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How do regulatory networks evolve and expand throughout evolution?

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Throughout evolution, regulatory networks need to expand and adapt to accommodate novel genes and gene functions. However, the molecular details explaining how gene networks evolve remain largely unknown. Recent studies demonstrate that changes in transcription factors contribute to the evolution of regulatory networks. In particular, duplication of transcription factors followed by specific mutations in their DNA-binding or interaction domains propels the divergence and emergence of new networks. The innate promiscuity and modularity of regulatory networks contributes to their evolvability: duplicated promiscuous regulators and their target promoters can acquire mutations that lead to gradual increases in specificity, allowing neofunctionalization or subfunctionalization.

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Introduction

Even in closely related species with highly similar genome sequences, gene expression patterns can be quite different [1,2]. This divergence in gene expression and regulation has been postulated to play a major role in evolution and is believed to be one of the primary sources of phenotypic variation between species [3–11].

Changes in transcriptional regulation can occur at different levels: through changes in DNA binding sites located around or inside target genes (so-called *cis* mutations) or by changes in *trans*, that is, differences in the abundance or activity of transcription factors (TF) — regulatory proteins that recognize and bind specific *cis*-regulatory sequences [12]. Comparative genomics studies have indicated a considerable amount of *cis*-regulatory sequence variation between species [13–15] and it has been argued for a long time that changes in *cis*-regulatory elements underlie most of the observed changes in transcriptional regulation [8,16,17]. Mutations in transcription factors were considered to be an unlikely source of variation, mostly because of the possible negative pleiotropic effects such mutations can evoke [16,18]. A mutation in a protein-coding region of a transcriptional regulator may simultaneously affect multiple target genes of this regulator (and thus can have widespread detrimental effects), whereas a mutation in a *cis*-regulatory element would only cause changes in the expression pattern of this particular gene and might thus be better tolerated by the cell [8].

Recent studies indicate that mutations in regulatory proteins may be more common than previously appreciated [19–21]. Moreover, these changes can play a prominent role in regulatory network evolution by altering expression, molecular interactions and post-translational modifications of the regulator [22–24,25••]. In keeping with this, it is well known that several transcription factors have DNA binding domains belonging to large paralogous families, although the transcription factors can differ extensively in sequence [26]. Hence, evolution through TFs appears to be a successful strategy for regulation of gene expression, although the exact nature and extent to which this mechanism has contributed to gene expression regulation has remained unclear [27••].

Duplication of a gene encoding a transcription factor was suggested to be the least complicated way for a transcription factor to evolve without significantly decreasing the fitness of an organism [28]. For example, one of the gene copies can retain the ancestral function (thus avoiding any negative pleiotropic effects), while the other is released from negative selective pressure, can mutate and in some cases evolve a different function [23]. Indeed, many transcription factors are known to arise by gene duplication, and a number of them have acquired a new function [29–31]. In addition, duplication of target genes — both small-scale and whole-genome — and subsequent diversification of the resulting duplicates have been shown to

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be important contributors to the evolution of transcriptional networks [1,32•,33,34]. Gene duplication has been widely recognized as the prime sources of novel genes in genomes: 50% of the genes in prokaryotes and around 90% of the genes in eukaryotes are the result of duplication [31,35–39]. Since these new genes need to be regulated correctly, the adaptation of gene regulation (and thus of regulatory networks) is particularly important [1,2,31].

In this review, we discuss recent insights in how duplication of a transcription factor gene can propel the rewiring and expansion of regulatory networks. We specifically focus on how gene duplication and subsequent divergence allows circumventing the potential negative effects associated with pleiotropy of a single copy transcription factor that could lead to misregulation of target genes.

Gene duplication is an important driver of regulatory network evolution

Gene duplication is increasingly recognized as the chief mechanism underlying evolutionary innovation. Whereas the exact evolutionary pathways and forces are often complex, a simplified model explains how duplication of a gene allows one of the two copies to retain the ancestral function whereas the other copy is relieved from negative selection and is allowed to mutate and explore novel functions [35,40]. Such duplication events are often associated with genes encoding enzymes, but they may also occur for the regulatory genes [23,41,42••,43••,44]. Duplication of a transcriptional regulator, its target gene(s) or duplication of both may establish novel interactions in the regulatory network or even lead to the emergence of a novel regulatory cascade [31] (Figure 1).

Comparative genomics reveals that many transcription factors, as well as their target genes, arose by duplication [29–31]. After duplication of a regulatory gene, the two identical copies are likely redundant, recognizing the same binding sites, responding to the same signal and, therefore, regulating the same set of target genes as the ancestral pre-duplication regulator. During subsequent divergence, one or both of the duplicated transcription factor paralog genes may acquire mutations that change the DNA binding domain and switch to regulating different target genes [42••]. Alternatively, the two paralogs can continue to regulate the same target genes as their ancestor but respond to a different signal, or bind different protein partners (cofactors) [45••,46,47]. A seemingly frequently occurring scenario is that of subfunctionalization (or 'division of labor'), where each paralog evolves to regulate a subset of the target genes originally regulated by the single ancestral transcription factor [40,45••,48] (Figure 2). Such subfunctionalization might not seem to contribute much to evolution, but in reality, division of labor among paralog regulators followed by specific mutations may allow a more precise and specific regulation of target genes. Another possible fate for duplicated genes is neofunctionalization, where one of the duplicates acquires a novel function that was not present in the pre-duplication protein. Such neofunctionalization could explain the emergence of completely new pathways that regulate new gene functions (Figure 2).

