holland et al., 2007; Kissinger et al., 1990; Qian et al., 1993)
C2H2 zinc finger (ZNF)	This family contains one β -sheet (β -hairpin) and one α -helix, stabilised by a zinc ion (Zn^{2+}) between two cysteine and two histidine (C2H2) residues. In this 'classic' ZNF, three amino acid residues within the α -helix bind a major DNA groove triplet. Multiple ZNF domains allow binding of extended DNA sequences.	CTCF, PRDM1, GLI1, MTF1, HIVEP1	(Chen et al., 1999a; Elrod-Erickson et al., 1996; Keller and Maniatis, 1992; Klug, 1999; Maekawa et al., 1989; Najafabadi et al., 2015; Pavletich and Pabo, 1991, 1993; Radtke et al., 1993)
Basic helix-loop-helix (bHLH)	Two amphipathic α -helices are separated by a linker region (the loop) that can vary in length. TFs containing this domain can form both homo- and heterodimers by interaction of hydrophobic residues with the corresponding surfaces of each helix.	TCF4, MYC, MYOD1 ASCL1 (MASH1)	(Cao et al., 2002; Davis et al., 1990, 1987; Krause et al., 1990; Meredith and Johnson, 2000; Murre et al., 1989; Sun and Baltimore, 1991)
Basic leucine zipper (bZIP)	DNA binding takes place via basic 'arms' adjacent to the bZIP protein-dimerization domain. Dimerization is crucial for DNA binding and transcriptional regulation. For this, the leucine residues between the right-handed amphipathic α -helices of the two domains form a coiled-coil dimer. These TFs can form homo- or heterodimers.	FOS, JUN, CREB3L4 (CREB4), BACH1, MYC, C/EBPA (zebrafish), GCN4 (yeast)	(Bohmann et al., 1987; Dang et al., 2008; Ellenberger et al., 1992; Landschulz et al., 1988; O'Shea et al., 1991; Oyake et al., 1996; Petrovick et al., 1998; Sassone-Corsi et al., 1988)
Nuclear hormone (steroid) receptors	Receptor TFs bear a conserved DNA-binding region, comprising two zinc fingers, and a specific hormone-binding region in their C termini. Ligand binding can cause nucleocytoplasmic translocation and may affect their ability to bind DNA. α -helices of the DNA-binding domain contact DNA half sites. Homo- or heterodimers regulate transcription and act either as co-activator or co-repressor complexes.	<u>Homodimeric receptors:</u> glucocorticoid (NR3C1), mineralocorticoid (NR3C2), progesterone (NR3C3), estrogen (ESR1, ESR2) receptors; <u>androgen receptor isoforms A and B (AR-A and AR-B), triiodothyronine receptor (THRA/THRB)</u> <u>Heterodimeric receptors:</u> retinoid X receptors alpha, beta and gamma (RXRA, RXRB, RXRG), vitamin D receptor (VDR)	(Chandra et al., 2008; Giguere et al., 1987; Green et al., 1986; Hollenberg et al., 1985; Lippman et al., 1973; Luisi et al., 1991; Mangelsdorf et al., 1990; Miesfeld et al., 1986; Petkovich et al., 1987)

Jabs et al., 1993) and Greig cephalopolysyndactyly syndrome (Hui and Joyner, 1993; reviewed by Dang et al., 2008). Disease phenotypes have been attributed to ~ 300 HI genes and range from primary immunodeficiencies (Franco-Jarava et al., 2018; Torgerson and Ochs, 2014) to ribosomopathies (Kyritsis et al., 2019) and cancer (Inoue and Fry, 2017; Jennings et al., 2012; Largaespada, 2001). Interestingly, a significant proportion of dosage-sensitive genes encode TFs, with higher relative numbers of these TF genes exhibiting HI compared to other human genes (Ni et al., 2019).

Over 1600 human TF genes have been grouped into gene families (Lambert et al., 2018), allowing for in-depth analysis of HI incidence in specific subsets of TFs (Table 1). Overall, 122 TFs from 32 gene families were identified and designated as the most reliable dosage-sensitive (MRDS) genes (Ni et al., 2019). This categorisation of TF families has attempted to explain the sensitivity – or insensitivity – of different TFs to dosage and found HI to be predominantly correlated with small TF families. Such small TF families – comprising fewer than eleven members, e.g. the Grainyhead and C2H2-ZNF/homeodomain families – are present

in the MRDS dataset, whereas the largest subgroup of the zinc-finger TF family Krüppel-associated box (KRAB) domain-containing zinc finger proteins (KZFPs), is found to be dosage-insensitive and is not present in the MRDS dataset (Ni et al., 2019). This observation is yet to be explained, with evolutionary pressure being a plausible cause for limiting TF family size (Ni et al., 2019). Why other evolutionary mechanisms, such as gene duplication and divergence (Gehring et al., 2009), have not been deployed to expand these small families of TFs and alleviate HI remains unknown. Additional mechanisms that could account for such bias may relate to the regulatory, activating or repressing, behaviour of such TFs, or to the numbers of downstream-regulated genes. Thus, more work is needed to identify possible structural or functional similarities between dosage-sensitive TFs to further pinpoint the underlying disease-causing mechanisms of HIs. This Review summarises how the advanced quantitative microscopy methodologies aid in the study of biophysical and functional properties of TFs, such that TF dosage-sensitivity at the molecular level may be linked to development and disease.

Factors resulting in HIs

Insufficient TF protein levels are the common denominator of haploinsufficient TF genes. In these cases, the abnormal phenotypes are triggered in a dosage-dependent manner (Gidekel et al., 2003; Gilchrist and Nijhout, 2001; Nishimura et al., 2001; Takeuchi et al., 2011). At the molecular level, altered TF numbers impair their functionality by interfering with their dynamic behaviour in cells and, thereby altering their transcriptional programmes (Bottani and Veitia, 2017; Veitia, 2003). We examine the following molecular and functional properties of TFs, which may underlie TF HIs in a tissue-specific manner (for a schematic representation, see Fig. 1):

- absolute TF numbers;
- stoichiometry of TFs and their interacting proteins;
- availability and binding kinetics to their TF target sites;
- stochastic nature of gene transcription;
- biophysical properties, such as their ability to form condensates.

How these parameters vary simultaneously between or within cells, tissues or entire organisms adds to the sheer complexity in characterising the causes of dosage sensitivity and HI in humans (Elowitz et al., 2002; Veitia, 2002). HI may manifest in a range of related phenotypes in humans. Moreover, it is gradually becoming clear that not one single molecular mechanism is responsible for TF HIs; rather, parameters that determine TF function or dysfunction act in concert.

Studying the mechanisms of TF concentration and function in TF HIs is becoming increasingly possible thanks to the continuous advancement of microscopy methods. Table 2 briefly outlines these methodologies and their potential applications to investigate TF biology, and selected methodologies are depicted in Fig. 2.

Absolute TF numbers

Most TFs are expressed and, typically, act in a cell type- or tissue-specific manner. RNA sequencing (RNA-seq) analyses of human

TFs found over a third of them to be enriched in specific tissues (Lambert et al., 2018; Uhlen et al., 2015). Spatiotemporally precise TF levels are key to driving cell specification and differentiation. Particularly during early development, different levels of stemness TFs control the fate of individual cells. For example, in mouse blastocysts, the distribution of SOX2 and POU5F1 (hereafter, referred to as OCT4), two of the key stemness TFs, confers an initial differentiation bias at the 4-cell stage (Goolam et al., 2016; Kaur et al., 2013; White et al., 2016). Later in embryonic development, differential expression of NANOG and GATA6 in a ‘salt-and-pepper’ pattern in the mouse embryonic inner cell mass controls the segregation of the epiblast from primitive endoderm lineages (Chazaude et al., 2006). Stemness TF levels continue to control the pluripotency of different stem cell populations in development (Adachi et al., 2013). For instance, the intrinsically fluctuating amounts of OCT4 and SOX2 contribute to the lineage commitment of mouse embryonic stem cells (ESCs) during differentiation (Strebinger et al., 2019). When *Pou5f1* and *Sox2* become downregulated, mouse ESCs can differentiate and activate the trophoblast stem cell TF networks required for normal embryonic development (Adachi et al., 2013; Masui et al., 2007; Niwa et al., 2000, 2005). This continued dependence on concentration for the precise function of TFs during development explains why aberrant TF gene dosage can have detrimental effects on development and physiology. Changes to the required concentration of TFs at different points in development can elicit diverse effects in gene regulation and, thus, result in different pathogenic phenotypes.

At the subnuclear level, local concentrations of TFs affect gene expression by controlling the kinetics of TF binding to chromatin, recruitment of RNAPolII and transcriptional bursting (Nelson et al., 2004; Senecal et al., 2014). In mouse ESCs, the clustering of SOX2-bound enhancers modulates the search for local SOX2 target sites and facilitates gene transcription (Liu et al., 2014). RNAPolII activity at the *POU5F1* enhancer is regulated by the accumulation of

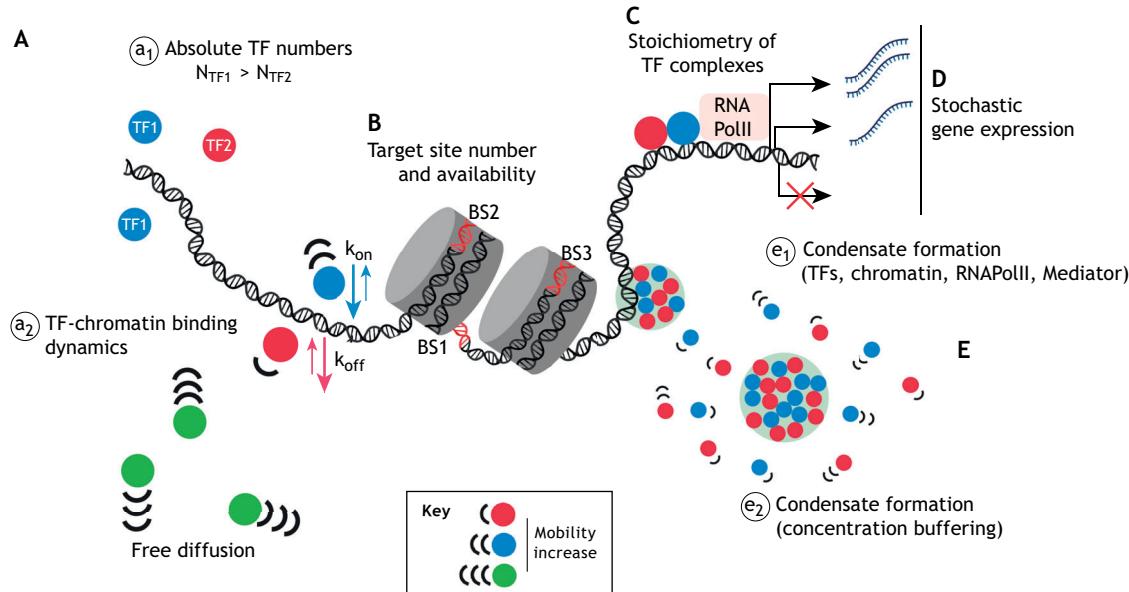


Fig. 1. Quantitative mechanisms that affect TF function. (A) a₁: N, absolute numbers of TF molecules. a₂: Quantitative TF-chromatin binding dynamics. (B) Number and availability of TF-binding sites on chromatin (red DNA stretches represent TF-binding sites). (C) Stoichiometry of TF-interacting proteins. (D) Stochastic gene expression. (E) e₁: TF co-condensate formation with chromatin, Mediator and RNA Pol II. e₂: Intranuclear buffering of TF concentration caused by formation condensate. BS, binding site. Blue, red and green circles represent different TFs; grey cylinders represent nucleosomes; circles with a light green background represent condensates.

