OXFORD

Briefings in Functional Genomics, 17(2), 2018, 116–137

doi: 10.1093/bfgp/ely004
Advance Access Publication Date: 26 February 2018
Review paper

ChIP-ping the branches of the tree: functional genomics and the evolution of eukaryotic gene regulation

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Abstract

Advances in the methods for detecting protein–DNA interactions have played a key role in determining the directions of research into the mechanisms of transcriptional regulation. The most recent major technological transformation happened a decade ago, with the move from using tiling arrays [chromatin immunoprecipitation (ChIP)-on-Chip] to high-throughput sequencing (ChIP-seq) as a readout for ChIP assays. In addition to the numerous other ways in which it is superior to arrays, by eliminating the need to design and manufacture them, sequencing also opened the door to carrying out comparative analyses of genome-wide transcription factor occupancy across species and studying chromatin biology in previously less accessible model and nonmodel organisms, thus allowing us to understand the evolution and diversity of regulatory mechanisms in unprecedented detail. Here, we review the biological insights obtained from such studies in recent years and discuss anticipated future developments in the field.

Key words: ChIP-seq; evolution; chromatin; transcription factors; eukaryotes

Introduction

The locations of transcription factor occupancy and the distribution of chromatin marks along the genome constitute indispensable information for understanding the mechanisms of gene expression and its regulation. Chromatin immunoprecipitation (ChIP) has been the main tool for their characterization since its invention in the 1980s [1–3]. ChIP and its numerous variants rely on the chemical cross-linking (typically using formaldehyde) of proteins to DNA, the subsequent enrichment of cross-linked fragments bound to the protein of interest, followed by reversal of cross-links and measurement of the purified DNA. Improvements in the tools for carrying out the latter task has made ChIP a progressively more powerful assay over the years. Initially, qPCR was used to assay enrichment over a small number of predefined sites [4]. Later, ChIP was coupled with microarrays (ChIP-on-Chip/ChIP-Chip) [5–10], which allowed a large number of targets to be probed. ChIP-Chip assays could in principle encompass the whole genome if tiling arrays were used, but in practice, this was relatively straightforward to accomplish only for small genomes such as those of yeast, *Arabidopsis* [11], flies [12] or worms [13], and most array studies focused on promoters and subsets of noncoding regions. In addition to usually falling short of fully genome-wide coverage, arrays also had numerous other issues having to do with noise levels, detailed resolution, signal range, interoperability of different platforms and others.

Early efforts to overcome these limitations by moving the ChIP readout from arrays to direct sequencing in the form of ChIP-PET (paired-end tagging) [14, 15] also suffered from difficulties related to low throughput, cumbersome library construction protocols and the short read tags being generated by these methods.

The advent of high-throughput sequencing in the mid-00s overcame most of these limitations, leading to the development of ChIP-seq [16–19] in 2006–07, which provided truly genome-wide coverage (with the exception of highly repetitive areas of

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the genome), with unprecedented sensitivity and resolution. In the decade since, ChIP-seq has become the workhorse of functional genomic studies of gene regulation. The genomic occupancy of hundreds of human, mouse, worm and fly transcription factors and the genomic distribution of histone marks in hundreds of human cell types have been systematically mapped using ChIP-seq by large-scale efforts such as the ENCODE [20], mouseENCODE [21, 22], modENCODE [13, 12], Roadmap Epigenomics [23], BLUEPRINT Epigenome and International Human Epigenome Consortium [24] and others, in addition to the method having been used in thousands of other scientific publications.

But ChIP-seq did not just resolve the technical limitations preventing the comprehensive high-resolution characterization of protein occupancy in the genomes of model organisms, it also opened the door to doing the same in any species for which a genome sequence is available. It did so by removing the major initial barrier of designing and manufacturing arrays, a slow and expensive process that was out of reach for many labs in the past. This enabled several lines of research that were not viable in the prior years. Studies of comparative analysis of transcription factor occupancy across species have now begun to unravel the biochemical and evolutionary factors behind its conservation and divergence. The direct mapping of regulatory elements in nonmodel species is helping us understand morphological changes during embryonic development. Finally, we have the tools to study transcriptional and regulatory biology across the whole tree of life, allowing us to track the deep evolutionary roots of its logic.

While these studies are still largely in their early stages in groups other than human, mouse, *Drosophila* and *Caenorhabditis elegans*, we review some of the most important insights they have delivered so far and discuss future directions for work in the field.

The evolution of transcription factors occupancy

Gene expression in the vast majority of eukaryotes is regulated largely by the orchestrated action of sequence-specific transcription factors binding to proximal and distal *cis*-regulatory elements. Knowledge of the mechanisms of evolution of transcription factor binding is therefore vital for a complete understanding of gene regulatory networks (GRNs), as they exist and function in extant organisms.

An intuitive expectation following from the importance of gene regulation for the proper functioning of the cell is that *cis*-regulatory elements would be highly conserved in evolution. This is indeed generally true. The sequencing of the human, mouse and other vertebrate genomes revealed that only not much $>\sim 2\%$ of their sequence consists of the exons of protein coding and noncoding genes [25], but whole-genome comparisons between these genomes identify a considerably higher fraction of conserved sequence, up to 10% [26, 27], with conserved noncoding elements (the bulk of which are thought to likely be *cis*-regulatory elements) making up the difference. However, the absence of conservation does not necessarily imply absence of function, and overall sequence conservation does not necessarily mean strict functional conservation (i.e. the same transcription factors need not be associated with a given conserved element in different species), meaning that the question to what extent regulatory networks themselves are conserved or divergent between species cannot be answered based on sequence alone.

Patterns of regulatory element evolution

In the late 1990s and early 2000s, initial approaches toward answering these questions used sequence comparisons between well-characterized loci in different species, tracking the conservation of individual transcription factor-binding sites, and experimental *in vivo* examination of expression patterns driven by orthologous or synthetic *cis*-regulatory elements. Application of these techniques to the *endo16* gene in the sea urchins *Strongylocentrotus purpuratus* and *Strongylocentrotus droebachiensis* [28], to even skipped enhancers in multiple *Drosophila* and other fly species [29–31] and to promoters of mammalian genes [32] revealed that functional conservation can be maintained despite considerable divergence at the sequence level, by means of compensatory mutations and of turnover of nonconserved regulatory elements (Figure 1A).

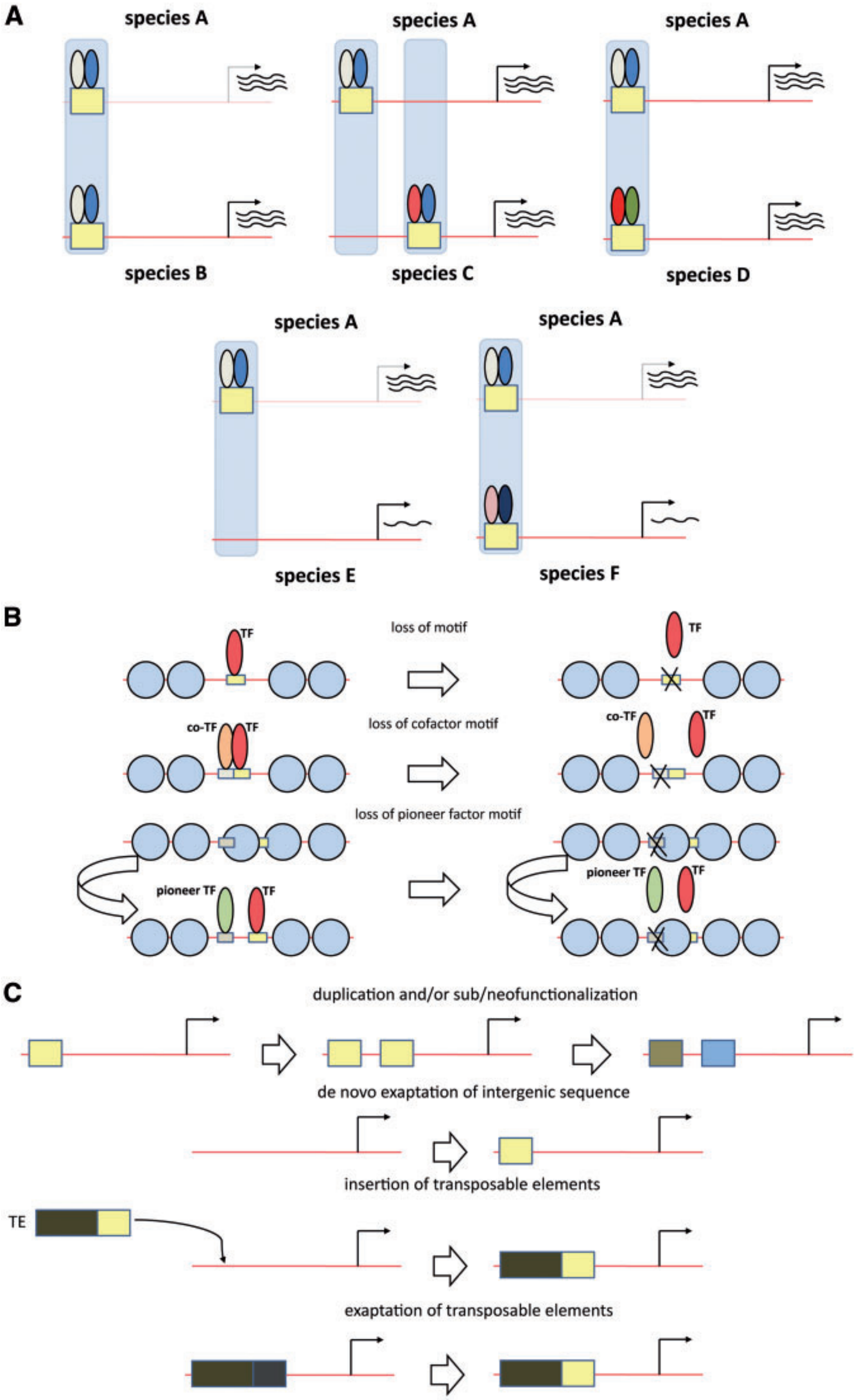
Yet to what extent such observations generalize could only be answered using genomic techniques. Pioneering studies addressing the question first appeared in 2007 [33], using ChIP-Chip over a set of orthologous human and mouse putative regulatory regions to compare the occupancy of the FOXA2, HNF1A, HNF4A and HNF6 transcription factors in hepatocytes of the two species. Strikingly, depending on the factor, between 41 and 89% of binding events appeared to be species-specific, and conserved occupancy around promoters was frequently observed in the absence of direct orthologous conservation of binding events. In a different study, only 20% of E2F4 sites were found to be conserved between human and mouse [34].

ChIP-Chip was also used to map the occupancy of Ste12 and Tec1 in the yeasts *Saccharomyces cerevisiae*, *Saccharomyces mikatae* and *Saccharomyces bayanus*, revealing only 20–21% conservation across all three species [35]; large turnover was also observed for Mcm1 in *S. cerevisiae*, *Kluyveromyces lactis* and *Candida albicans* using the same approach [36].