Table 2. Overview of the main advanced microscopy methodologies used to study TF dynamic behaviour, concentration, stoichiometry and localisation

Microscopy methodologies (technical information)	TFs (Key questions/findings)	Advantages	Disadvantages
Fluorescence correlation spectroscopy (FCS)	This method can quantitatively characterise in live specimens (live cells in culture, <i>ex vivo</i> tissue/organ and the whole organism) TF concentration, mobility and the extent of TF binding to chromatin through changes in TF mobility (Chen et al., 2014b; Ehrenberg and Rigler, 1974; Mazza et al., 2012; Mir et al., 2017; Normanno et al., 2015; Papadopoulos et al., 2019; Papadopoulos et al., 2010; Vulkojevic et al., 2010; White et al., 2016; Yao et al., 2006). It can also provide information about TF stoichiometry, i.e. homo-oligomerisation by brightness analysis (Gaglia et al., 2013).	FCS allows the detection of TF molecules with the ultimate, single-molecule sensitivity. It is quantitative and non-destructive, enabling measurements in live specimens. Due to its high temporal resolution, FCS is suitable for the study of fast dynamical processes, e.g. diffusion of small molecules and fast reaction kinetics. FCS measurements are best performed when the system is in a steady-state. This is advantageous for the study of reaction kinetics, as one does not need to establish initial concentration gradients (Elson, 2013).	FCS measurements are best performed when the system is in a steady-state. While this is advantageous for the study of reaction kinetics (see Advantages), it is disadvantageous for the study of transient dynamical states. FCS is not best suited for the study of slow reactions, e.g. long TF residence time on chromatin (low 'off' rates). FCS does not 'see' immobile molecules; therefore, it is not best suited to study binding to large/stationary intracellular structures. For providing spatial information, FCS measurements have to be acquired sequentially at different locations. Interpretation of the measurements may be difficult since more than one process with similar characteristic times can give rise to the observed fluorescence intensity fluctuations. Conventional single-point FCS is not an imaging methodology.
Fluorescence cross-correlation spectroscopy (FCCS)	This method can be used to study the efficiency and stoichiometry of TF complex formation, such as hetero-/homo-dimers, as well as the chromatin-binding behaviour of these complexes (Bacia and Schwille, 2007; Krieger et al., 2014; Langowski, 2017; Papadopoulos et al., 2015; Reho et al., 2020).	Co-diffusion, which is detected by FCCS, indicates the presence of molecular interactions with no requirement for the proximity and orientation of individual fluorophores. FCCS provides information about both the concentration of free and bound TF molecules without the need to separate the two fractions. The TF-chromatin binding equilibrium dissociation constant (K_d) can be measured in live specimens.	False positive results may be obtained in the cases of signal bleed-through (cross-talk) from one detector to the other. False negative results may be obtained if the system alignment is not optimal. Conventional, single-point FCCS is not an imaging methodology.
Massively parallel FCS (mpFCS)	Interacting proteins are each labelled with spectrally distinct fluorophores. Separate detectors enable simultaneous recording of temporal fluctuations of fluorescence intensity from both fluorophores. Temporal autocorrelation analysis is performed for each signal separately. In addition, covariance in fluorescence intensity fluctuations from the two different fluorophores is analysed to assess the co-diffusion of the investigated molecules (Bacia and Schwille, 2007; Krieger et al., 2014; Langowski, 2017; Rika and Binkert, 1989; Schwille et al., 1997).	All advantages as for FCS, with the addition of spatial information. Quantitative confocal imaging without scanning is enabled (Krnpat et al., 2019). Study of fast dynamical processes is made possible due to the high temporal resolution of the SPAD camera with a temporal resolution of (~20 µs) (Krnpat et al., 2019), which is more than an order of magnitude better than for the best EMCCD cameras.	mpFCS has the same disadvantages as FCS. Additionally, it has reduced sensitivity, because out-of-focus light, originating from molecules in remote focal planes above/below the focal plane, can pass through adjacent pinholes and increase the background signal. The disadvantage of SPAD matrix detectors compared to EM-CCDs stems mainly from the variability in dark count rates between individual detectors, which, if not accounted for, gives a non-uniform signal-to-noise-ratio over an image frame.
Selective plane illumination microscopy (SPIM)-FCS	Unlike conventional fluorescence microscopy where both illumination and fluorescence are collected along the same axis, the collection objective in SPIM is placed perpendicularly to the excitation path. A thin multiple observation volume elements.	This allows 3D imaging of biological samples with a high frame rate and micron-scale spatial resolution in the axial direction.	Sample preparation is more complex than for confocal microscopy and requires special holders to enable the use of two objectives. Structural inhomogeneities in the sample produce 'shadows'

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Microscopy methodologies (technical information)	TFs (key questions/findings)	Advantages	Disadvantages
light sheet (0.5–1.5 μm thick) is created using a cylindrical lens to focus light in one direction only (the sheet thickness), while leaving it to spread in the other two axes (the sheet width). Fluorescence intensity fluctuations are recorded from a plane in the sample using fast scientific complementary metal oxide semiconductor (SCMOS) or EM-CCD cameras (Capoulade et al., 2011; Krieger et al., 2015; Krieger et al., 2014; Langowski, 2017; Wohland et al., 2010).	ICS and ICCS transcription factors in live cells (Pernus and Langowski, 2015).		behind the obstructions, creating a light sheet of uneven intensity. Non-uniformity of light sheet thickness over longer distances limits the area from which a uniform signal can be recorded.
Image correlation spectroscopy (ICS) and image cross-correlation spectroscopy (ICCS)	Intra- and inter-cellular analysis of TF movement and <i>in vivo</i> flow was characterised in a multicellular organism (Clark et al., 2016). Additionally, ligand-induced TF translocation and diffusion maps have been obtained (Milkuni et al., 2017).	Quantitative characterization of TF concentration, mobility and homo-oligomerisation can be achieved using well-established techniques and non-specialised microscope systems.	ICS and ICCS perform well only on relatively homogenous samples, since the presence of regions that are significantly brighter than the average fluorescent species biases the correlation function (Hendrix et al., 2016). Large number of pixels, $n>64$, need to be considered for an accurate spatial correlation analysis.
ICS methodologies extract quantitative information about molecular concentration, mobility and oligomerisation by calculating a correlation function from fluorescence intensity fluctuations as a function of space across an image acquired by confocal laser scanning microscopy (CLSM) or across space and time from a CLSM imaging time series (Digman et al., 2005; Mets et al., 2020; Wiseman, 2013; Wiseman, 2015). The dual-colour variant of ICS, Image cross-correlation spectroscopy (ICCS), can quantify molecular interactions from the analysis of two-channel CLSM images (Comeau et al., 2008), whereas pair correlation function (pCF) analysis can be used to study long-range intracellular molecular diffusion, detect the presence of barriers to diffusion and produce a map of transport of chemically inert molecules in live cell nuclei (Hinde et al., 2010).		SPT visualises the displacement of individual molecules. Because only a small subset of TF molecules are fluorescent in the sample, the total TF concentration is not a limiting factor for the success of the measurements.	The spatial and temporal resolutions of this method are limited by the resolution and image acquisition speed of the microscopy setup. Analysis of TF displacement relies on the diffusion and kinetics model chosen to fit the data, such as the number of different diffusion behaviours (Mazza et al., 2012).
Single-particle tracking/single-molecule tracking (SPT/SMT)	By tracking individual TF molecules in a specified/observation area/volume, the trajectories are visualised and displacements of TFs can be measured. Models are used to derive information about the chromatin-binding kinetics of single or multiple TFs, e.g. free diffusion or chromatin-bound (Ball et al., 2016; Chen et al., 2014a; Elf et al., 2007; Gebhardt et al., 2013; Hansen et al., 2017; Izeddin et al., 2014; Mire et al., 2017; Normanno et al., 2015). Similarly, the fractions chromatin-bound or free-diffusing TF molecules can be derived (Chen et al., 2014b; Hipp et al., 2019; Liu et al., 2014; Menta et al., 2018; Morisaki et al., 2014; Teves et al., 2016).		FRAP is used to determine the freely diffusing or chromatin-binding behaviour of TF molecules, even at higher concentrations (that are not accessible by FCS) and can, therefore, be used to investigate TF diffusion even in highly expressing cells and within condensates. FRAP is easy to perform and easy to understand intuitively.
Fluorescence recovery after photobleaching (FRAP)	Within a small selected region of the sample, intense laser light quickly renders the fluorescent molecule unable to fluoresce. The rate of fluorescence recovery in the photobleached region is recorded using a low-intensity laser beam to excite the not-bleached molecules, and fitted with suitable models to derive the diffusion and/or the off-rates (Axelson, 1981; White and Stelzer, 1999).	FRAP allows the measurement of TF diffusion and chromatin-binding behaviour and is used to derive the mobile (freely diffusing) versus immobile, e.g. chromatin-bound, TF fractions (Li et al., 2019; Mazza et al., 2012; Phair and Misteli, 2000; Sprague et al., 2004; Teves et al., 2016).	The imaging instrumentation used may be too slow for the recording of rapidly diffusing molecules. The shape and the size of the photobleached area need to be accounted for in the analysis of FRAP curves, which may be intricate. Photochemical and/or thermal cell injuries are possible. Incomplete fluorescence recovery due to the presence of obstacles that obstruct diffusion and presence of reversibly photobleached fluorophores may lead to false interpretations.