The advent of ChIP-seq eventually allowed truly genome-wide corroboration of these initial results in addition to much more detailed insights into the mechanisms driving conservation and divergence of occupancy. Mapping of transcription factor binding in the livers of five vertebrates (human, mouse, dog, opossum and chicken, separated by as much as 300 My of evolution) [37] showed conservation levels of 10–22% between placental mammals and of 6% between humans and opossum for CEBPa and HNF4A, and only 2% between humans and chicken for CEBPa. Half of lineage-specific losses of binding are associated with lineage-specific occupancy events in the nearby genomic vicinity, suggesting widespread regulatory element turnover through the evolution of compensatory binding, with novel nonorthologous regulatory elements taking over the function of the ones having been lost.

At the opposite extreme of phylogenetic distances, HNF4A, CEBPA and FOXA1 were profiled in livers from rat and five closely related mouse strains and species [38]. Again, significant divergence of occupancy was observed, with 30% conservation of FOXA1 binding events for the 20 My phylogenetic distance between mouse and rat, 40% within 6 My and 70% for divergence within 1 Mya.

Large-scale divergence of occupancy has been reported by all other studies in mammals. A comparative study of HNF4A, CEBPA, ONECUT1 and FOXA1 in human, macaque, mouse, rat and dog found 21 and 37% conservation between human and macaque, 21–31% between mouse and rat and as little as 7% for more distal relationships [39]. Comparison of PPARγ ChIP-seq profiles between human and mouse adipocytes also revealed that only $\sim$20% of occupancy sites are shared [40].


A
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species F
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co-TF
TF
loss of pioneer factor motif
pioneer TF
TF
pioneer TF
TF
C
duplication and/or sub/neofunctionalization
de novo exaptation of intergenic sequence
insertion of transposable elements
TE
exaptation of transposable elements

Figure 1. Patterns and mechanisms of regulatory element, transcription factor occupancy and gene expression evolution. (**A**) Patterns of conservation and divergence. Functional conservation of gene expression (top row) can be achieved through conservation of both regulatory elements and transcription factor occupancy (left), but it can also be maintained in the presence of significant turnover, both of occupancy and at the sequence level, either through replacement of individual transcription factors occupying an orthologous regulatory region (right) or by the evolution of nonorthologous regulatory regions (middle). Loss of regulatory elements and alterations in transcription factor occupancy can also lead to changes in gene expression (bottom row). (**B** and **C**) Mechanisms of transcription factor occupancy and regulatory element evolution. (**B**) Mechanisms of loss of transcription factor binding: loss of cognate motif (top), loss of cofactor binding (middle), loss of pioneer factor binding (bottom); (**C**) Mechanisms of gain of transcription factor binding and *de novo* evolution of regulatory elements: duplication and/or sub/neofunctionalization, direct *de novo* exaptation of existing intergenic space, insertion of TEs, exaptation of ancestral TE sequences.

The mouseENCODE project carried out comparative analysis of several dozen factors between mouse and human [22, 41, 42]. In this analysis, while up to half of identified occupied sites were not alignable to the other species at the sequence level, an even smaller fraction (varying between different factors but generally between 15 and 33%) of conserved occupancy was observed, underscoring the larger extent of sequence conservation relative to occupancy conservation.

Widespread turnover has also been observed even for polymerase (Pol) III occupancy. RNA Pol III primarily transcribes transfer RNAs (tRNAs) plus a few other classes of small RNAs; accordingly, it associates with localized genomic regions around these genes. Of note, many copies of genes for the same tRNA isotype can be found in mammalian genomes. Comparison of Pol III binding between six mammals demonstrated that a large fraction of occupancy sites are species-specific, but overall binding at the tRNA isotype level was largely conserved [43].

Mechanisms of regulatory element evolution

Comparative ChIP-seq studies have thus at this point firmly established that regulatory element turnover is a fundamental feature of the functional evolution of mammalian genomes. Gene expression regulation in functionally equivalent cell types is frequently conserved, even though substantial fractions of the regulatory elements driving it have diverged. But what exactly are the mechanisms through which individual factor occupancy and larger regulatory elements are gained and lost?

Divergence of transcription factor sequence specificity does not appear to be the answer. Although orthologous transcription factors can on occasions evolve distinct sequence preferences [44], large-scale characterization of transcription factor sequence specificity using methods such as HT-SELEX [45] has shown that it is usually conserved across deep evolutionary distances, going back even as far as the dawn of metazoan evolution.

Genomic alterations in *cis* are therefore the main drivers of regulatory evolution, a conclusion supported by the observation that human chromosome 21 transplanted in mice preserves human-specific gene expression and transcription factor occupancy profiles [46].

The most obvious mechanism for losing occupancy is loss-of-function mutations in the corresponding transcription factor motif. One of the surprising trends to emerge from comparative ChIP-seq studies, both in mammals and in flies, is that such changes often do not account for even a majority of lineage-specific occupancy losses [37, 38, 47, 48]. Instead, the explanation for a significant portion of such cases appears to be the loss of motifs of cofactors or pioneer factors (Figure 1B). This conclusion (substantial binding differences without obvious sequence changes) is also corroborated by studies of the effects of within-population genetic variation on transcription factor occupancy [49–51], as well as by observed patterns of binding conservation, such as the finding that cobinding factors frequently lose or gain occupancy in concert in different species [38, 41].

Some of the key known mechanisms of regulatory element gain include the duplication in *cis* of existing elements followed by the subfunctionalization of the two new copies or the neofunctionalization of one or both, exaptation from ancestral DNA and derivation from either recent or more ancient transposable element (TE) insertions, as TEs often contain functional transcription factor-binding sites and active promoter/enhancer elements (Figure 1C). Comparative ChIP-seq analyses have helped clarify the relative roles of these processes in evolution.

Exaptation of TEs turns out to be particularly important for certain kinds of regulatory elements and factors, a conclusion that emerged early in the history of global transcription factor mapping studies [52]. For example, CTCF binding sites often arise thanks to insertions of short interspersed nuclear elements (SINEs), which contain sequences with high affinity for CTCF [53]. Other important examples involve the neuronal-gene repressor NRSF/REST, which frequently evolves new binding sites through the exaptation of low-affinity sequences found in TEs such as ERV1 [54], and the contribution of TEs to the rewiring of the core regulatory network in mammalian embryonic stem cells [55]. Of note, TE exaptation seems to be most important for factors with long recognition motifs (to which group both CTCF and NRSF/REST belong), as binding sites for such factors are naturally more difficult to evolve through random drift from ancestral sequence.

Several efforts have compared regulatory elements as a whole (rather than individual transcription factors, using ChIP-seq against the enhancer-associated H3K27ac and the promoter-associated H3K4me3 histone marks) in multiple mammalian species [22, 41, 56–59], which has allowed first, a comparison of the rates of evolution between different types of elements, and second, an assessment of the relative contribution of the different mechanisms for their gain. Remarkably, promoters are much more conserved than enhancers; most promoters are highly conserved between any two mammals, while in contrast enhancer elements turn over rapidly. Exapted TEs (long terminal repeats and SINEs) are enriched in newly evolved promoters, both in promoters arising from recent and from ancestral DNA regions, with the former constituting the majority [57]. In contrast, most new enhancers appear to evolve from exaptation of ancestral DNA sequences, and TEs are highly enriched only in enhancers evolving from phylogenetically recent sequences [57, 59].

The conservation of regulatory elements unsurprisingly appears to be correlated to functional constraints. That such constraints acting on promoters are higher than those applying to any individual enhancer is fairly obvious, thus it is not surprising that promoters are more highly conserved. Probably related to such constraints is the higher conservation of individual transcription factor occupancy in promoters and promoter-proximal regulatory regions than in distal ones, observed both in mammals and insects [41, 48, 60], as is the observation by the ENCODE and mouseENCODE consortia that regulatory elements active in multiple tissues and likely having pleiotropic effects exhibit higher degrees of occupancy conservation, as do transcription factors that co-associate with many other factors.

To what extent these principles of regulatory conservation apply to other phylogenetic groups is of considerable interest, and remains to be answered by future expansions of the range of lineages covered by comparative functional genomic studies.

Evolutionary dynamics of regulatory element turnover

Data for lineages other than mammals are also of key importance for answering the other major question regarding regulatory evolution: What are the evolutionary forces driving it, i.e. what are the relative contributions of mutation, selection and genetic drift to the observed patterns of conservation and divergence?

The only other group in which extensive comparative ChIP-seq studies have been carried out is flies, and the picture painted by these studies has generally been rather different from what is seen in mammals, mostly returning much higher estimates of occupancy conservation. An early analysis of six developmental transcription factors in *Drosophila melanogaster* and *Drosophila yakuba* reported that fewer than 1–5% (depending on the factor) of ChIP-seq peaks observed in one species were clearly absent or displaced in the other [61]. Mapping of Twist occupancy in six *Drosophila* species [48] estimated a 60% conservation of binding events between *D. melanogaster* and *Drosophila pseudoobscura*, which are estimated (according to substitutions per neutral site) to be as equally diverged as humans and chickens. On the other hand, comparative analysis of four transcription factors (BCD, GT, HB and KR) involved in early anterior–posterior patterning in *D. melanogaster*, *D. yakuba*, *D. pseudoobscura* and *Drosophila virilis* returned lower conservation estimates, with 15–38% of regions bound in *D. melanogaster* also bound in *D. yakuba* and *D. pseudoobscura* [60].

The one notable difference between flies and mammals clearly going in the other direction in terms of conservation concerns the insulator protein CTCF. CTCF occupancy is generally the most conserved of any transcription factor in mammals [53, 62, 63], likely reflecting its critical role in partitioning the spatial organization of the genome but has been reported to diverge rapidly in *Drosophila* [64]. This is possibly because flies contain multiple insulator proteins (see further discussion below), of which CTCF is not the most important, relaxing the constraints on the evolution of its occupancy.

How are the observed patterns of conservation and divergence to be interpreted? Transcription factor-binding sites and regulatory element evolution have attracted considerable attention from theoretical geneticists in the past [65, 66, 67]. Based on population genetic considerations and available data on key population genetic parameters across eukaryotes [28, 29, 67], the following model, centered on the balance between selection and genetic drift, has been proposed. The power of natural selection to eliminate maladaptive alleles and fix adaptive ones is limited by the effective population size (N_e) of real-life populations. More specifically, when $|s| \leq \sim 1/N_e$, where s is the selection coefficient associated with them, alleles behave effectively neutrally. Thus, in populations with large values of N_e, selection is highly efficient, and even small selective differences are 'visible' to it, and *vice versa*—in populations with small N_e, slightly maladaptive genomic changes can drift to fixation because of the relaxed strength of selection. Most mutations that eventually lead to regulatory turnover are expected to be maladaptive to some extent because they can lead to misregulation of gene expression. It is thus natural to expect lineages with small effective population size to exhibit increased rates of regulatory turnover (Figure 2A). More specifically, N_e is highest in prokaryotes, and then generally decreases from prokaryotes to unicellular eukaryotes to smaller invertebrates, with large-bodied complex multicellular organisms having the smallest effective population sizes (Figure 2B). Following this line of reasoning, faster *cis*-regulatory evolution would be expected in vertebrates than in flies because of the significantly lower N_e of the former (Figure 2C).

Does available data confirm these theoretical expectations? A review of published comparative studies appeared in 2014 [47], and it found concordance with the model presented above, with reported transcription factor occupancy conservation being higher in flies than what is observed in mammals (Figure 2C). However, it was based on compiling estimates of the fraction of conserved occupied sites directly taken from individual studies, which used different analysis methods and therefore might not have been directly comparable with each other. A reanalysis of a subset of several factors was published later in 2015 [70]. It used uniform data processing and analysis procedures across flies and mammals and reported that in fact the two lineages exhibit similar rates of regulatory evolution (Figure 2D). However, while it did apply an uniform analytical pipeline, it did not fully control for all potential relevant variables (such as, for example, organismal generation times), and it also focused on only a small set of transcription factors.

The issue of how empirically observed rates of regulatory divergence fit with theoretical considerations will have to be resolved in the future by a much expanded compendium of transcription factors, the application of uniform data processing pipelines and, most importantly, the inclusion of a much wider coverage of lineages, preferably with different population genetic environments (such as, for example, unicellular eukaryotes with high effective population sizes [71]), and covering a wide spectrum of evolutionary distances. Such studies are beginning to emerge in other metazoan systems. For example, ChIP-seq was recently used to compare occupancy by the developmental transcription factor Tbrain (Tbr) in the sea star *Patiria miniata* and the sea urchin *S. purpuratus* [44], which diverged ∼450–500 Mya; ∼10% of sites appear to be conserved in this case. Much more data will certainly become available in the future, but more systematic approaches to experimental design and data collection will undoubtedly also be helpful for its integration into a coherent picture of evolutionary dynamics.

Mapping the rewiring of gene regulatory networks

The development of multicellular organisms is driven by GRNs, primarily composed of transcription factors acting in concert to specify developmental patterning. GRNs are understood to have an ultimately hierarchical structure, beginning with the fertilized zygote. Each developmental phase specifies the events happening in the subsequent stages through the activation or repression of certain GRN sub-circuits in defined regions of the embryo, with individual terminally differentiated cell types residing at the bottom of these hierarchies [72, 73].

The key to understanding the evolution of organismal morphology and development therefore lies in large part in studying the rewiring of GRNs over evolutionary time. As a well-known example, the pelvic apparatus of the threespine stickleback fish (*Gasterosteus aculeatus*) usually contains prominent spines, but in multiple freshwater stickleback populations, these structures have been partially or entirely lost (possibly because of the absence of predatory fish, nutrient limitations or other factors). Such losses can be tracked to the recurrent loss of an enhancer for the *Pitx1* gene [74], which encodes a homeodomain transcription factor and is involved in the specification of hindlimb development in vertebrates.

Painstaking work over many years aimed at picking apart the details of the *cis*-regulation of individual developmental genes has resulted in detailed GRNs maps in several systems, with the one of early sea urchin embryonic development being perhaps the most famous example [75]. Such GRNs have often been presented in the form of network circuit diagrams, in which different factors have explicit binary relationships with each other. The conceptual clarity of depictions of this sort is highly attractive, and finding a way for regulatory relationships to be comprehensively mapped onto such diagrams over the whole genome is the ultimate goal/holy grail for the field.

Based on a naive understanding of the relationship between transcription factor occupancy and gene expression, ChIP-seq promised to provide maps of these circuits on a genome-wide scale, and across lineages, something especially attractive given that many striking developmental and morphological innovations in metazoans are to be found outside of the traditional model systems. The reality has turned out to be significantly more complicated though. First, transcription factor occupancy exhibits a continuum, with many more weaker sites than strong

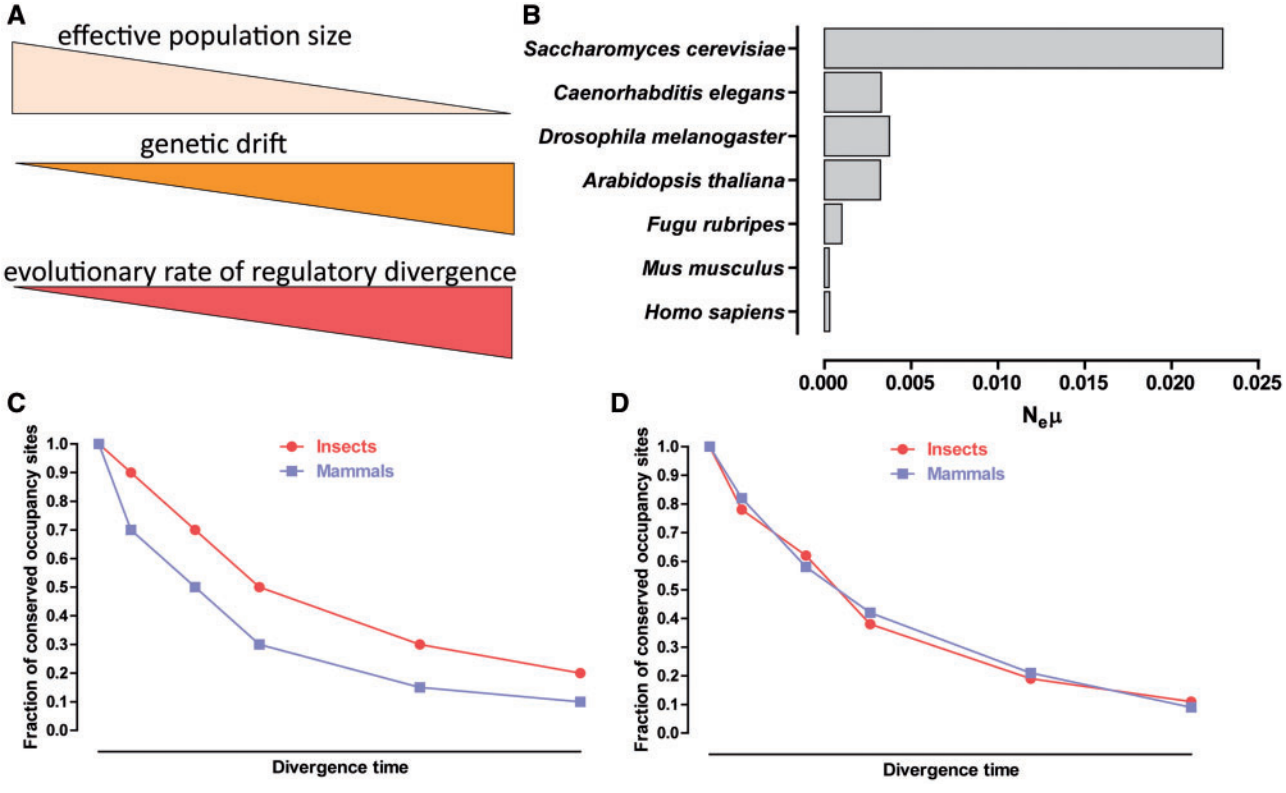


Figure 2. Theoretical expectations regarding the evolutionary dynamics of transcription factor occupancy and interpretations of existing data sets. (**A**). Population genetic theoretical considerations lead to an expectation of faster turnover of regulatory sites in organisms with low effective population sizes (N_e) than in species with large N_e. (**B**) Distribution of effective population size values in some of the main model systems [68]. Shown is the product $N_e\mu$ of N_e and the mutation rate μ, which can be most directly estimated empirically, unlike N_e alone [68, 69]. (**C**) The compilation of individual studies summarized in [47] suggested higher rates of regulatory site turnover in mammals than in flies. (**D**). In contrast, a reanalysis of several data sets in flies and mammals using a uniform data processing pipeline found similar rates of regulatory divergence within the two groups [70].

ones, and without a clear delineation between true binding events and background [76, 77]. Second, while there is some evidence that low-level occupancy sites are more frequently nonfunctional [78], there is no absolute relationship between ChIP signal strength and functional effects on nearby genes or a clear way to clarify that relationship on a per-site basis from ChIP data alone. Third, the combinatorial complexity of transcription factor binding has turned out to be high, and it is not always clear which of all the factors associated with a given putative regulatory element are of critical importance for its functioning. Similar redundancy seems to be also widespread at the level of the set of regulatory elements associated with a given gene [79]. Finally, and perhaps most important, most putative regulatory sites in metazoans with large genomes are distally located from promoters. Consequently, identifying which putative enhancers regulate which promoter is not a trivial task without performing additional experiments.

These inherent complexities of transcription regulation in eukaryotes are perhaps the reason why while traditional circuit diagrams are by no means obsolete, deriving them from functional genomic data alone has been difficult, and why mapping their rewiring on a genome-wide across evolution has not been widely done yet. However, ChIP-seq provides highly useful lists of candidate regulatory elements, which can be subsequently characterized in more depth, especially with the now widespread CRISPR-based tools for genome and epigenome editing [80, 81] provided that the organism studied is amenable to such manipulations. ChIP-seq is also informative about global trends at the genomic level. Several studies using it to track regulatory changes during evolution and/or development of nonmodel species have been published over the past few years.

An example is the mapping of epigenomic and transcriptomic difference between forelimbs and hindlimbs in bat development using *Miniopterus natalensis* as a model system [82] (Figure 3A). Unlike most tetrapods, bat forelimbs and hindlimbs develop into morphologically different from each other structures. The forelimbs become elongated webbed wings, while the hindlimbs develop into fairly short legs. The integrated analysis of H3K27ac and H3K27me3 ChIP-seq, transcriptomic and comparative genomic data identified pathways involved in the differential patterning of the two structures, including the Wnt/β-catenin, Wnt-PCP and BMP signaling pathways as well as several ribosomal proteins. Increased forelimb mesenchymal condensation apparently driven by diminished β-catenin signaling seems to be particularly important for forelimb growth and patterning.

ChIP-seq has also provided insights into vertebrate limb development in a different context. Mapping of H3K27ac enriched regions in mouse and lizard developing embryos and comparative genomic analysis relative to available snake genomes has revealed that numerous limb-specific enhancers are retained in snakes, even though snakes are limbless and have been so for nearly 100 million years [83] (Figure 3B). A major reason for this relatively unexpected conservation appears to be that many limb enhancers are also used during the development of genital structures, which, unlike limbs, are not reduced in snakes.