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Table 2. (Continued.)

Microscopy methodologies (technical information)	TFs (key questions/findings)	Advantages	Disadvantages
Förster resonance energy transfer (FRET)	Interacting molecules are fluorescently labelled with distinct fluorophores selected so that the emission spectrum of one of the fluorophores, called a donor, overlaps with the absorption spectrum of the other fluorophore, called an acceptor. The transfer of energy from an excited donor to an acceptor molecule leads to acceptor molecule excitation and subsequent emission of fluorescence. This allows us to measure the physical proximity (≤ 10 nm) between the molecules (Forster, 1946).	This method – although strictly speaking being a proximity assay – is often used to study binding and complex-formation between different TFs, such as the formation of hetero- and homo-dimers, and interactions with chromatin (Evans et al., 2007; Mivelaz et al., 2020; Musselman et al., 2013; Robertson et al., 2018; Vancreaenbroeck and Hofmann, 2018; Wang et al., 2006; reviewed by Day et al., 2001). FRET has also been used to study ternary TF complexes (Demarco et al., 2006; Shyu et al., 2008; Sun et al., 2010). However, FRET is indispensable for the study of conformational changes, in which case the donor and acceptor molecules reside on the same TF molecule (Vamosi et al., 2008).	FRET imaging is the first super-resolution fluorescence microscopy technique, as it allows imaging of distances <10 nm. FRET is simple to configure on wide-field/CLSM microscopes that are available in most labs.
Bimolecular fluorescence complementation (BiFC)	Interacting molecules are tagged with complementary, symmetrically/asymmetrically split fluorescent protein halves that are, on their own, not fluorescent. Formation of complexes leads to assembly of fluorescent proteins; interactions are inferred from the reconstitution of fluorescence (Hu et al., 2002; Hu and Kerppola, 2003).	This method can be used to study direct interactions between TFs or TFs and other proteins (hetero- and homodimers) (Dard et al., 2019; Hu et al., 2002; Hu and Kerppola, 2003; Kerppola, 2006; Papadopoulos et al., 2015; Shokri et al., 2019).	The hetero- and homodimers are visualised whereas the monomeric partners are not.
Confocal spinning disc (CSD) microscopy	Multipoint excitation is obtained by using a pair of rotating disks. The collector disk contains micro-lenses, whereas the pinhole disk contains pinholes. This way, each micro-lens has an associated co-aligned pinhole, which is axially positioned at the focal plane of the micro-lens. As the discs spin, an array of focused laser beams scans across the specimens (Hayashi and Okada, 2015; Jonkman and Brown, 2015; Petran et al., 1986).	CSD is used for TF imaging and localisation, and it can be combined with other methodologies to study TF mobility and binding dynamics (Hayashi and Okada, 2015; Jonkman and Brown, 2015; Petran et al., 1986; Schueder et al., 2017; Zhan et al., 2014).	CSD provides higher temporal resolution as compared to classic point scanning confocal microscopy, thus allowing for imaging of fast dynamic processes in live specimens.
2-/multi-photon excitation (2PE/MPE) microscopy	Out-of-focus excitation of fluorophores in the axial direction is significantly reduced, compared to that of single-photon excitation, by using two (or more) lower-energy photons (Denk et al., 1990; reviewed by So et al., 2000).	This is used for imaging and localisation of TFs and it can be combined with methods to measure TF mobility and chromatin-binding dynamics (Denk et al., 1990; Ferguson et al., 2012; Guan et al., 2015; Yao et al., 2006).	The use of multi-photon excitation enhances penetration depth, thus facilitating imaging deeper within tissues. Background fluorescence is reduced by decreasing out-of-focus excitation.
Selective plane illumination microscopy (SPIM), lattice light-sheet microscopy (LLSM), reflected light-sheet microscopy (RLSM) and highly inclined laminated optical sheet (HILLO)	A group of methodologies that deploy different strategies to create a thin ($0.5\text{--}1.5\ \mu\text{m}$) sheet of light that is used to illuminate the sample. This reduces out-of-focus light and improves signal-to-noise ratio, and enables imaging deeper into the sample.	TF imaging and localisation studies can be combined with TF imaging in multiple-focal planes to enable <i>in toto</i> (whole tissues or embryos) with significantly reduced photobleaching and phototoxicity to live specimens. Imaging is not limited to the cellular plasma membrane at the adhesive surface, but can be performed deep within cells.	Lateral spatial resolution is somewhat worse than in As in mpFCS, out-of-focus light, originating from molecules in remote focal planes above/below the focal plane, can pass through adjacent pinholes and increase the background signal.
			Sample preparation and mounting may be challenging. The full numerical aperture (NA) of the imaging objective cannot be utilised, as objectives with a lower NA are used to reimage the sample plane, limiting the NA to <1.0 .

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Table 2. (Continued.)

Microscopy methodologies (technical information)	TFs (key questions/findings)	Advantages	Disadvantages
Total internal reflection fluorescence (TIRF) Evanescent waves only penetrate a short distance, ~200 nm, into the sample, significantly reducing out-of-focus light (Axelrod, 1981; Douglass and Vale, 2008).	TIRF can be combined with other imaging methodologies to measure TF mobility, chromatin-binding dynamics and subnuclear localisation (Axelrod, 1981; Bonham et al., 2009; Douglass and Vale, 2008; Horn et al., 2016).	TIRF similarly allows for less phototoxicity and less photobleaching, and high signal-to-noise ratio.	The limited penetration depth of the evanescent wave only permits imaging and studying of fluorescent proteins at the cell surface, the plasma membrane at the adhesive surface or <i>in vitro</i> . As a result, structures located deeper within cells can usually not be visualised. Therefore, for cells bearing nuclei deeper within the cell, the study of TF behaviour can be challenging.
Structured illumination microscopy (SIM, 3D-SIM) A super-resolution fluorescence imaging technique where a movable diffraction grating is placed in the path of the laser beam and the sample is excited with a known, spatially structured pattern of light. Several images – the number of which depends on width and spacing of the pattern lines – are acquired by changing the angle and orientation of the pattern, processed and mathematically deconvolved to yield a super-resolution image (Gustafsson, 2000; Gustafsson, 2005).	This method is used for TF imaging, localisation and the measurement of TF molecular movement and dynamic binding to chromatin, in combination with other methodologies (Gustafsson, 2000; Gustafsson, 2005; Patel et al., 2013; Penraad-Mobayed et al., 2018).	SIM is based on wide-field microscopy. By altering the pattern frequency, the axial resolution can be adjusted. The lateral resolution in SIM is ~100 nm, and the axial resolution is ~250 nm (in 3D-SIM). Fluorophores used for conventional microscopy can be used for SIM imaging. Fast processes can be studied in live cells and dual-colour SIM imaging is possible.	Errors in the grating and mismatch of refractive indexes can lead to errors in image reconstruction. SIM is very sensitive to out of focus light and the signal is noisier. Cells are overexpressing fluorescently tagged TF molecules cells are being imaged. Image reconstruction is slow, requiring ~1–30 s of processing time.
Stimulated emission depletion (STED) and 3D STED An excitation laser beam with a Gaussian intensity profile is well-aligned with a red-shifted STED laser beam, characterised by a ‘doughnut-shaped’ intensity profile that is close-to-zero at the centre and of high intensity at the circumference. These superimposed laser beams are raster-scanned across the sample. The Gaussian beam excites fluorescent molecules. However, before the excited molecules emit a fluorescence photon, the STED laser stimulates them to return to ground state by emitting a photon with exactly the same energy as the STED laser. This, effectively, reduces the probability of fluorescence emission and only molecules located in the very centre of the STED beam – which are not efficiently stimulated to return to the ground state, since there the STED beam intensity is low – can emit fluorescence. This, in turn, increases the resolution past the diffraction limit (Donnert et al., 2007; Hell and Wichmann, 1994; Willig et al., 2007).	This method can localise TFs with a precision that is, for measurements in live cells, up to an order of magnitude better than confocal microscopy (50–100 nm) (Donnert et al., 2007; Hell and Wichmann, 1994; Heller et al., 2013; Li et al., 2019; Willig et al., 2007). In combination with other methodologies, e.g. STED-FCS (Egeling et al., 2009; Kastrup et al., 2005), TF movement and dynamic binding to chromatin can be characterised.	STED, in its original realisation, greatly improves the lateral resolution, leaving the axial resolution unchanged. Axial resolution and lateral resolution are improved in 3D-STED. STED images are acquired directly, and image reconstruction and processing are not required, although processing is often applied to enhance the contrast. STED microscopy imaging can be performed in live cells.	The power of the STED laser beam determines the spatial resolution, and laser power of 5–200 MW/cm ² is needed to achieve the highest possible spatial resolution. This, in turn, may lead to photobleaching and phototoxicity, when measurements are performed in live cells. Only some fluorophores are sufficiently photostable and compatible with the STED lasers available in STED microscopes. STED microscopy is a raster scanning methodology. The temporal resolution of STED microscopy imaging is, therefore, low.
Stochastic optical reconstruction microscopy (STORM), direct STORM (dSTORM), photoactivated localisation microscopy (PALM) and fluorescence PALM (fPALM) This group of super-resolution fluorescence microscopy methodologies relies on the use of photo-switchable fluorophores that can undergo reversible changes in their intrinsic properties through the use of light as an external stimulus. At the beginning of the imaging process, all fluorophores are in the so-called ‘off’ state, which is non-fluorescing (dark). A small subset of molecules residing at longer intermolecular distances than	These methods can locate TFs with a precision in the tens of nanometres range (~50 nm) (PALM Cai et al., 2019; Gebhardt et al., 2013; Hansen et al., 2017; Izeddin et al., 2014; Liu et al., 2014); fPALM (Akamatsu et al., 2017; Spacek et al., 2017); dSTORM (Gao et al., 2017; Hansen et al., 2017).	PALM and STORM allow for the highest spatial resolution of all optical microscopy methodologies discussed and are quantitative, as they are able to detect single molecules and count their numbers. Multi-colour imaging is also possible.	These methods require particular fluorescent labelling, e.g. photo-activatable and photo-switchable fluorophores or dyes. Reconstruction of the images necessitates many on-off cycles (>10,000), which limits the application of these methodologies to slow processes. Image processing is time consuming. Very high sample labelling density is needed – the Nyquist-Shannon sampling theorem states that the minimum

Continued

Table 2. (Continued.)