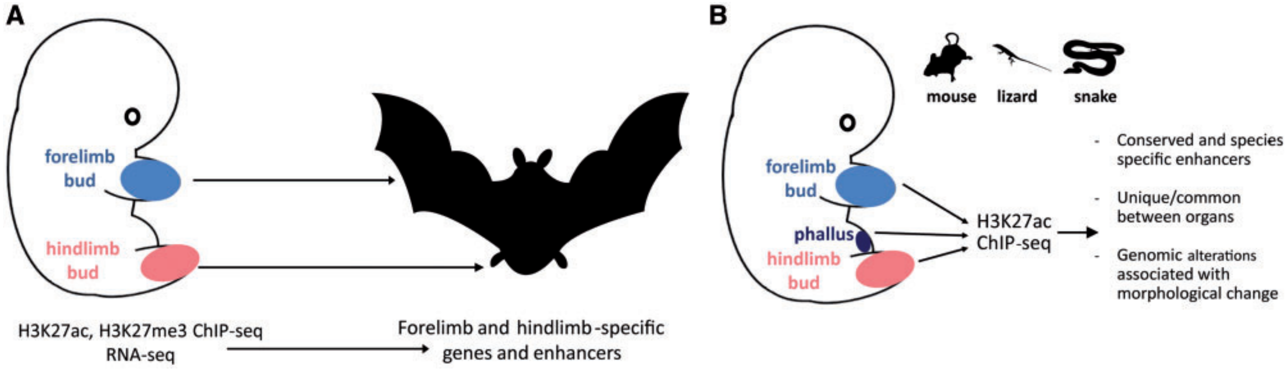

A
forelimb bud
hindlimb bud
H3K27ac, H3K27me3 ChIP-seq
RNA-seq
Forelimb and hindlimb-specific genes and enhancers
B
mouse
lizard
snake
forelimb bud
phallus
hindlimb bud
H3K27ac ChIP-seq
- Conserved and species specific enhancers
- Unique/common between organs
- Genomic alterations associated with morphological change

Figure 3. Examples of using ChIP-seq to map GRNs in development and evolution. (**A**). Cataloging enhancers involved in patterning the morphology of bat limbs. The forelimb bud of bats develops into elongated webbed wings, while the hindlimb bud produces much shorter legs. H3K27ac and H3K27me3 ChIP-seq and RNA-seq were applied to developing limb buds to chart the developmental enhancer landscape involved in the specification of these structures [82]. (**B**) Understanding the role of limb-specific enhancers in snake evolution. Snakes lack legs, but H3K27ac ChIP-seq in mouse and lizard embryos and comparative genomics reveal that a substantial portion of limb enhancers are in fact conserved in snakes, one major reason for which is their role during phallus development, where a similar developmental program is deployed [83].

Detailed functional testing of an enhancer of the *Tbx4* gene (which is important for hindlimb development) showed loss of its hindlimb activity in snakes as well as its importance for both hindlimb and urogenital development in mice.

The application of functional genomic approaches to developmental questions in nonmodel systems is still in its early days, but we can expect its future expansion to shed light on the genomic foundations of developmental gene regulation, including in metazoan lineages outside vertebrates such as arthropods and echinoderms. Insects in particular provide an endless diversity of morphological innovations. With large numbers of sequenced genomes expected to come online through efforts such as the i5K Consortium [84], the infrastructure for research in that direction will become available in the near future. Epigenomic maps were already used to annotate the regulatory genome in the context of head development in the butterfly *Heliconius erato* [85], and many more studies of this type will surely be carried out moving forward.

Charting the evolution of gene regulatory mechanisms

Transcription and transcriptional regulation in mammals, in which the relevant processes have been studied in greatest detail, is driven by the interplay of certain classes of regulatory elements—chiefly promoters, enhancers acting at a distance and insulators blocking long-range interactions and the spread in *cis* of epigenetic states. These mechanisms of regulation fit well with the overall architecture of large-sized vertebrate genomes, with their sparsely distributed coding regions, and large expanses of intergenic and intronic space. Different classes of regulatory elements are marked by specific subsets of chromatin-binding proteins and histone marks, and so are genomic regions associated with active transcription, heterochromatin, etc., defining characteristic chromatin states [86]. Well-known examples include the association of H3K27ac and H3K4me1 with enhancer elements [87–89], of H3K4me3 with active promoters of genes [90, 91], of H3K36me3 with active transcription [92, 93], of H3K27me3 and H3K9me3 with repressed facultative and constitutive heterochromatin [94–96] and the demarcation of insulator elements by CTCF and cohesin [97, 98]. More generally, specific highly choreographed arrays of dynamic histone modifications are associated with all aspects of chromatin biology, including transcription [99], splicing [100], replication and mitosis [101], DNA repair [102] and others. Histone modifications serve as platforms for the recruitment of effector proteins containing corresponding recognition domains, and thus constitute what is often referred to as the 'histone code' [103]. The complexity of histone marks is dizzying [104]; hundreds of them have been identified in mammalian cells by proteomics studies, but so far only a fraction have been carefully studied and are well understood in mechanistic detail.

A remarkable feature of histone proteins is their extreme conservation across eukaryotes, especially at sites carrying key modifications involved in the processes listed above [105, 106]. This implies an accordingly strong general (but not necessarily absolute) conservation of the corresponding biochemical processes these residues are associated with, and that at the least a core set of histone marks involved in the transcriptional cycle, heterochromatin formation and a few other areas date back to the last common eukaryotic ancestor (LECA). It also makes the biology of the few organisms that represent major exceptions from the usual rules all the more interesting.

LECA may have had a small and compact genome, as did the more recent ancestors of metazoans, with the baroque mammalian genome and transcription regulation architecture evolving later in the process of metazoan diversification. What the deep evolutionary factors behind these developments are and what their relationship to the phenotypic complexity of mammals is, i.e. is the regulation-from-a-distance, enhancers/promoters/insulators model a prerequisite for building a highly complex multicellular organism such as us, remains an open question.

As we discuss below, understanding of chromatin biology across the eukaryotic phylogeny will be of crucial importance for answering these questions.

The known eukaryotic diversity in the 2010s

Traditional presentations of eukaryotic diversity have focused on the three main lineages where complex multicellularity evolved—land plants, fungi and metazoans—with other eukaryotes lumped into the overlapping categories of 'algae' and 'protists' (depending on whether they photosynthesize). The phylogenomic era has radically transformed this simplistic understanding. We now know of in the neighborhood of a hundred distinct eukaryotic lineages that are as deeply divergent (and should be thought of as equivalent in rank clades) as metazoans [107] (see Figure 4 for a partial overview), and new lineages of major phylogenetic importance are still being regularly discovered [108, 109]. These are tentatively grouped in several more or less phylogenetically coherent groups—Opisthokonta, Amoebozoa, Excavata, Archaeplastida, Hacrobia, Rhizaria, Alveolata and Stramenopiles. Opisthokonta and Amoebozoans are clearly grouped together in the Unikonts; Stramenopiles, Alveolates and Rhizaria are tentatively grouped in the SAR (Stramenopiles-Alveolates-Rhizaria) superclade; and some poorly studied lineages are still difficult to confidently link with any of the major groups. Where exactly the root of the eukaryotic tree lies is still unclear; different topologies have been proposed by various studies [110, 111]. The root might lie between Excavata and the rest of eukaryoties, or it may even be located within the current Excavata assemblage (making it paraphyletic), with the Discobeans (the clade containing the Jakobids and Kinteoplastids; Figure 4) on one branch and all other eukaryotes on the other [110].

The Opistokonts feature both metazoans and fungi, and the model systems in which the bulk of cellular and molecular biology research is being carried out. In addition, *Monosiga brevicolis* and *Capsaspora owczarzaki* are emerging as model systems in the two phylogenetically closest to metazoans groups, Choanoflagellata and Filasterea, respectively. Among the Amoebozoans, the mycetozoan *Dictyostelium* has long been studied as a model for the origins of multicellularity. The Archaeplastida assemblage features all groups with a primary plastid (i.e. derived from the original endosymbiotic event back in the Precambrian), including land plants (*Arabidopsis* being the main model system for plants) and green algae (with *Chlamydomonas* and *Volvox* being popular model organisms). The Excavates include several clades featuring important disease agents, which have accordingly attracted significant attention from researchers such as *Trypanosoma* and *Leishmania* in Kinetoplastida, *Trichomonas* in Parabasalia, *Giardia* among the diplomonads and *Naegleria* in Heterolobosea. The Alveolates include the Apicomplexans, the major parasitic lineage featuring the malaria plasmodium, *Toxoplasma* and a number of other pathogens and the free-living ciliates, among which *Tetrahymena* and *Paramecium* have a long history of being model organisms. Finally, model systems are emerging in the diatomes and the brown algae (Phaeophyta) among the Stramenopiles.

However, the vast majority of the eukaryote diversity is still extremely poorly studied, and the discipline of evolutionary cell and molecular biology [114], which aims to understand not only how exactly cellular processes function in different organisms but also why they came to be organized in the way they are, is still in its infancy because of the limited amount of data available.

The evolution of eukaryotic chromatin organization

The ancestral eukaryote genome, the appearance of complex genomes and the origins of morphological complexity

As outlined above, the organization of chromatin and the logic of gene expression regulation in metazoans are generally well known. But while rapid advances are being made in understanding the detailed mechanistic workings of this system, how and why it came to be remain open questions. Also not understood at present is the relationship between the enhancer/promoter/insulator model and the emergence of complex multicellularity. The genomes of unicellular yeasts, which have served as a model system for studying transcriptional regulation for many decades, are highly compact, with little intergenic space, and appear to use primarily promoter-proximal mechanisms of regulating transcription. While the transcriptional biology of most of them has not been studied in detail, the genomes of many (but by no means all) other unicellular eukaryotes are also similarly compact. In contrast, animal genomes typically contain large expanses of intergenic space, and long introns, as do the genomes of land plants, the lineage that has achieved the second highest level of morphological multicellular complexity as meausured by the number of distinct cell types in the organism [115].

The expansion of the genomes of multicellular eukaryotes is usually explained as a consequence of the lower efficiency of natural selection in lineages with decreased effective population size [69], which in turn is a natural consequence of the increased physical size of multicellular organisms. In such a population genetic environment, noncoding DNA and TEs, whose presence is usually nonadaptive and which are efficiently eliminated by natural selection in microbial lineages, can proliferate leading to large genomes full of repeats, introns and other noncoding DNA. As discussed above, these conditions have also been proposed to accelerate GRN evolution through duplication and sub/neofunctionalization of regulatory elements [67]. In turn, this might have enabled morphological complexification by facilitating the appearance of new cell types. The utilization of different distal regulatory elements to drive the expression of the same gene in different contexts may have been particularly useful for that purpose, which fits well with the on average a dozen or more putative such elements per gene that have been observed in mammalian genomes by large-scale candidate regulatory elements mapping efforts such as ENCODE and mouseENCODE.

One plausible view of how complex multicellularity developed that emerges based on these considerations sees regulatory complexification, genome expansion and organismal complexification as going hand in hand in a feed-forward loop relationship. More complex organisms tend to be physically larger and with lower effective population sizes, which allows for proliferation of noncoding DNA and the appearance of more complex GRNs, which in turn enables further organismal complexification. Gene regulatory mechanisms centered around distal regulatory elements and working in 3D space play a central role in this model. The diversity of gene expression programs in which an individual gene may participate is most likely substantially more limited if the gene is to be regulated exclusively through promoter-proximal means. But if regulatory elements are physically decoupled from the gene's promoter, the constraints on their number and subfunctionalization are lifted, and they can more freely evolve in ways allowing the gene to be expressed in different developmental contexts and cell types.

A prediction this model makes is that long-range gene regulation and large genomes should be prevalent in large-bodied organisms with complex multicellularity. Such level of complexity has been achieved in metazoans and plants, on more than one occasion in fungi, in red and in brown algae, and it is indeed frequently accompanied by expanded genomes. To what extent long-range gene regulation is also common will be elaborated in more detail further below.

Before that, we will note that this model for the origin of complex gene regulation (or its alternatives) does not necessarily explain when exactly it evolved and what its molecular foundations are. Phylogenomic studies over the past couple decades have converged on the view that eukaryotes arose from within

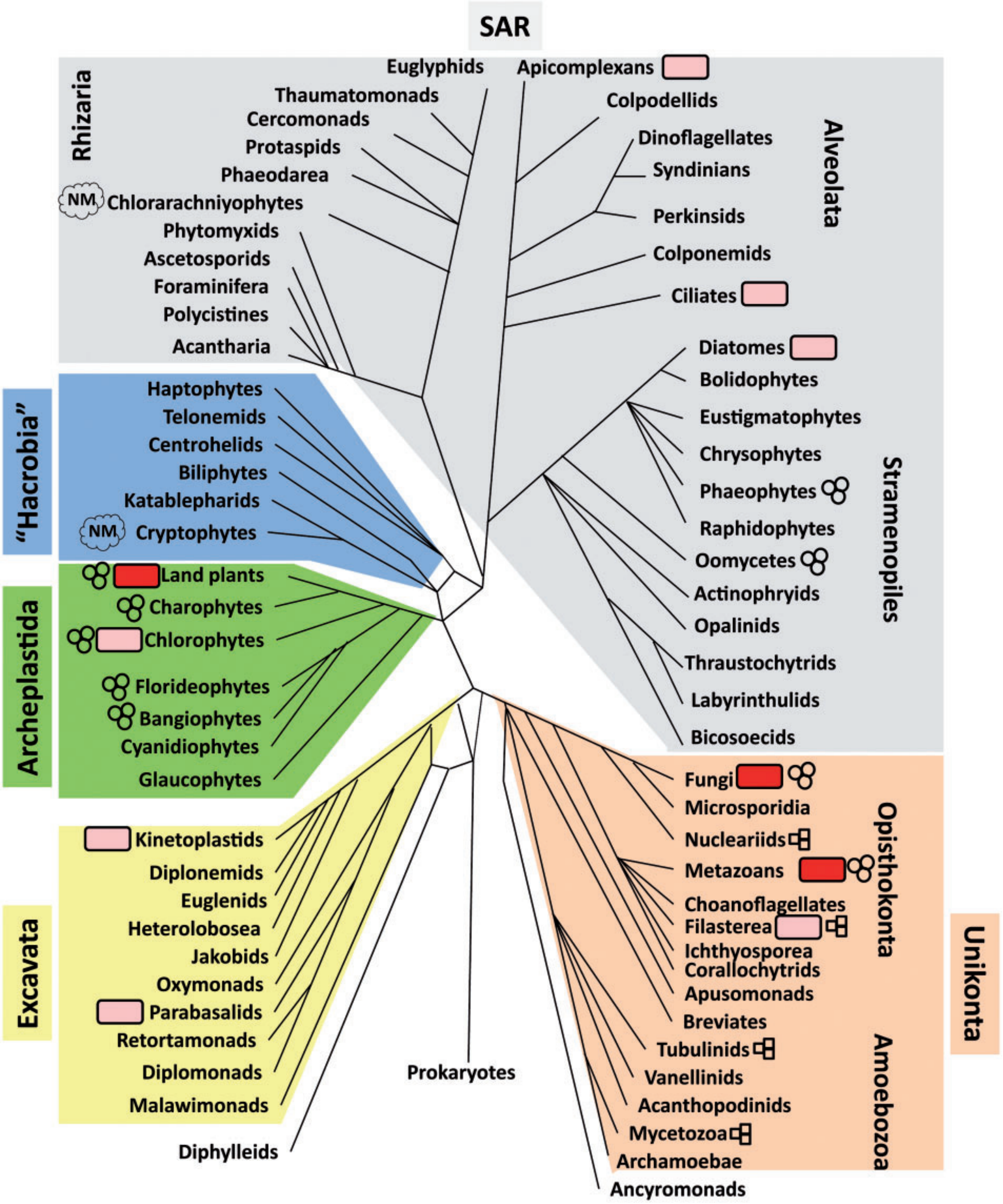


Figure 4. Major eukaryotic clades, their epigenomic characterization and the origins of multicellularity. The tree shown follows previously published topologies [112], but it should be noted that the precise deep branching is still to be fully resolved by future phylogenomic studies. Red rounded rectangles are placed next to the lineages in which chromatin and transcriptional biology have been studied in considerable detail. Pink rounded rectangles are placed next to clades for some representatives of which initial epigenomic studies have been carried out in some detail. Clades in which multicellularity has evolved are indicated with three circles where multicellularity results from cell division, and by three triangles where multicellularity is aggregative [113]. The lineages containing nucleomorphs (the chlorarachniyophytes and the cryptophytes) are indicated with 'NM'.

archaea. Most likely this happened as the direct result of an endosymbiotic event with a member of the α-proteobacteria, from which the modern mitochondrion derives [116, 117]. Thus, the ancestral eukaryotic genome probably looked much like the genomes of modern archaea, i.e. following the typical prokaryote organization of genes into operons and being small, compact and free of spliceosomal introns. It is tempting to think that the LECA genome was also streamlined, and this would be the more parsimonious explanation given the distribution of genome sizes across modern eukaryotes (most of which are unicellular species with compact genomes).

However, LECA seems to have been an essentially modern eukaryote, with all core characteristics that define the eukaryotic cell and sharply separate it from prokaryotes, and no clear intermediates, stemming from the period before the radiation of the known modern eukaryote supergroups, have ever been found. Thus, some time seems to have passed between eukaryogenesis and LECA. One thing we can say with some confidence about the LECA genome is that it was intron-rich, as comparisons of intron positions across eukaryotes have shown that many introns are ancestrally shared by all modern groups and must therefore date back to the time before LECA [118]. Combined with the clear relationship between eukaryotic spliceosomal introns and prokaryotic Group II self-splicing introns, and with the observation that the latter are phylogenetically restricted to bacteria but absent from archaea, an attractive model has been proposed, according to which spliceosomal introns originated as a result of the invasion of the archaeal genome by Group II self-splicing introns from the α-proteobacterial endosymbiont [119, 120]. Spliceosomal introns evolved later as a result of the loss by individual Group II introns of the ability to autonomously splice in *cis* and the transfer of that function in *trans* to the spliceosome. As Group II introns are rather large ribozymes, that would imply that the original eukaryote lineage might have had a larger genome than what is seen in modern eukaryotes with compact genomes. Whether that was also the case for LECA or the genome had already shrunk considerably by that time cannot be known with certainty at the moment though. In addition, the presence of large introns does not necessarily imply the existence of large intergenic spaces.

Still, the core of the machinery mediating long-range enhancer-promoter interactions seems to have been present in LECA. The Mediator complex, which plays an important role in the process of establishing such loops [121], is ancestral to all eukaryotes, as is the cohesin complex [122] (even if its primary ancestral role might have been chromatin cohesion during mitosis and meiosis).

The main multicellular lineages, however, have still apparently all emerged from ancestors with compact genomes, such as green algae, and the various holozoan opisthokont lineages sister to metazoans. On the other hand, large genomes are not unique to multicellular eukaryotes. The famous 750 Gb genome size estimates for some amoebas [123] are possibly artifacts of polyploidy or methodology, but a number of protists do indeed have large genomes. Dinoflagellate genomes are notorious for their enormous sizes, with the smallest ones being ∼1.5 Gb (for example, *Symbiodinium* [124]) and most being considerably larger (for example, the ≥100 Gb genome of the dinoflagellate *Prorocentrum micans* [125]). Giant genomes much larger than those of mammals have also been reported in diatoms [126] and in other protozoan lineages. Unfortunately, but for understandable reasons, genome sequencing efforts have so far targeted representative of each lineage species with small genomes. As a result, little is known about the organization of large protist genomes. Similar constraints have also applied to genome sequencing projects in fungi. Hundreds of genomes have been sequenced, but no sequences are available for the largest ones, which approach 1 Gb [127]. Yet, based on genome size estimates, it seems that increases in genome size relative to a more compact ancestral state can be found in all multicellular lineages: red algae genomes range between 100 Mb and 2.8 Gb [128], and those of brown algae range between 200 Mb and 3.6 Gb [129].

Several major questions thus emerge. Is a sparse genomic organization with distal regulatory elements tightly phylogenetically linked to the emergence of multicellularity? If yes, are the two causally related? If it evolved independently on multiple occasions did it evolve through similar modifications of the same preexisting components, and did it converge onto similar mechanisms everywhere or to divergent states operating under somewhat different principles? Are the identity and characteristics of distal regulatory elements the same in all eukaryotes? What can such similarities/differences tell us about the logic of mammalian gene regulation and its origins?

A separate set of similar questions concerns the conservation and divergence of chromatin biology. As mentioned above, histone proteins are generally extremely highly conserved across eukaryotes, with the key residues on which the best studied histone modifications are deposited being ancestrally present in all lineages [105]. The core histone marks themselves also appear to be ancestral as evident by their presence in both metazoans, plants and ciliates, as do some histone variants such as H2A.Z [130]. However, conservation of histone marks does not necessarily mean conservation of associated biochemical processes, much less conservation of combinatorial sets of histone marks and resulting chromatin states. What exactly the ancestral state of the histone code was, how it came it to be and how conserved and constrained all of its aspects are among eukaryotes is still not entirely clear.

These questions can only be fully answered through comprehensive functional genomic studies of eukaryotic lineages throughout the tree of life. We are far from achieving that goal, but initial insights have already been obtained from a number of clades.

The three most important multicellular lineages all contain well-studied model systems; thus, most information for such comparisons is naturally available from those species.

Functional genomic studies in non-yeast fungi

In the case of the fungal lineage, all model systems are yeasts or molds with compact genomes, but the wealth of sequenced genomes includes a number of complex multicellular species, which appear to have compact genomes too (for example, the *Amanita muscaria* mushroom has a ∼40 Mb genomes with ∼18 000 genes [131]), and are likely using primarily promoter-proximal regulatory elements. Of note, unicellular yeasts lack H3K27me3, the developmentally regulated heterochromatin histone modification, while that modification is present in the mold *Neurospora* [132]. A curious feature of *Neurospora* biology that has been revealed by functional genomics is that it appears to use a unique mechanism for organizing its chromatin in 3 D space. Unlike the CTCF/cohesin-driven looping used in metazoan genomes, studies using ChIP-seq and 3 D genome structure mapping techniques such as Hi-C have shown that heterochromatin regions marked by H3K9me3 and H3K27me3 organize most 3 D interactions in *Neurospora* nuclei [132, 133].

Functional genomics and the chromatin biology of land plants

It is remarkable given their economic importance, but the study of enhancers in plants is still in its infancy. Plants do have enhancers, but only a small number of individual enhancer elements have been characterized in any depth [134, 135], and functional genomic studies have so far painted a rather inconclusive picture regarding their properties. DNAse hypersensitivity maps have identified thousands of intergenic and intronic open chromatin regions in rice, in *Arabidopsis* and in *Brachyopodium* [136–139]. Similarly, comparative genomics efforts have identified large numbers of conserved noncoding sequences, exhibiting enhancer-like characteristics [140, 141]. Typical features of eukaryotic chromatin, such as the association of H3K4me3 with promoters, of H3K9me3 and of Polycomb-deposited H3K27me3 with heterochromatin, are also present in plants.