Microscopy methodologies (technical information)	TFs (key questions/findings)	Advantages	Disadvantages
those imposed by the diffraction of light are stochastically switched 'on' through photoactivation. After a precise readout of their location, these fluorophores switch back to the dark state or are photobleached with the readout light. A new subset of fluorophores is then switched on. By reading out the location of the stochastically switched photoactivated subsets of molecules, and using computational methods to calculate and overlay the identified positions of individual molecules, a high-resolution image is constructed. Irrespective of whether photoactivatable proteins ([PALM (Beuzig et al., 2006); iPALM (Hess et al., 2006)] or organic dyes [STORM (Churchman et al., 2005; Rust et al., 2006); dSTORM (Heilemann et al., 2008)]) are being used, the localization accuracy of a single molecule depends on the number of photons being detected from that molecule.		sampling frequency of a signal that will not distort its underlying information, should be double the frequency of its highest frequency component. According to this theorem a dye molecule should be present at least every 10 nm to achieve an image resolution of 20 nm. Thus, immuno-based labelling approaches may result in a spotty image due to the size ($\sim 20\text{--}30\text{ nm}$) of the primary and secondary antibodies.	
Atomic force microscopy (AFM)	AFM is the collective term for an ever growing number of techniques characterised by the use of a mechanical probe to perform force measurement, topographic imaging or controlled sample manipulation (e.g. local stimulation of cells). In the most common AFM realization, a cantilever moving over the sample is deflected by changes at the sample surface. This allows the measurement of topology and forces due to binding (Binnig et al., 1986; Butt et al., 1990; Dufrene et al., 2017; Scheuring et al., 2001).	AFM is restricted to surfaces. Measurement artefacts from the cantilever tip or the applied force upon contacting the sample are common. The temporal resolution of AFM imaging is low – in the seconds-to-minutes range (Dufrene et al., 2017).	AFM is characterised by a high spatial resolution at the nanometre level and a high signal-to-noise ratio. It neither requires fluorescent labelling of the studied TFs nor sample fixation. Assessment of multiple physical, chemical and biological parameters is possible (Binnig et al., 1986; Butt et al., 1990; Kierzek et al., 2001; Sanchez et al., 2011; Scheuring et al., 2001; Shaffer et al., 2017; Yamamoto et al., 2014).

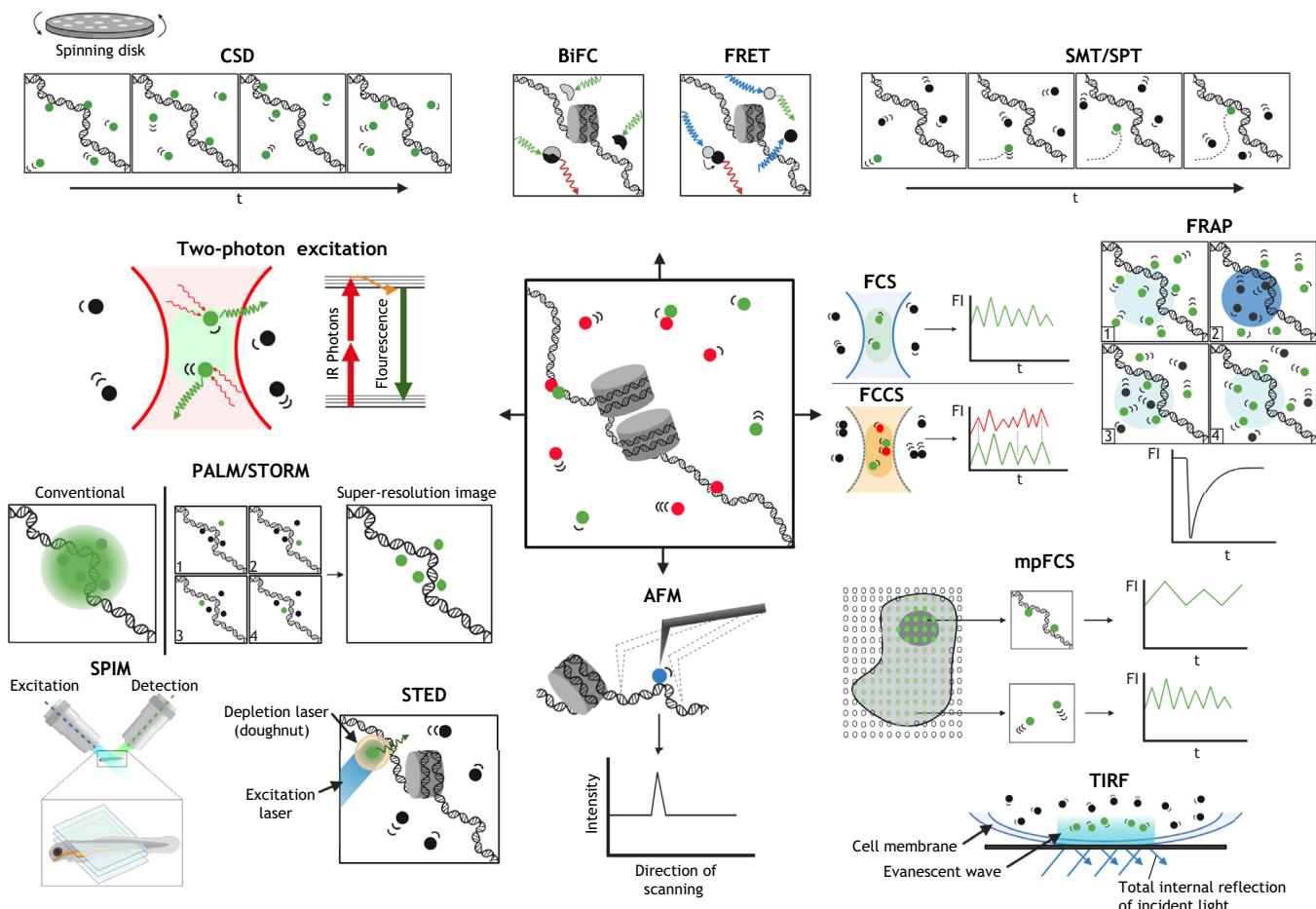


Fig. 2. Outline of quantitative microscopy methodologies used to study the concentration, dynamic behaviour, stoichiometry and subnuclear localisation of TFs. For details on each approach, see Table 2.

SOX2 and the chromatin reader BRD4, their chromatin-binding dynamics, as well as their interactions with the Mediator complex and the elongation controller P-TEFb, a cyclin-dependent kinase consisting of CDK9 and one of several cyclin subunits (Li et al., 2019). The subnuclear localisation of TFs may also vary between cell cycle stages and many TFs (including SOX2) become enriched on mitotic chromosomes, a phenomenon referred to as ‘mitotic bookmarking’ (Caravaca et al., 2013; Dufourt et al., 2018; Kadauke et al., 2012; Young et al., 2007). This local enrichment is mediated by the active nuclear import of TFs (Teves et al., 2016). TF binding persists through cell divisions, thus conferring transcriptional ‘memory’. At the cellular level, the concentration of TFs may provide cells with a molecular ‘readout’ of their relative positions in a tissue. For example, in *Drosophila* embryos, the chromatin-binding activity of Bicoid (Bcd), a maternally provided TF and key morphogen, which determines embryonic anteroposterior polarity, remains unaltered despite its low levels within posterior nuclei. This is achieved when Bcd binds hubs together with the pioneer TF Zelda (Zld), which results in increased local concentrations (Mir et al., 2017). Thus, while distinct concentrations of TFs may elicit differential cell fates, cells can deploy alternative mechanisms to preferentially counteract the effect of different TF concentrations when needed.

Although TF function has been extensively studied in flies, the latter are a rather inappropriate model to study TF HIs, as

the majority of heterozygous mutations do not cause observable developmental defects. This may be explained by the fact that the *Drosophila* genome is ~20 times smaller than the human genome, but the average concentration of TFs regulating this smaller genome is not proportionately decreased. Additionally, extensive endoreplication, which takes place in several fly tissues, produces polyploid cells during development, which are expected to be able to better buffer low TF gene dosage than diploid cells (Sher et al., 2013). However, gene regulation by TFs has been studied very extensively in this organism, therefore, we discuss crucial findings from *Drosophila*. Taken together, the discussed findings support the notion that the absolute number of TFs is an important determinant affecting gene expression by means of various mechanisms.

The importance of TF numbers and their subnuclear localisation for normal function are an enticing subject of research. The absolute number of TFs can be measured in living or fixed cells by fluorescence correlation spectroscopy (FCS) or super-resolution microscopy (SRM), respectively (Table 2 and Fig. 2). FCS allows the measurement of TF numbers and their molecular mobility with high temporal resolution (Ehrenberg and Rigler, 1974; Papadopoulos et al., 2010; Vukojevic et al., 2010). Additionally, the spatial variability of TF numbers within or between cell nuclei can be simultaneously analysed by employing massively parallel FCS (mpFCS) that uses an array of detectors coupled to diffractive optical elements (EM-CCDs) (Capoulade et al., 2011; Krmpot et al., 2019; Papadopoulos et al.,

2015), or by selective plane illumination microscopy (SPIM) coupled to fast cameras, such as electron-multiplying charge-coupled devices (EM-CCDs) (Capoulade et al., 2011; Krieger et al., 2015, 2014; Wohland et al., 2010). FCS has been used to study the dynamic behaviour of TF binding to chromatin, such as in the case of the formation of the Bcd gradient during early *Drosophila* development (Abu-Arish et al., 2010), the TF-chromatin binding dynamics of SOX2-OCT4 (Chen et al., 2014b), TetR (Normanno et al., 2015), the specific and non-specific binding of the Hox TF Sex combs reduced (Scr) (Papadopoulos et al., 2010; Vukovic et al., 2010) and the variable dynamic binding behaviour of the TF MYC (Rosales et al., 2013). FCS has also been employed to study the variability in TF concentration and how this leads to the acquisition of differential developmental fates as, for example, in the case of the TF Senseless in the *Drosophila* wing imaginal discs (Giri et al., 2020), FOS and JUN in HeLa cells (Szalóki et al., 2015) or the transcriptional co-activator Yorkie (the ortholog of the human YAP transcriptional co-activator) in different subcellular compartments of the developing *Drosophila* airways (Skouloudaki et al., 2019). Taken together, these studies show how diverse FCS methodologies can provide information on TF dynamic behaviour in live cells.

SRM methodologies, however, provide higher spatial resolution to visualise the distribution of subnuclear TFs and, therefore, complement FCS. Despite lacking the temporal and quantitative information, which FCS allows to derive by studying live samples, SRM methodologies provide excellent spatial information, which FCS methodologies cannot. Single-molecule spatial resolution may be achieved by limiting the number of fluorescent light-emitting molecules through selective illumination and/or sparse excitation (see Table 2 and Fig. 2). In these cases, TFs can be located – with high precision – on chromatin, whereas the concentration of TFs can still be inferred semi-quantitatively, by comparing their fluorescence intensity levels with those derived from a sample of known TF concentration (Lasker et al., 2020; Li et al., 2019; Reisser et al., 2018; Wollman and Leake, 2015). Furthermore, the quantification of TF transcripts can provide information about relative gene expression levels and, in some cases, may be used as a proxy to quantify TF abundance in a population of cells. Such relative measures of TF messages, their target transcriptomes and/or cell-to-cell heterogeneities of transcript numbers can be obtained by detection of RNA in single-cells. These measures scale with, albeit do not necessarily predict, TF variability at protein level and include single-molecule fluorescence *in situ* hybridisation (smFISH) and single-cell RNA sequencing (scRNA-seq). smFISH has been used to measure levels of *FOXO* mRNA (Bllice-Baum et al., 2019), *NKX2-2* mRNA distribution in mouse pancreatic islet cells (Cui et al., 2018) and *OCT4/SOX1/T-BRACHYURY* transcription in differentiating mouse ESCs (Lanctôt, 2015). A main advantage of scRNA-seq is that it can distinguish between cells on the basis of their expressed mRNA repertoire (transcriptome profiling), and can measure the levels of individual TF messages, such as of *OCT4/SOX2* in developing mouse embryos (Goolam et al., 2016), or *PAX6* in a subclass of mouse cortical cells (Zeisel et al., 2015). The combination of microscopy-based and transcriptomics modalities allows researchers to precisely quantify the cell-to-cell variability of TF concentration at the protein or mRNA level, as well as to assess how such variability affects their target transcriptome. Overall, one can link the absolute amount of TFs to their function in transcriptional regulation. To understand how aberrant levels of TFs may lead to HI phenotypes, the absolute number of TFs needs to be efficiently measured in single cells and between different cells in a tissue. Therefore, quantitative methodologies need continuous

advancement to become routinely accessible and accommodate a continuously increasing demand for precision.

Stoichiometry of TF complexes

TFs rarely act alone. They predominantly bind target enhancers jointly with other TFs or cofactors to regulate the transcription of their target genes (Golan-Lagziel et al., 2018; Veitia, 2002). Hence, the formation of TF complexes depends on TF numbers, a phenomenon that appears to be conserved in evolution (Papp et al., 2003; Sopko et al., 2006; Veitia, 2002, 2003). A study by Ni et al. showed that dosage-sensitive TFs – which often drive HI phenotypes – interact with a total of 851 proteins, 25% of which are other TFs (Ni et al., 2019). Dosage-insensitive TFs, by contrast, interact only with 263 proteins, of which 17% are other TFs (Ni et al., 2019). Whether such TF-TF interactions are required for TF function and whether HIs of some TF genes are partially caused by abnormal TF stoichiometry within complexes remain to be shown. However, this disparity is striking and might shed light into the widespread downstream transcriptional effects triggered by reduced levels of individual TFs in HIs.

Many TF complexes display cooperative binding to chromatin, rendering the maintenance of stable cell type-specific transcriptional programmes reasonably sensitive to TF numbers (Bartman et al., 2016; Fukaya et al., 2016). This might explain why either a reduction or increase in individual TF protein levels can have deleterious effects on normal cellular function (Chen et al., 2008; Cox et al., 2010; Gao et al., 2012; Papp et al., 2003; Veitia, 2002; Wang et al., 2006; Wuebbgen et al., 2012). An extensively studied example of this is regulation of the ESC state by distinct TF combinations and levels (Chen et al., 2008; Cox et al., 2010; Gao et al., 2012; Wuebbgen et al., 2012). A second example is the cooperative binding between PAX6 and SOX2 during optic field induction. Here, both *Pax6* overexpression (e.g. through a third-copy allele) and heterozygous pathogenic mutations result in ocular developmental defects that are similar between mice (Ouyang et al., 2006; Schedl et al., 1996) and humans (Gerth-Kahlert et al., 2013; Hall et al., 2019) – see Fig. 3 for developmental abnormalities in the eye, caused by heterozygous *PAX6* and *SOX2* mutations. Such strong dependence on TF abundance, in which either increased or decreased TF numbers trigger a similar abnormality, is particularly interesting and may hold true for other, less well-studied TF genes. In addition to cooperative binding, TFs may recruit – or assist the binding of – other factors to chromatin. ‘Pioneer’ TFs, such as the HI-associated forkheadbox A1 and 2 (FOXA1/2) and SPI1 (also known as PU.1), bind ‘closed’ chromatin, making it accessible to non-pioneer TFs that act either alone or through interactions with chromatin remodelers (Barozzi et al., 2014; Cirillo et al., 2002; Heinz et al., 2010; Li et al., 2012; Zaret, 2018).

The functional impairment of protein complexes in disease has been recently summarised by Bergendahl et al., 2019, who discuss possible mechanisms by which disease-causing mutations alter protein structure. This, in turn, may result in aberrant complex formation by affecting the protein stoichiometry or by inhibiting other protein interactions, thus causing abnormal cell and tissue functions, ultimately leading to disease. Therefore, the number of TFs may influence the function of other TFs when transcriptional regulation depends on TF-cofactor complexes.

Single-molecule analysis of the stoichiometry of TF complexes can be performed in both living or fixed cells. The proximity of molecules, as an implicit measure of complex formation, can be analysed by Förster resonance energy transfer (FRET) (Dikovskaya et al., 2019; Szalóki et al., 2015) or bimolecular fluorescence complementation (BiFC) (Hu and Kerppola, 2003; Moustaqil et al.,

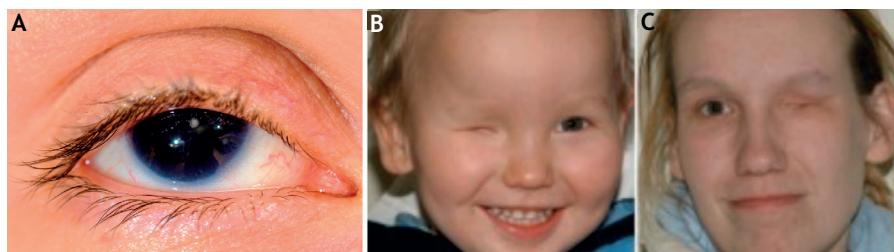


Fig. 3. Abnormal TF numbers and TF HI have important phenotypic consequences. Human ocular developmental abnormalities can be caused by heterozygous mutations in the *PAX6* and *SOX2* TF genes. (A) Eye of a patient with a phenotype typical of *PAX6* HI, showing near-complete aniridia (absence of the iris) and mild ptosis (drooping of the eyelid). The patient had cataract surgery in young adulthood (Hall et al., 2019). Image courtesy of David Hall, Critical Care, Royal Infirmary of Edinburgh, UK. (B,C) An affected son (B) and his mother (C), both bearing a *SOX2* mutation that causes HI, which results in unilateral anophthalmia (absence of the eye). Although they carry the same mutation, their phenotypic abnormalities only affect the right or the left eye, respectively. Image adapted with permission from Gerth-Kahlert et al. (2013) under the terms of the CC-BY 3.0 license.

2018; Papadopoulos et al., 2015) (see Table 2 and Fig. 2). However, steric hindrance and the need for correct three-dimensional orientation of TF complexes are main limiting factors of these methods, and may cause false-negative results. FRET, FCS and combined SPIM-fluorescence cross-correlation spectroscopy (SPIM-FCCS) have been used in combination to show that FOS forms homodimers that are also capable to bind chromatin when JUN is not present in equal concentrations to FOS (Szalóki et al., 2015). As the formation and transcriptional regulatory activity of FOS homodimers were suggested to play a role in oncogenesis, the stoichiometry of TF complexes and their activity have been investigated by combining different quantitative approaches in the context of *FOS* overexpression (Szalóki et al., 2015). TF complexes at specific nuclear compartments or gene loci, which are rendered visible in BiFC or undergo energy transfer in FRET, may also be imaged by using SRM (Kwon et al., 2017; Yamamoto et al., 2014) (see Table 2 and Fig. 2). Furthermore, one can analyse how the stoichiometry of TF complexes changes during development or varies across cells in the same tissue. This is possible by using FCS, as in studies regarding the homodimerization of Ser on chromatin (Papadopoulos et al., 2015; Papadopoulos et al., 2019; Rosales et al., 2013; Skouloudaki et al., 2019; Szalóki et al., 2015; Vukojevic et al., 2010) and *FOS* (Szalóki et al., 2015). FCS has also been applied to study the formation of heterocomplexes between MYC and the transcriptional regulator MAX (Rosales et al., 2013). Oligomerisation of the mitochondrial TF TFAM on chromatin was similarly investigated using fast-scanning stimulated emission depletion (STED) microscopy (Heller et al., 2013). Moreover, TF complexes have been studied by using dual labelling strategies. For example, Savatier and co-workers used two-photon FCCS to investigate the interactions between alpha and beta estrogen receptors (ESR1 and ESR2, respectively) or between ESRs and TIF2 (Savatier et al., 2010). The colocalisation of glucocorticoid receptor (NR3C1) and its interacting protein GRIP1, as well as of the transcriptional activators BMAL (also known as ARNTL) and CLOCK was similarly visualised by using dual-colour single-particle tracking (SPT) (Gebhardt et al., 2013). Thus, TF complexes can be quantitatively analysed in time and in space to provide molecular insights into how their interactions and concentrations affect entire regulatory networks. To uncover the roles of TFs in HI, their interactome needs to be thoroughly investigated and quantitative microscopy methods should be used to characterise the potentially abnormal TF function based upon aberrant TF stoichiometries.