However, despite numerous studies using ChIP-seq to map histone marks and chromatin states in the main plant systems [142–146] as well as the moss *Physcomitrella patens* [147], clear chromatin signatures of active enhancers have not yet emerged [132, 133]. H3K27ac appears to correlate with active enhancers [148], as it does in metazoans. However, the association is weaker, and to what extent the additional features that seem to distinguish mammalian enhancers (H3K4me1, eRNA transcription, poised enhancers being marked by H3K4me1 alone but not by H3K27ac and others) are shared by their plant counterparts is also not clear. Part of the challenge is that there are few gold-standard enhancer elements in plants around which detailed studies are to be built. Another is that ChIP experiments are usually done on whole parts of plants, containing a mixture of cell types, thus producing a convolution of signals and decreasing the signal-to-noise ratio. Methods for the specific isolation of pure cell populations will be needed to resolve these issues [149].

How enhancer–promoter interactions in 3 D space are regulated in plants is at present also not understood and remains to be elucidated in the future. A remarkable feature of *Arabidopsis* chromatin, in stark contrast to what is observed in most metazoans, is the absence of topologically associated domains (TADs; regions of increased 3 D interaction frequency, which are also generally not crossed by promoter–enhancer interactions) [150–152]. On the other hand, the existence of insulator elements in plants has been postulated [153]. Of note *Arabidopsis*, with its fairly compact genome (27 655 genes in ∼ 135 Mb [154]), may not be the ideal system for such studies. Other plants with sparser genomes, such as rice and maize, are potentially more informative. Indeed, a more recent study [155] found that about a quarter of the rice genome is organized into TADs, but data from a number of other lineages will be needed to fully understand plant chromatin organization.

No epigenomic data providing information about the regulatory organization of multicellular red algae and brown algae are available at the moment, but their future study will be highly informative regarding the origin and phylogenetic distribution of distal regulatory elements and its relationship to multicellularity, as will be the study of the closest algal relatives of land plants.

A number of protozoan lineages have, however, been examined in some detail, and these efforts have revealed some of the extent of conservation and divergence of chromatin biology in eukaryotes.

Functional genomics and the chromatin biology of apicomplexans

A relative wealth of information has become available in recent years from the apicomplexan *Plasmodium falciparum* [156–158], whose epigenome exhibits some curious features. Unlike most other eukaryotes, in which H3K4me3 is localized around promoters, as is the histone variant H2A.Z, in *Plasmodium* H3K4me3 and H2A.Z, together with the apicomplexan-specific histone variant H2B.Z, demarcate intergenic regions in their entirety. In addition, the H3.3 variant, which is typically deposited around sites of active transcription, has been suggested to also associate with subtelomeric regions in *Plasmodium* [159]. Additional intriguing properties of *Plasmodium* chromatin include strong nucleosome positioning over splice sites and transcription termination sites (TTSs), but an absence of the typical [160] tightly positioned nucleosomes immediately around transcription start sites (TSSs). It is possible that intergenic regions are occupied by a different type of nucleosomes because of the extreme AT-richness of the *Plasmodium*, which reaches and sometimes even exceeds 90% in intergenic regions.

Functional genomics and the chromatin biology of kinetoplastids

Another interesting group that has attracted attention because of its medical relevance is kinetoplastids. As mentioned above, it is possible that the discobeans as a whole are the deepest diverging eukaryotic clade [110], which may or may not be related to kinetoplastids exhibiting a number of divergent features. The most remarkable among them is the apparently complete loss of gene regulation at the transcriptional level [161]. Instead, genes are organized into long constitutively expressed polycistronic units, pre-mRNAs are *trans*-spliced with splice leader sequences to produce mature mRNAs and gene expression regulation happens primarily at the posttranscriptional level through mechanisms regulating RNA stability and translation.

Kinetoplastids also have more divergent than usual histone tails, missing a number of key histone code residues [162, 163], such as H3K9. The kinetoplastid epigenome is far from comprehensively characterized, but multiple ChIP-seq studies have revealed some aspects of its organization. It appears that the boundaries of polycistronic transcription units in trypanosomatids are demarcated by nucleosomes containing four histone variants, H2AZ, and the unique to kinetoplastids H2BV, H3V and H4V [164] (reminiscent in some ways to what is observed in apicomplexans) as well as by histone acetylation [165]. Kinetoplastid genomes are also remarkable for containing a unique elaborate DNA modification, glucosylated hydroxymethyluracil, also known as base J. Base J has been mapped genome-wide and appears to be a marker for transcriptional termination at the end of polycistronic units [166, 167].

How this strikingly divergent genomic organization came to be is an open question. Parasitism is a defining theme of kinetoplastid biology (although free-living kinetoplastids do exist), and parasitic lineages are often highly derived. However, the sequencing of the genome of *Bodo saltans*, one of the free-living kinetoplastids, suggested that polycistronic organization is ancestral to the groups [168]. How much deeper this feature extends within the Discobea clade is not known, as little data exist for the other lineages in the group, except for the Heteroloboseans/Percolozoa, where a couple of *Naegleria* genomes have been sequenced [169, 170] and appear to have a conventional organization. However, not much is known about their functioning beyond that, and even less is known about other related lineages, even though the fascinating biological questions regarding them are plenty, such as the organization of permanently condensed chromosomes in euglenids [171].

Functional genomic studies of other protozoan lineages

Within excavates, limited epigenome mapping has also been carried out in *Trichomonas vaginalis* (Parabasalia) [172], targeting only two histone marks, H3K4me3 and H3K27ac, and revealing the usual enrichment around TSSs of active genes. Interestingly, chromatin in the diplomonad *Giardia lamblia* has been reported to contain an HU-like protein (HU-type proteins are histone-like nucleoid-associated nonspecific DNA-binding proteins typically found in bacteria, although they can also bind to RNA [173, 174]) in addition to the linker and the four core histones [175]. In addition, its promoters have been suggested to produce highly abundant transcripts in both orientations, unlike in other eukaryotes [176], but detailed studies using more modern functional genomic tools are lacking.

More comprehensive histone mark profiling has been carried out in two algal lineages. Five histone marks (H3K4me3, H3K27ac, H3K9me3, H3K36me3 and H3K27me3) were mapped along the compact genome of the classic chlorophyte model species *Chlamydomonas reinhardtii* [177]. Surprisingly, H3K9me3, which is generally strongly associated with heterochromatin, was reported to associate with the promoters of most genes, forming a bivalent signature with the H3K4me3 and H3K27ac marks, while H3K27me3, also a heterochromatin mark, was found to form bivalent domains along gene bodies with the transcription elongation mark H3K36me3. Both of these are unusual and unexpected chromatin states not found in other eukaryotes.

In the diatom *Phaeodactylum tricornutum*, H3K4me2, H3K9me2, H3K9me3, H3K9/K14Ac and H3K27me3 were profiled [178], providing the first glimpse into the epigenome of a stramenopile and revealing largely shared features with what is observed in most other eukaryotes.

No comprehensive epigenomic characterization has been carried out so far for any lineage of the Hacrobians, the Amoebozoans or the Rhizarians.

Functional genomics and the chromosomal biology of ciliates

Within the alveolates, aside from apicomplexans, it is the ciliates that include several established and emerging model systems (*Paramecium*, *Tetrahymena*, *Oxytricha*, *Euplotes*, *Stentor*). Except for MNAse-seq-based nucleosome positioning studies in *Tetrahymena* [179, 180], epigenomes have not yet been mapped in ciliates, but ChIP-seq has proven highly useful in understanding the unique biology of their chromosomes. One of the defining features of ciliates is their nuclear dualism. Ciliates have a somatic macronucleus (MAC), which arises from a germline micronucleus (MIC) through the elimination of internal eliminated segments (IESs; largely containing repetitive elements) from the MIC chromosomes. Macronuclear destined segments (MDSs) in the developing MAC are retained and stitched together into somatic chromosomes. In addition to the differences in sequence content, the MAC is also highly polyploid, with tens to thousands of copies of each chromosome. How this transformation is accomplished varies between different ciliate lineages, but common between all of them is the major role that small RNAs play in the process.

In *Paramecium* and *Tetrahymena*, the MIC genome is fully transcribed from both strands, generating double-stranded RNA (dsRNA), from which small RNAs (called scnRNAs) are produced by Dicer proteins, loaded onto Piwi-family Argonaute proteins and exported to the old MAC. There they are ‘scanned’ against transcripts generated from the rearranged chromosomes present in it. If matches to scnRNAs are found, they are degraded, leaving only scnRNAs targeting IESs, which are then exported to the developing MAC, where they guide the excision of IESs. Once initial IESs are excised, a secondary population of small RNAs is produced, as a feed-forward loop to ensure the complete elimination of IESs from the developing MAC. In *Paramecium*, where IES excision is mostly precise, so-called iesRNAs are generated through the formation of circular DNA segments from the excised IESs, which are then transcribed bidirectionally resulting in dsRNAs further processed into iesRNAs [181]. But in *Tetrahymena*, where IESs are mostly in intergenic regions and excisions can be imprecise, the mechanism is chromatin-based. Early scnRNAs guide the establishment of heterochromatin (marked by H3K9me3 and H3K27me3) and recruitment of HP1 (heterochromatin protein 1 [182, 183]) homologs along IESs; heterochromatin formation spreads in *cis* and results in the production of late scnRNAs. Mechanisms for restricting its spreading into MDSs must exist, however, and ChIP-seq maps have helped establish that this is accomplished through the binding of a different HP1-like protein, Coi6p, together with several interaction partners and histone demethylases to MDSs [184, 185]. This mechanism appears to be analogous to how insulator elements block the spreading of heterochromatin in other eukaryotes.

Genome rearrangement is most striking in *Oxytricha* and related spirotricheans. In these organisms, MDS segments in the MIC are not collinear and have to be unscrambled during MAC formation (a process apparently guided by long RNA templates from the old MAC). Remarkably, the end result of unscrambling is the formation of nanochromosomes just a few kilobases long, typically containing only a single gene [186, 187]. The structure of nanochromosomes poses a number of interesting questions regarding the organization of chromatin in these nuclei, as they contain extremely short (only a few tens of bases) stretches of DNA between TSSs and TTTs and telomeres. How transcription factor binding around promoters, nucleosome positioning and histone marks in *Oxytricha* deviate from the typical eukaryotic norms remains to be studied in the future. Of note, nanochromosomes seem to have evolved independently on multiple occasions within the ciliates [188, 189], but their features have not been studied outside of *Oxytricha*.