Target site number, accessibility and binding

The cell- and tissue-specific sensitivity to TF levels also depend on the number of TF target-binding sites on chromatin and their

accessibility – which might be subject to developmental control, as well as how TF molecules dynamically move in nuclei and undergo various interactions with chromatin, until they find and bind to their target sites. On average, the number of TF molecules per cell is between thousands and tens-of-thousands (Simicevic et al., 2013). This is roughly one order of magnitude higher than the number of specific TF binding sites, which is on average several hundreds to a few thousands (Ganapathi et al., 2011; Johnson et al., 2007; Palii et al., 2011; Robertson et al., 2007). Therefore, the number of binding sites of a given TF in a certain cell type cannot always explain why reduced TF amounts may not suffice for proper TF function. Additionally, genes controlled by a single TF may be more sensitive to molecular numbers than those controlled by two or more TFs, making the correlation between the ‘number of TF molecules’ and the ‘number of TF-binding sites’ far from straightforward. The complexity increases when we consider the existence of mechanisms, which overcome the dependence of transcriptional control on the stoichiometry of TF molecules to binding sites, i.e. by allowing the expression of important developmental genes to also depend on collaborating TFs. This is achieved by the evolutionary ‘addition’ of such ‘collaborating’ TF-binding sites into enhancers. For instance, the presence of both Zld- and Dorsal (Dl)-binding sites in an enhancer of the *short gastrulation (sog)* gene guarantees the potentiation of *sog* transcription in the early *Drosophila* embryo, such that all cells express the same amount of *sog* – even in positions where the Dl morphogen TF concentration is low (Papadopoulos and Tomancak, 2019; Yamada et al., 2019). In this case, additional Zld-binding sites convert *sog* expression from ‘analog’ – i.e. proportional to Dl concentration along the Dl dorsoventral gradient – to ‘digital’, whereby Zld guarantees efficient *sog* expression as long as Zld-binding sites are present in the *sog* enhancer (Papadopoulos and Tomancak, 2019; Yamada et al., 2019). Thus, studies from the fly shed light onto how some target genes maintain robust expression, even upon limiting amounts of the TFs that regulate them. Such collaborating and ‘potentiating’ TFs may themselves be expressed in a tissue-specific manner. In this case, expression of the same target gene across different tissues or of different target genes in the same tissue both depend on the amount of TF. This might explain why certain TFs result in HIs in one tissue but not in another. Generally, the number of TF-binding sites may strongly influence the expression of specific genes in the same tissue or of the same gene in some tissues only and not in others, making gene expression less, or more, sensitive to TF numbers. Therefore, TF HIs that result in developmental abnormalities or disease frequently manifest in a subset of tissues, at distinct

developmental stages or are due to the mis-regulation of only some of their target genes.

The binding affinity of TFs on their DNA-binding sites is another important factor required for normal gene regulation (Levine, 2010). This chiefly depends on the binding site sequence and defines how ‘strongly’ a TF binds to this site. Low-affinity binding sites are, thus, less likely to be bound when TF concentrations are low and, therefore, may require higher numbers of TFs and/or cofactors for transcriptional regulation (Arthur et al., 2017; Tsai et al., 2017). This may serve as an additional control mechanism for differential gene expression in space and time. In *Drosophila*, the Hox TF Ultrabithorax can bind low-affinity target sites on certain *shavenbaby* enhancers when complexed with the Hox cofactors Extradenticle and Homothorax. This confers region-specific control for the formation of epidermal denticle belts in the embryonic cuticle (Crocker et al., 2015; Tsai et al., 2017). While low-affinity binding sites render gene expression more sensitive to TF levels, their existence can be important for safeguarding the specificity of TF binding. This is of particular importance for the specification, development and differentiation of body structures. Additionally, it is an important mechanism to control and exploit the function of TFs that exhibit very similar binding behaviours, such as Hox TFs. As a result, the transcriptional output of genes with multiple low-affinity binding sites can be dosage dependent (Crocker et al., 2015; Driever and Nüsslein-Volhard, 1988; Giorgetti et al., 2010; Lorberbaum and Barolo, 2013; Ochoa-Espinosa et al., 2005; Ramos and Barolo, 2013; Stewart-Ornstein et al., 2013; Struhl et al., 1989; Tsai et al., 2017). This, in turn, enables cells to acquire distinct developmental fates based on their position along a TF concentration gradient (Driever and Nüsslein-Volhard, 1988; Lorberbaum and Barolo, 2013; Ochoa-Espinosa et al., 2005; Ramos and Barolo, 2013; Struhl et al., 1989). For instance, the homeodomain TF CUX1, a tumour suppressor, binds distal target enhancers in cultured human cells in concert with co-activators and cohesin. Loss of one *CUX1* allele abolishes CUX1 binding, and genes that contain multiple low-affinity CUX1-binding sites become mis-regulated (Arthur et al., 2017). Furthermore, Louphrasitthiphon et al. (2020) recently investigated the binding between microphthalmia-associated transcription factor (MITF) and chromatin, and suggested that low-affinity binding sites also act as a TF ‘reservoir’ in the genome. Chromatin immunoprecipitation sequencing (ChIP-seq) and single-molecule tracking (SMT) were used to show that the binding affinity between MITF and chromatin is increased by MITF acetylation, and that an acetylation-mutant MITF exhibits increased numbers of transient interactions with chromatin at low-affinity binding sites (Louphrasitthiphon et al., 2020). The authors suggested that low-affinity sites act as a reservoir for non-acetylated MITF, which is released upon acetylation in order to bind high-affinity sites (Louphrasitthiphon et al., 2020). Although both high- and low-affinity binding sites can favour TF specificity and facilitate differential gene expression, the number of TF-binding sites in enhancers, as well as their affinity, may also strongly affect the sensitivity of gene expression relative to TFs and, therefore, may result in TF HI.

Yet, cell- or tissue-specific transcriptional responsiveness of genes relative to the level of certain TFs may stem from the differential accessibility of their binding sites, and the set of TFs and cofactors targeting them. Both processes contribute to the kinetics of TF target site search and binding (Liu et al., 2014; Tsai et al., 2017). To find their specific binding sites, TFs ‘search’ chromatin by diffusion and transient, non-specific electrostatic interactions (Chen et al., 2014b; Elf et al., 2007; Liu et al., 2014; Louphrasitthiphon et al., 2020; Papadopoulos et al., 2010;

Raccaud et al., 2019; Slutsky and Mirny, 2004; Voss et al., 2011; Vukojevic et al., 2010). Specific binding to their cognate sites depends on the strength of weak dipole interactions, such as hydrogen bonds and Van der Waals forces, which are exercised between amino acid (aa) residues of the TF and the DNA bases. Thus, the balance between non-specific and specific chromatin interactions determines the amount of TF molecules available to bind their cognate sites – an important determinant of how efficiently TFs find these specific binding sites.

The accessibility of binding sites depends on chromatin conformation and nuclear organisation, and may modulate the ability of TFs to efficiently search for and find their target sites. Chromatin organisation within cell nuclei is dynamic, cell type-specific and has been extensively studied with respect to its effect on gene expression. The degree of chromatin compaction affects TF binding and gene expression (Akhtar et al., 2013). Additionally, topologically associated domains (TADs), i.e. regions of self-interacting chromatin, are thought to regulate gene expression by establishing chromatin contacts within and between TADs, as well as by generating transcriptional microenvironments (Dixon et al., 2015; Tsai et al., 2017; reviewed by Gonzalez-Sandoval and Gasser, 2016). For example, Tsai and collaborators suggested that, in *Drosophila*, clustering of enhancers with low-affinity binding sites permits the formation of high local concentrations of the TF Ultrabithorax, which potentiates its binding interactions with chromatin (Tsai et al., 2017). TADs are also dynamic and cell type-specific, and have been studied with regard to differentiation (Kaur et al., 2013; Plachta et al., 2011; White et al., 2016), reprogramming (Beagan et al., 2016) and their role in *HOX* gene expression during limb development (Gebhardt et al., 2013; Langowski, 2017; Paakinaho et al., 2017; Rehó et al., 2020; Savatier et al., 2010; Vámosi et al., 2008). Nucleosome positioning further influences chromatin accessibility and, thereby, TF binding to gene regulatory elements (Chen et al., 2014a,b; Gebhardt et al., 2013; Hansen et al., 2017; Li et al., 2019; Mir et al., 2017; Normanno et al., 2015; Tirosh and Barkai, 2008; White et al., 2016; Wollman et al., 2019; Yao et al., 2006; Zhao et al., 2017). At the whole-genome level, histone modification signatures dictate chromatin accessibility and are subjected to developmental control (Bell et al., 2010). As such, the effect of binding site accessibility on TF-chromatin binding kinetics may trigger differential gene expression and differentiation biases between seemingly identical cells, even when the TFs mainly responsible for such processes are expressed in all cells at the same levels. In mouse preimplantation embryos, OCT4 exhibits differential chromatin-binding kinetics as early as at the 8-cell stage, which is one of the first determinants of differentiation bias towards inner cell mass or trophectoderm (Plachta et al., 2011). Moreover, the chromatin-binding kinetics of SOX2 and OCT4 can be used to predict cell fate (White et al., 2016). In pluripotent cells, OCT4 and SOX2 bind chromatin more stably than in extraembryonic cells (Kaur et al., 2013). Long-lived SOX2-chromatin binding is regulated by methylation of histone H3 at arginine 26 (H3R26), such that reduction in H3R26 methylation decreases the lifetime of SOX2-bound complexes. As a result, SOX2 target expression declines, and so do the numbers of pluripotent cells (White et al., 2016). These are excellent examples of how the kinetics of TF binding and the epigenetic landscape can influence development. Therefore, the dependence of gene expression on TF concentrations in a cell- and tissue-specific manner also relies on the number, affinity, accessibility and chromatin-binding kinetics of TF-binding sites.

It follows that quantitative analyses of TF-chromatin interaction kinetics and studies of cognate site configuration provide a deeper insight into gene- and cell-specific sensitivity to TF levels. FCS and SPT are used to obtain the fractions of freely diffusing TF molecules that seem to facilitate cognate site search, as well as of TF molecules bound to chromatin (see Fig. 2 and Table 2). For example, FCS studies of Scr in flies suggest that slowly diffusing TFs predominantly engage in rapid electrostatic interactions with chromatin, thereby reflecting the molecular movement TF molecules exhibit while searching for their specific DNA-binding sites (Papadopoulos et al., 2010; Vukojevic et al., 2010). Interestingly, the diffusion of MYC and P-TEFb – as measured by SPT – suggests that a binding site search may be TF- and target site-specific (Izeddin et al., 2014). By using SPT, enhancer clustering has been suggested to facilitate target-site search and binding (Liu et al., 2014). Chen and collaborators combined multi-focus SMT and FCS to study how mutant and wild-type SOX2 and OCT4 in mouse ESCs display differential binding dynamics on enhanceosomes, as well as the effects chromatin modifications have on TF molecules searching for their cognate DNA sites (Chen et al., 2014b). Once bound, the DNA-residence time of a TF reflects its binding affinity. SPT is better suited to measure long-lived – presumably specific – TF-DNA interactions. Monitoring of these interactions does not require very high temporal resolution and photobleaching can, thus, be avoided by time-lapse imaging experiments with longer ‘dark’ intervals between rounds of image acquisition. For example, by using reflected light-sheet microscopy (RLSM)-SPT, Gebhardt and colleagues compared the DNA-residence time of NR3C1 and of ESR monomers and dimers (Gebhardt et al., 2013). Mazza and collaborators combined SPT with FCS and FRAP to identify different proportions and residence times of mutant p53 (Mazza et al., 2012). FRAP can also be used to characterise TF-chromatin interactions by investigating their kinetic on- and off-chromatin rates, for instance, how TFs bind mitotic chromosomes. Here, the TFs exhibit high on-rates and reduced mobility during interphase, resulting in a more efficient search of TFs for binding sites (Raccaud et al., 2019). Furthermore, application of FRAP and SPT showed that SOX2-chromatin binding behaviour during mitosis is more dynamic than during interphase (Teves et al., 2016). FRAP experiments also identified that mutations in the high-mobility group domain of SOX2 largely abolished its clustering at the *POU5F1* enhancer, again suggesting that TFs can engage in different modes of chromatin-binding behaviour (Li et al., 2019). In addition, the chromatin-binding behaviour of TF heterocomplexes with other TFs has been studied by FCCS; particularly in cases where TF heterodimerisation is known to be required for DNA binding, such as for the dimers between FOS and JUN (Langowski, 2017), retinoic acid receptor (RAR) and retinoid X receptor (RXR) (Rehó et al., 2020), and ESR1/2 and TIF2 (Savatier et al., 2010). FRET has similarly been used to study chromatin binding of TF dimers and complexes, in some studies in combination with FCS or FCCS. Dimerisation and chromatin-bound intermediates of the basic helix-loop-helix leucine zipper (bHLH-LZ) domains of the TFs MYC and MAX were investigated by single-molecule FRET (smFRET) and FCS (Vancraenenbroeck and Hofmann, 2018). FCCS and FRET have also been used to study the chromatin-binding dynamics and intermolecular distance between FOS and JUN in homo- and heterodimers (Szalóki et al., 2015; Vámosi et al., 2008). Additionally, ternary complexes between FOS, JUN and the NF κ B subunit p65 have been investigated by BiFC-FRET (Shyu et al., 2008).

The signal-to-noise ratio in FCS and SPT can be increased by reducing the illumination volume, for example, by using lattice light-sheet microscopy (LLSM) in combination with SPT (LLSM-SPT) (Chen et al., 2014a; Mir et al., 2017), LLSM-FCS (Mir et al., 2017), RLSM-SPT (Gebhardt et al., 2013), highly inclined laminated optical sheet (HILO)-SPT (Hansen et al., 2017) or spatial light interference microscopy (SLIM)-SPT (Wollman et al., 2019), as well as by implementing sparse-excitation methodologies, such as 3D-STED (Li et al., 2019), single-particle tracking photo-activated localization microscopy (sptPALM) (Normanno et al., 2015), photo-activatable (PA)-FCS (White et al., 2016; Zhao et al., 2017) and multiphoton FCS (Yao et al., 2006) (see Table 2 and Fig. 2). The interaction of the GTPase RAP1 with nucleosomes and the resultant local opening of chromatin have also been studied *in vitro* by a combination of smFRET and TIRF (Mivelaz et al., 2020). Moreover, the spatial intranuclear variability in TF concentrations and chromatin-binding behaviour can simultaneously be addressed by novel methods, such as mpFCS (Krmpot et al., 2019; Papadopoulos et al., 2015) and 3D-SPT (Chen et al., 2014a). However, linking TF dynamics and concentration to their function by visualising specific genomic loci and their conformation has so far proven challenging.

To address these challenges, CRISPR/dCas9- and TALE-based labeling of specific DNA sequences can be used in live cells to visualise TF dynamics. However, the low signal-to-noise ratio has so far restricted this application to repetitive sequences (Chen et al., 2013; Knight et al., 2015; Ma et al., 2013; Thanisch et al., 2014). In a recent study, Li et al. targeted dCas9 to distal enhancers of the *Pou5f1* and *Nanog* genes, to link clusters of BRD4 and enhancers with active transcription (Li et al., 2020). However, the authors did not address the dynamic behaviour that might underlie such interactions. Specific conformations of TF-binding sites can be analysed using SRM in combination with chromatin markers associated with accessibility and active transcription (Cai et al., 2019). The kinetics and variability of TF-chromatin interactions can be analysed by quantitative microscopy at high temporal or spatial resolution. These methodologies allow to link the intercellular variability of TF-chromatin interactions and gene expression to gene- and cell-specific target site availability, and – from there – to further understand the role of TF-binding dynamics in the context of HIs.

Stochastic gene expression

Although highly significant, TF-chromatin interactions alone do not sufficiently explain the significance of TF stoichiometry underlying HIs. Aberrant TF numbers can also affect gene expression because this in itself is an inherently stochastic process. Transcription is a ‘noisy’ process and, for a plethora of genes, it occurs in bursts, resulting in temporally fluctuating levels of mRNA (Bothma et al., 2014; Chubb et al., 2006; Fukaya et al., 2016; Larsson et al., 2019; Lee et al., 2019; Rodriguez et al., 2019). Because the frequency of transcriptional bursts depends on TF concentration (Senecal et al., 2014), when TF numbers are low, transcriptional noise can become a limiting factor for the normal expression of some genes and may result in abnormal function. The aforementioned stochasticity further results in temporally and spatially variable gene expression within a cell, and elicits heterogeneity between cells in both prokaryotic (Kierzek et al., 2001; Ozbudak et al., 2002; Wolf and Arkin, 2002) and eukaryotic organisms (Blake et al., 2003; Raser and O’Shea, 2004). In mammals, gene expression noise occurs both during development (Abranches et al., 2014; Mohammed et al., 2017; Olsson et al., 2016; Trapnell et al., 2014) and in disease (Avraham et al., 2015; Shaffer et al., 2017; Tirosh et al., 2016).

In bacteria, auto-activation of the competence TF ComK confers resistance to environmental stress by transcribing genes involved in the uptake of DNA (Maamar and Dubnau, 2005; Smits et al., 2005; Suel et al., 2006). Perturbing ComK levels by upregulating the rate of transcription and downregulating translation decreased the noise of ComK-dependent gene expression and reduced overall competency. This observation underlines that a subset of cells may exploit this cell-to-cell variability – introduced by transcriptional noise – to confer a group advantage (Maamar et al., 2007; Ozbudak et al., 2002; Suel et al., 2007; Thattai and van Oudenaarden, 2001). Albeit shown in a simple system, this indicates that stochasticity can result in transcriptional heterogeneity and increase the level of ‘fitness’ in a population. Similarly, in multicellular organisms, perturbations in TF numbers may amplify the cell-to-cell variability of transcriptional programmes. For example, in humans, deletions of one allele of the TF-encoding gene *NKX3.1* have been described in ~40% of prostate cancers (Bova et al., 1993; Bowen et al., 2000; Macoska et al., 1995). Mouse models recapitulating this deletion display NKX3.1 HI, aberrant target gene expression and progressive prostate diseases (Abdulkadir et al., 2002; Magee et al., 2003). Transcriptional ‘bursting-like’ behaviour and the resulting cell-to-cell variability have been specifically associated with HIs of tumour suppressor genes (Kemkemer et al., 2002). The inactivation of a single allele of *NF1* results in neurofibromas in humans (Fahsold et al., 2000; Hoffmeyer et al., 1998). Furthermore, cultured melanocytes derived from patients with a single functional *NF1* allele exhibited variable dendritic outgrowth, owing to increased stochastic transcriptional noise compared to normal melanocytes (Kemkemer et al., 2002). Overall, such variabilities can lead to excessive divergence in gene expression, even among cells of the same lineage that feature very similar transcriptional programmes. Such paradigms underscore the complexity of TF HIs. That is, gene transcription might only become intolerably impaired in a subset of functionally identical cells – yet still lead to abnormal organ function and disease. Therefore, although it is established that low TF concentrations significantly influence ‘bursty’ gene expression, more studies will be needed to identify which target genes become the most severely impaired.

The inherently stochastic nature of transcription, discussed above, can be quantified by using single-molecule and single-cell methodologies and, thus, be linked to TF HIs. The dynamics of transcription can be observed *in vivo* by incorporating labelled nucleotides to monitor the production of nascent transcripts (Morisaki et al., 2014) or it can be visualised at specific loci by smFISH (Hsu et al., 2017; Kochan et al., 2015; Mehta et al., 2018; Titlow et al., 2018). Tagging the untranslated regions of endogenous genes with stem-loop-encoding sequences of bacteriophages MS2 or PP7, fused to a fluorescent reporter, allows the visualisation of nascent RNA and, thus, is suitable to track the expression of genes of interest (Bothma et al., 2014; Katz et al., 2018). Intercellular variability of transcription can be quantified by smFISH and scRNA-seq (Beach et al., 1999; Bertrand et al., 1998; Guo et al., 2017; Halpern et al., 2017; Hocine et al., 2013; Treutlein et al., 2016; Yan et al., 2013). Further, advances in scRNA-seq have allowed the analysis of allele-specific ‘bursting’ to be used as a means of identifying imbalances of gene expression (Borel et al., 2015; Chen et al., 2016; Deng et al., 2014; Faddah et al., 2013; Jiang et al., 2017; Kim and Marioni, 2013; Marks et al., 2015). In other studies, the cellular transcriptional activity has been inferred by examining localisation and dynamics of the transcriptional machinery (Cho et al., 2018; Cisse et al., 2013; Li et al., 2019; Steurer et al., 2018). From this, it becomes clear that to link TF behaviour to gene expression at high temporal and spatial

resolution, studies often have to utilise both live- and fixed-cell methods to obtain complementary information. For example, the local concentration and dynamics of SOX2, CDK9, BRD4 and MED22 were measured by 3D STED and FRAP, and nascent *POU5F1* transcripts were visualised by MS2-MCP labelling (Li et al., 2019). This correlation between local concentrations of TFs and gene expression leads to the final TF HI-influencing mechanism explored in this Review: the formation of condensates.