Poorly studied groups with radically different chromatin organization

It is the third major alveolate lineage, the dinoflagellates, where the most striking nuclear organization among all eukaryotes that have been studied in any detail is to be found, so divergent in fact, that decades ago it was thought that they may represent an intermediate state between prokaryotes and eukaryotes [190]. The packaging of chromatin by nucleosomes is a universal eukaryotic feature except for special cases such as mammalian sperm cells, during the development of which histones are largely replaced by protamines [191]. However, in dinoflagellates, chromatin appears to not be packaged by histones, and chromosomes are permanently condensed throughout the life cycle, existing in a liquid crystalline state [192, 193]. Dinoflagellates do, however, possess histone genes, although they are highly divergent in sequence, and they do also have a number of traditional chromatin modification enzymes and remodeling factors, including the FACT histone chaperone, which plays a major role in cotranscriptional nucleosome disassembly in all eukaryotes [106]. Thus, histones likely are of some importance for dinoflagellate chromatin biology, and transcription through nucleosomal arrays is probably happening, but nothing is known about the group beyond that. Research on dinoflagellates has been hampered by the massive size of their genomes (usually much bigger than those of humans), which has until recently precluded genome assembly efforts. But sequences for several *Symbiodinium* species, whose genomes are relatively small, have recently become available [194, 195], and the mysteries of these nuclei should be resolved in the future through the application of functional genomic tools.

Another curious case of likely highly divergent chromatin structure is provided by nucleomorphs. Secondary endosymbiosis, i.e. the engulfment of one photosynthetic eukaryote by another, has happened on numerous occasions in evolution [113], and it usually leads to the eventual disappearance of the endosymbiont's nucleus, leaving only the plastid. However, on two independent occasions, in the chlorarachniophytes and the cryptophytes (Figure 4), the nucleus has been retained in the form of a nucleomorph, with chlorarachniophyte nucleomorphs being of green algal and cryptophyte ones of red algal origin. Nucleomorph genomes are extremely small (a few hundred kilobases in length) and highly compact, and exhibit a strikingly convergent organization in both lineages [196]. Interestingly, nucleomorph histone tails are divergent in chlorarachniophyte nucleomorphs (and more conserved in cryptophytes) [197]. Key histone modifications ancestrally involved in the transcriptional cycle in eukaryotes have been lost, presenting numerous questions regarding the conservation of the corresponding biochemical processes in these nuclei.

The deep evolution of chromatin organization in the context of current knowledge

While the precise rooting of the eukaryotic tree of life and functional genomic studies of early diverging lineages, particularly in excavates, may change the picture significantly, we can, based on current knowledge, draw some conclusions about LECA's chromatin and about the evolution of chromatin biology following the eukaryotic radiation. A core set of histone marks and associated processes is likely ancestral to all eukaryotes. These include the association of histone acetylation with open chromatin (which may follow simply from the resulting relaxation of the electric charge of histone molecules and not be position-specific), the demarcation of promoters by H3K4me3 and the H2A.Z histone variant, the transcription-associated deposition of H3K36 methylation and the use of H3K9 and H3K27 methylation for gene repression and heterochromatinization. Given the close connection between heterochromatin and small RNA-mediated mechanisms for TE suppression [198–200], found throughout diverse eukaryotes, it seems plausible that the latter were part of the solution to the problem of keeping TEs in check in LECA too. It is likely that numerous other histone marks also date back to LECA, but currently available data encompassing a sufficient diversity of lineages are still limited to only a few modifications. It is also likely that different eukaryotes possess diverse repertoires of lineage-specific marks, especially on the faster evolving H2A, H2B and H1 histones, remaining to be understood in depth in the future.

The conservation of histone marks and their functions does not, however, appear to imply strict conservation of chromatin states, and divergent modes of partitioning the genome have evolved, as shown by the examples of Trypanosomatids, *Plasmodium*, *Chlamydomonas* and others. Some evolutionary plasticity with respect to chromatin organization appears to exist, considerable in some lineages, with a common set of chromatin states (as defined by a limited set of histone modifications) shared by most eukaryotes, and additional lineage-specific states appearing during evolution, through novel combinations of histone marks, addition of novel histone variants and other chromatin-associated proteins and even DNA modifications The diversity and functional significance of these variations remains to be characterized in detail.

Also not yet fully resolved at present is the question of the relationship between organismal complexity and regulatory architecture. As discussed above, enhancers in plants are still poorly understood, and no data are available for other complex multicellular lineages such as red and brown algae. Relevant data do, however, exist regarding metazoans and some of their closest relatives.

The origins and evolution of metazoan regulatory architecture

The holozoan lineage comprises metazoans, and their closes unicellular relatives, which are, in order of phylogenetic proximity, the choanoflagellates, the filastereans, the ichtyosporeans and corallochytrids (Figures 4 and 5). The ancestor of metazoans was most likely a colonial organism similar to choanoflagellates. The striking resemblance between choanoflagellates and the choanocyte cells of sponges was noticed as far back as the mid-19th century [212] and has been extensively confirmed by modern genomics [213].

The availability of genome sequences from these groups has allowed the tracing of the evolution of the metazoan transcription factor toolkit, which appears to have been assembled gradually but beginning before the appearance of multicellular animals, with many TF families already present in unicellular holozoans [214]. Their genomes are fairly compact (but not as compact as those in yeasts such as *S. cerevisiae*) leaving open the question when exactly enhancers and other conserved features of metazoan genomes appeared in the evolution of animals.

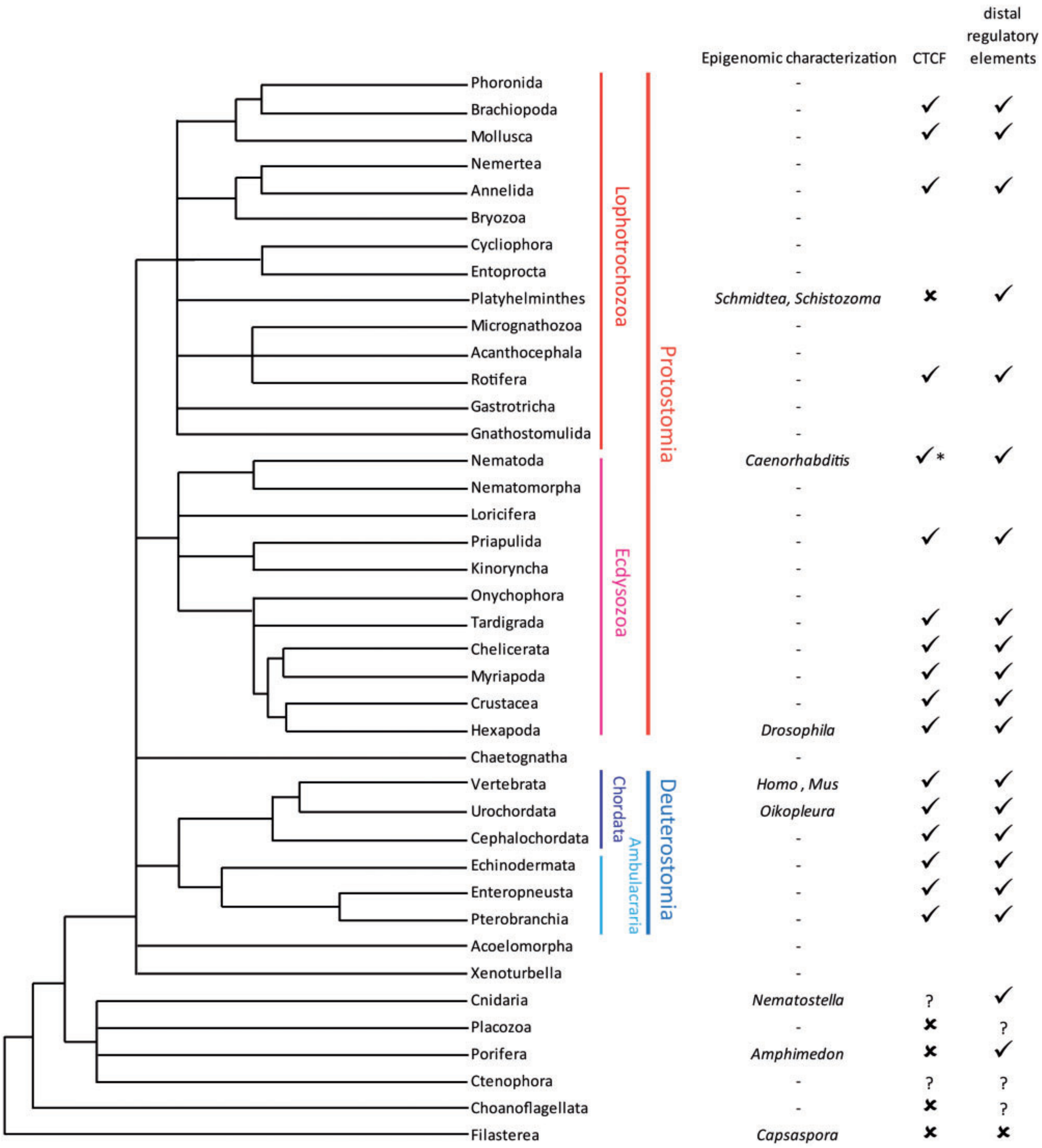
A comprehensive mapping of histone marks and open chromatin regions was carried out in *C. owczarzaki*, the best studied filasterean [215]. It did not find apparent distant regulatory elements, with potential such regions being promoter-proximal. Remarkably, putative filasterean regulatory elements appear to smaller in size than in metazoans, indicating that during metazoan evolution, both their combinatorial and individual complexity increased. Curiously, the repressive H3K9me3 and H3K27me3 modifications were not observed in *Capsaspora*.

The early non-bilaterian metazoan groups include the Placozoa, sponges/Porifera, Cnidaria and Ctenophora. Owing to their deep and probably rapid divergence, it has been difficult to conclusively determine their relationships [201–206]. Different studies have suggested strongly conflicting topologies: rootings of the metazoan tree between Ctenophora and all other phyla, between Porifera and all other groups, within Porifera and others have all been proposed.

Recently, ChIP-seq has been used to map histone modifications in the sponge *Amphimedon queenslandica* [216] and the chidarian *Nematostella vectensis* [217]. These studies revealed the presence of distal regulatory elements and an overall typically metazoan regulatory architecture. Therefore, metazoan enhancer elements seem to have originated somewhere between filastereans and poriferans/cnidarians, but when and how exactly remain to be answered by analysis of other non-bilaterians and in choanoflagellates, and by the hoped for future firm establishing of the correct topology of the metazoan tree.

Interestingly, while they do seem to have enhancers, it is not clear whether cnidarians and sponges have typical insulator elements. The phylogenetic distribution of CTCF, the classic insulator protein in metazoans, appears to be restricted to bilaterians, with some possibility for the existence of distant homologs in Cnidaria and Ctenophora [218, 219] (Figure 5).

CTCF is also missing, as a result of secondary losses, in some nematodes, including *C. elegans*, as well as in flatworms. *Caenorhabditis elegans* does possess distal enhancers [220], but it appears to, remarkably, lack TADs on its chromosomes, perhaps as a consequence of losing CTCF, except, even more



	Epigenomic characterization	CTCF	distal regulatory elements
Phoronida	-		
Brachiopoda	-	✓	✓
Mollusca	-	✓	✓
Nemertea	-		
Annelida	-	✓	✓
Bryozoa	-		
Cycliophora	-		
Entoprocta	-		
Platyhelminthes	*Schmidtea, Schistozoma*	✗	✓
Micrognathozoa	-		
Acanthocephala	-		
Rotifera	-	✓	✓
Gastrotricha	-		
Gnathostomulida	-		
Nematoda	*Caenorhabditis*	✓*	✓
Nematomorpha	-		
Loricifera	-		
Priapulida	-	✓	✓
Kinoryncha	-		
Onychophora	-		
Tardigrada	-	✓	✓
Chelicerata	-	✓	✓
Myriapoda	-	✓	✓
Crustacea	-	✓	✓
Hexapoda	*Drosophila*	✓	✓
Chaetognatha	-		
Vertebrata	*Homo , Mus*	✓	✓
Urochordata	*Oikopleura*	✓	✓
Cephalochordata	-	✓	✓
Echinodermata	-	✓	✓
Enteropneusta	-	✓	✓
Pterobranchia	-	✓	✓
Acoelomorpha	-		
Xenoturbella	-		
Cnidaria	*Nematostella*	?	✓
Placozoa	-	✗	?
Porifera	*Amphimedon*	✗	✓
Ctenophora	-	?	?
Choanoflagellata	-	✗	?
Filasterea	*Capsaspora*	✗	✗

Figure 5. Relationships between metazoan phyla and their closest relatives and the extent of their epigenomic characterization. Taxons for which the correct phylogenetic relationships is still to be conclusively established (such as the position of Ctenophora relative to other metazoans [201–205], the monophyly of poriferans and the overall relationships between non-bilaterian clades [206] and the placement of Chaetognatha [207], Acoelomorpha [208, 209], Xenoturbellida [209, 210] and others) are incorporated as unresolved branches on the cladogram, with Orthonectida and Dicyemida (tentatively placed in Lophotrochozoa [211]) being omitted. Phyla in which at least some epigenomic studies have been carried out are indicated on the right-hand side with the corresponding genus names for the main taxa studied. Lineages without published sequenced genomes have been left blank. Note that CTCF has been lost in some nematodes such as *C. elegans* but is present in other members of the phylum.

remarkably, for chrX. On chrX the dosage compensation complex, a condensin complex that localizes to sequence-specific recruitment elements, plays a role analogous to the CTCF/cohesin combination in other bilaterians. How exactly enhancers are restricted to their cognate promoters on autosomes remains to be fully understood, as is any potential relationship between the loss of TADs and the organization of many *C. elegans* genes into polycistronic operons.

In the evolution of insects, on the other hand, the array of insulator proteins has expanded [221]. Six such factors in addition to CTCF operate in *Drosophila*. The mechanisms of metazoan insulation thus seem to exhibit a considerable degree of plasticity, but to fully understand the functional constraints on them and their relationship with 3 D nuclear organization, they need to be studied in representative species from a much wider sampling of animal phyla (Figure 5) than the limited data that exist at present includes.

In addition to vertebrates, insects, nematodes, *Amphimedon* and *Nematostella*, epigenomic studies have been carried out in a couple flatworms and in the urochordate *Oikopleura dioica*.

An important result to emerge from histone marks profiling in the parasitic trematode *Schistosoma mansoni* [222] and in the planarian *Schmidtea mediterranea* [223] is the existence of bivalent promoters, simultaneously marked by H3K4me3 and H3K27me3 in flatworm stem cells. These bivalent structures were first identified in mammalian embryonic stem cells [224], where they serve to poise the expression of developmentally regulated genes, and are resolved toward an active or repressed state with the progression of embryonic development. The presence of such domains in flatworms suggests deep conservation of this feature of animal stem cells.

The highly derived *Oikopleura* genome is intriguing for being extremely compact, at only 70 Mb, compared with the genomes of other chordates, which are at least an order of magnitude larger, and for genes being organized into operons. However, the profiling of 19 histone modifications and CTCF in *Oikopleura* revealed generally conserved epigenomic organization.Distal enhancers and insulators and typical chromatin states are observed, the main difference being the restricted extent of heterochromatin (because of the compacted nature of the genome) and sex-specific chromatin states on the Y chromosome [225].

Overall, the properties of metazoan chromatin present a pattern of general conservation, but, as is the case with the deeper evolutionary splits between protist groups, some notable divergence cases are observed too. The most comprehensive comparison of chromatin organization in metazoans was carried out by the ENCODE and modENCODE consortia using hundreds of human, fly and worm data sets [12, 13, 226]. It revealed an overall conservation of histone modification patterns around regulatory elements but also some differences. Examples of the latter include the enrichment of H3K23ac around expressed promoters in *C. elegans*, but across both active and inactive gene bodies in *Drosophila*, and the association of H4K20me1 with both active and silent genes in humans but only with expressed genes in fly and worm. Perhaps, the most significant such difference is the colocalization of the heterochromatin marks H3K27me3 and H3K9me3 in worms, in contrast to the distinct domains that they tend to form in mammals and flies [226].

How widespread such differences are between different metazoans and whether they are restricted to these marks awaits further research. The systematic functional genomic characterization of species from all phyla using comprehensive arrays of histone mark profiles should provide a much fuller and detailed picture of the evolution of gene regulation in metazoans.

Remaining technical challenges

While ChIP-seq has been successfully adapted to a wide diversity of lineages in the past decade, a number of technical challenges remain to be overcome to fully unlock its potential for understanding their biology, with many of the issues being specific to particular lineages.

For example, a major limitation to functional genomic studies of chromatin in clades such as the apicomplexans, ciliates, *Dictyostelium* and others is the extremes of GC-composition biases that can be found outside mammals. Such genomes can often approach or exceed an AT content of 80%. On its own this would not be an enormous problem; however, GC content is not uniform across the genome, with exons of protein-coding genes typically being relatively GC-rich (because they are under stronger purifying selection at the codon level) in contrast to the even more extremely AT-rich intronic and intergenic regions. Under such conditions, polymerase chain reaction (PCR)-based methods for sequencing library generation produce strong biases toward exons, making data generated in such ways difficult to interpret, as the true ChIP signal is often almost completely overwhelmed by the PCR bias. For example, it has been proposed that nucleosome positioning in the MAC of *Tetrahymena* is strongly driven by sequence features, in particular exons and GC content [179], but such a result is also what would be expected if the observed effect is because of PCR biases, and PCR is not even the only problematic step in a ChIP protocol, as cross-link reversal, typically carried out at 65°C, can bias against extremely AT-rich sequences, which become denatured even at such relatively low temperatures and are therefore underrepresented by library building protocols dependent on double-stranded DNA ligation steps. Linear amplification methods that resolve these issues have been developed [227–230], but they have not been widely adopted yet.

Another significant lineage-specific issue concerns background and artifactual signals in poorly studied (and often, not well assembled) genomes of emerging model and nonmodel species. ChIP-seq data sets in traditional model systems are now understood to often contain artifacts of several sources, such as a bias toward open chromatin regions [231–234] and the presence of strong but artifactual enrichment over so-called 'blacklist' regions [235]. Historically, it took several years for these sources of bias to be understood, classified and corrected for, and for a set of standard practices to be compiled [76]. Some sources of artifacts (such as the bias toward open chromatin) are likely to be common to all species, but nevertheless such accumulated experience is not available for nonmodel systems, and new genomes can always present unexpected surprises in terms of sources of artifacts [236]. It is not clear at present to what extent published results in insufficiently well-characterized species are affected by such factors.

It is also not clear how generally applicable analysis tools are, as evidenced by the discrepancy between the published metaanalyses of transcription factor occupancy divergence in metazoans. ChIP-seq processing and analysis pipelines have been designed primarily with mammalian genomes in mind, with their particular repeat and background structure, but most genomes have different properties. Strong transcription factor peaks are probably successfully captured regardless of these issues, but the impacts of the convolution of algorithms and genome and background properties on the overall sets of identified occupancy sites are currently not well understood.

A more general challenge is the lack of purified uniform cell lines for most nonmodel species. As discussed above regarding plants, this confounds interpretation, especially of histone modification data sets, because of the mixing of different cell types together in the same ChIP reactions. Such issues may exist even for single-celled organisms if substantial heterogeneity of cell states exists within the experimental population. In the light of these considerations and the ones regarding experimental artifacts outlined above, results reporting unexpected combinations of histone marks such as bivalency between H3K9me3/H3K27me3 and marks usually associated with active transcription may have to be taken with a grain of salt until independently verified. Of note, even H3K4me3/H3K27me3 bivalent domains in mammalian embryonic stem cells were not truly proven not to be the result of heterogeneity between cells or between individual chromosomes within the same cells until methods for directly mapping the combinatorial modification states of individual nucleosomes emerged recently [237]. Such approaches should prove invaluable in resolving chromatin state conundrums in other species.

Finally, reagent availability remains a major remaining challenge for ChIP-seq studies even in human and mouse cells, and it is even more of an issue in other organisms. High-quality reliable antibodies for most human transcription factors are not available, which has necessitated the application of epitope tagging methods toward the mapping of their occupancy sites. CRISPR/Cas9-based genome editing has eventually allowed such tagging to be carried out as knock-ins into the native locus [238], mitigating against possible overexpression artifacts. ChIP-grade antibodies are even less accessible for nonmodel organisms; thus, epitope tagging is likely to be the approach of choice in such cases, with its success depending on the particular details of the system in question.

Epitope tagging is a viable solution for transcription factors and other proteins, but perhaps even more substantial is the challenge of obtaining reliable histone modification antibodies. Most such antibodies have been polyclonal (and therefore nonrenewable), although monoclonal ones are becoming increasingly available, and of varying quality [239, 240]. Histone proteins are some of the most conserved in all eukaryotes, but some minor differences in histone tails nevertheless exist, and, as discussed above, in some of the most interesting groups, histones are in fact fairly divergent, making existing antibodies either inapplicable or unreliable. New reagents may have to be generated and extensively characterized in such cases; developing reliable systems for the large-scale screening and validation of reagents would prove highly useful toward that goal.

Conclusions

It may be overused at this point, but the saying that nothing in biology makes sense except in the light of evolution remains as true as ever, and it also applies just as strongly to transcriptional regulation and chromatin biology. If we are to truly grasp these aspects of our biology, we need to understand how and why they evolved to their current state. High-throughput sequencing has been a true game changer in this quest, by making accessible every organisms on the planet to functional genomic techniques. The past decade has seen many exciting insights and advances resulting from its application, with many more certain to come in the coming years, as we make our way around the branches of the eukaryotic tree.

Key Points

- Comparative analyses of transcription factor occupancy across species illuminate the mechanisms of and the driving forces behind the evolution of gene regulatory elements
- Access to nonmodel and emerging model organisms enables the mapping of GRN rewiring during evolution
- Epigenomic studies across the tree of life reveal the conservation and divergence of general regulatory mechanisms.
- Much of the known eukaryotic diversity is still to be sampled, including some of the most intriguing groups; comparative transcription factor occupancy studies have also been limited in scope so far.
- Unresolved technical challenges remain to be overcome to fully empower research in the field.

Funding

This material is based on work supported by National Institutes of Health awards (grant numbers 1DP2OD022870-01, 1UM1HG009436 and 1U01HG009431).

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