Control of TF function through the formation of condensates

In recent years, phase separation of proteins has gained substantial attention in biological research. The formation of biomolecular condensates has been implicated in a plethora of cellular functions. These are as diverse as membrane-less organelles, such as the nucleolus (Feric et al., 2016), as well as the normal and abnormal variants of widely studied proteins, such as FUS, G3BP1, TDP43 (officially known as TARDBP) and BRD4 (Han et al., 2020; McGurk et al., 2018; Niaki et al., 2020; Riback et al., 2020; Yang et al., 2020) which are implicated in disease pathogenesis. However, our understanding of the biological meaning of condensate formation, particularly by TFs, remains incomplete. As such, phase separation of TFs, aiding nuclear compartmentalisation and gene regulation, is a relatively novel concept (Boija et al., 2018).

Phase separation of proteins depends on physico-chemical conditions, such as protein concentration, charge, 3D structure and cellular pH (Taratuta et al., 1990; Wang et al., 2018). The thermodynamics of phase-separated systems predicts that protein concentration inside condensates is higher than in the surrounding dilute phase (Klosin et al., 2020). Condensates may possess liquid, gel or solid-like properties but the biological consequences of such phase-separated entities – and whether they are beneficial or not – remain ill-defined (Brangwynne et al., 2009; Conicella et al., 2016; Kato et al., 2012; Lin et al., 2015; Strom et al., 2017). Condensates exhibit rapid protein exchange with the surrounding dilute phase, movement within the dense phase, as well as fusion and fission phenomena during their formation and maturation (Brangwynne et al., 2009; Handwerger et al., 2005; Phair and Misteli, 2000; Strasser et al., 2008). Although the exact biophysical mechanism of condensate formation remains elusive, weak multivalent interactions between intrinsically disordered regions seem to be the main driver (Dzuricky et al., 2020; Kato et al., 2012; Lin et al., 2017; Sabari et al., 2018; Wei et al., 2017).

As previously discussed, the intranuclear concentration of some TFs substantially influences gene regulation and function. Since condensate formation buffers concentration and functionally compartmentalises the nucleus (Klosin et al., 2020), condensates can control local TF concentrations. Super-enhancers are clusters of enhancers that accumulate components of the transcriptional machinery. They are thought to harness the formation of condensates to favour biochemical reactions through local increases in TF concentration and the formation of compartmentalised reaction/diffusion networks, without excluding additional mechanisms. In this way, the molecular crowding brings regulatory sequences and promoters into close proximity (Hnisz et al., 2013), and such reversible local reaction/diffusion networks favour gene expression. Such a process can depend on nucleation events triggered by physiological (e.g. NF- κ B in inflammation; Nair et al., 2019), developmental (e.g. Prospero in neural differentiation; Liu et al., 2020) or molecular processes (e.g. depletion of proteins such as the Mediator complex and BRD4; Sabari et al., 2018). The high-density assemblies of transcriptional machinery components at enhancers is, by definition, substantially assisted by the formation of condensates.

This is because TFs, e.g. SOX2, OCT4 and NANOG (Boija et al., 2018), co-activators, e.g. BRD4 and MED1 (Sabari et al., 2018) and RNApolIII (Boehning et al., 2018; Cho et al., 2018) all form condensates. However, to what extent this depends on condensate formation alone or on clustered DNA-binding interactions, remains under investigation (Li et al., 2020). So far, studies of the function of TF condensates in the regulation of gene expression remain scarce and have mostly focussed on linking condensate formation to transcriptional output (Li et al., 2020; Sabari et al., 2018), examining silencing of genes, such as *prospero* in *Drosophila* (Liu et al., 2020), and investigating diseases, such as HOXD13-associated synpolydactyly (Basu et al., 2020). Alternatively, condensate formation has been recently proposed to be involved in buffering the concentration of TFs in the dilute phase (Klosin et al., 2020), thereby dampening the variability of TF concentration. Although the universality of such functions remains to be experimentally confirmed, it is intriguing that many TFs depend on a stringent regulation of their local concentration for normal function. Examples include PAX6/SOX2 during ocular development (Matsushima et al., 2011), OCT4 in the preimplantation embryo (Gerovska and Araúzo-Bravo, 2019) and NANOG during blastocyst formation (Bessonnard et al., 2014). Therefore, formation of condensates and how they control TF variability across cells warrant further investigation in the context of HIs.

Understanding the biological function of TF condensate formation requires the characterisation of their constituents, their subcellular location and their dynamic behaviour over time. Fast scanning microscopy – such as confocal spinning-disk (CSD) microscopy and LLSM – is used to observe cellular localisation and co-partitioning of proteins into condensates (Chong et al., 2018; Sabari et al., 2018), and can be quantified by SRM (Cai et al., 2019; Chong et al., 2018; Sabari et al., 2018) (see Table 2 and Fig. 2).

The mobility of TFs within condensates, and the exchange of molecules between condensates and the environment, have been measured by FRAP, FCS and SPT. Condensates formed by transcriptional machinery components, such as BRD4 and MED1 (Boija et al., 2018; Cai et al., 2019; Chong et al., 2018; Gibson et al., 2019; Guillén-Boixet et al., 2020; Klosin et al., 2020; Liu et al., 2020; Sabari et al., 2018; Teves et al., 2016; Zamudio et al., 2019), and TF condensates, such as OCT4 (Boija et al., 2018), have been also studied by FRAP. Additionally, the formation of TF condensates driven by low complexity domains has been studied by FRAP and SPT (Chong et al., 2018). Stress-induced condensates, e.g. YAP, have also been studied by FRAP (Cai et al., 2019), and the formation and maturation of G3BP1-related stress granules have recently been investigated by FRAP and FCS (Guillén-Boixet et al., 2020).

It will be interesting to further investigate the biological functions of TF condensates through a combination of single-molecule approaches to link TF numbers and their dynamic behaviour to gene expression and HIs.

Conclusions

Here, we have outlined how TF abundance and dynamic interactions with chromatin are required for normal development and how abnormalities in such regulation can result in disease.

We have discussed the key factors and cellular functions that control TF abundance in cells and tissues. TFs undergo complex kinetic interactions with chromatin; therefore, investigating TF numbers and molecular movement at the cellular and tissue levels is essential. Studied paradigms of dosage-sensitivity and HI emphasise how recent quantitative microscopy advances help

researchers to investigate such complex TF interactions and their malfunction in disease. To date, such information on TFs and TF-cofactor complexes can be obtained with high spatiotemporal resolution. When these methodologies are applied to multiple cell types and disease models, they help to understand the molecular underpinnings of HI-associated diseases. However, a remaining challenge is to simultaneously study the behaviour of collaborating TFs and TF-complexes in live cells and tissues. This shortcoming is likely to result from the limitations of fluorescence microscopy to faithfully investigate several differently labelled proteins at the same time. Mass spectroscopy, RNA-seq and ChIP-seq methods do provide much broader, albeit static, datasets of TF binding and protein and mRNA abundances in cells but lack the dynamic information of live systems. A further challenge is to combine temporal and spatial super-resolution methods so that the mobility and chromatin-binding dynamics of individual TFs can be investigated at specific genomic loci. As discussed, this is currently limited to repetitive loci, which can be studied in tandem using the same fluorescent probe. Non-repetitive DNA sequences require a large number of sequence-specific probes to sufficiently increase the signal-to-noise ratio for fluorescence imaging (Chen et al., 2013; Knight et al., 2015; Ma et al., 2013; Thanisch et al., 2014).

Nevertheless, when studying the function of fluorescently labelled TFs, microscopy methodologies have become sensitive enough to detect molecules at low, physiologically relevant concentrations and by using low excitation power, which largely preserves the normal cell function. In fact, smaller and brighter fluorescent tags have been identified (Govindan et al., 2018; Oliinyk et al., 2019) but tagging genes non-disruptively does necessitate functional, i.e. genetic, validation of the endogenous behaviour of TFs. Additionally, the generation of further tissue and live animal models will explain the aberrant function(s) of TFs at the molecular level, in a biologically relevant context. For example, heterozygous LOF mutations of the widely expressed transcriptional co-activator YAP1 cause diverse defects in patients, such as ocular abnormalities or craniofacial/intellectual disabilities, suggesting that the observed phenotypes are the result of tissue-specific HIs of *YAPI* (Williamson et al., 2014). The generation of animal models to study the underlying mechanism of these defects will facilitate understanding the phenotypic variability observed in human patients by means of quantitative microscopy methodologies.

From a translational perspective, new animal models will enable the detection of early developmental transcriptional and signalling defects that normally lead to disease onset and progression. This will be possible by combining high spatial and temporal resolution microscopy – as discussed in this Review – with *in vitro* quantification of TF transcriptomes and interactomes. To date, most studies on TF function have addressed the impact of their mutations in disease only qualitatively. The information on intracellular TF concentrations and chromatin-binding behaviour is scarce, and may have been underappreciated. Until now, computational and bioinformatics analyses, coupled to machine learning and high-throughput screening, have been predominantly deployed to predict TF binding sites (Elmas et al., 2017; Shen et al., 2018) or TF-binding behaviour based on DNA structure (Zhou et al., 2015). Further approaches need to be developed to investigate the levels and molecular behaviour of TFs, and to predict transcriptional outcomes. To this end, the ample availability of current and future single-cell transcriptome datasets should be exploited to study how TF dosage affects gene transcription. This will enable us to understand the

underlying mechanisms and the causal relationships between abnormal TF numbers, impaired TF behaviour and the manifestation of disease.

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