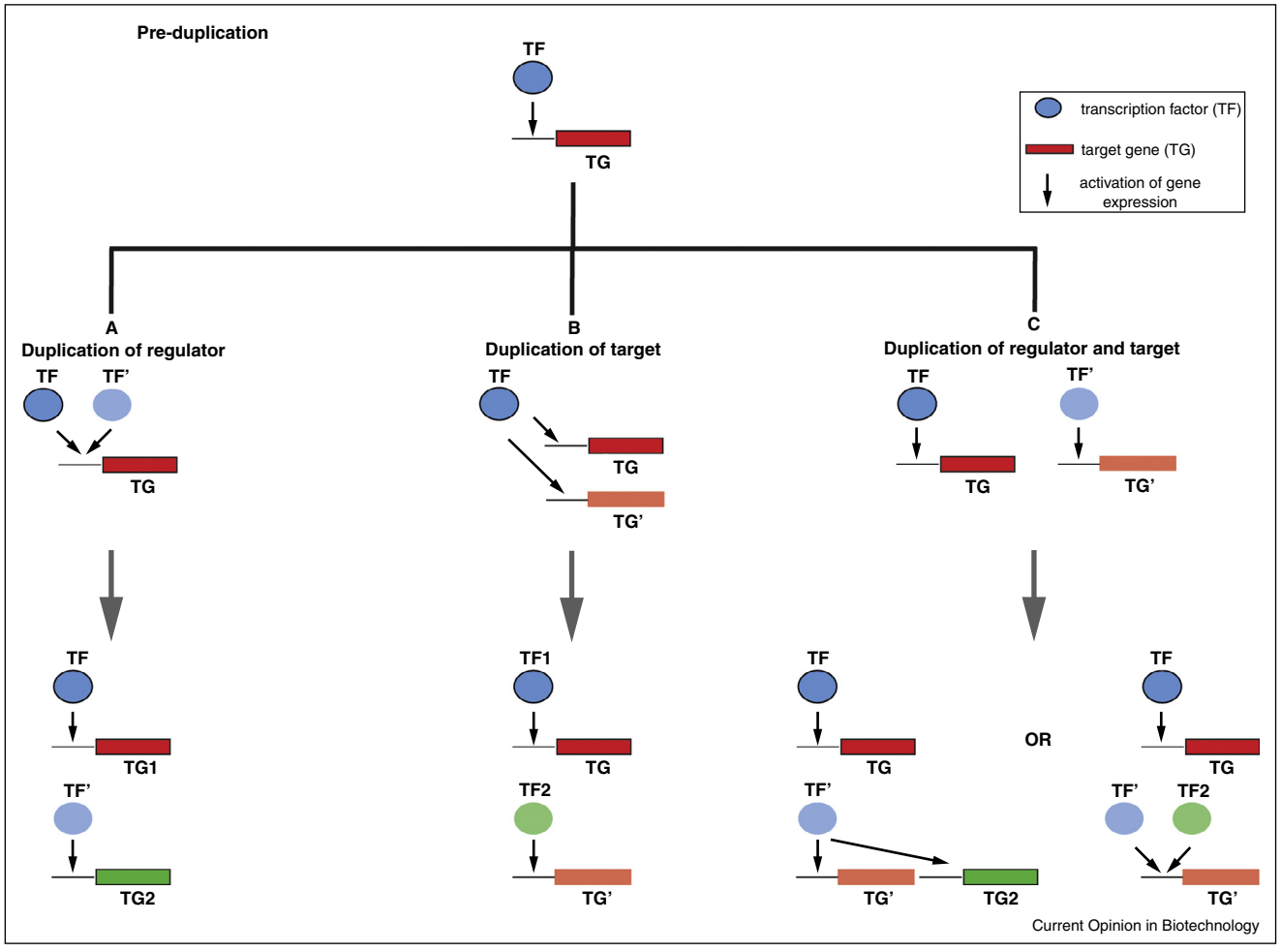
Interestingly, despite the multitude of examples of how gene and whole-genome duplications have contributed to the evolution and expansion of gene regulatory networks, the exact molecular details and mutational pathways are not yet well understood. How can two identical transcription factors gradually diverge into two distinct proteins, each responding to a specific input and each regulating a specific set of targets? It is important to note that this is a complex problem, because evolution generally happens gradually, and during the entire process, fitness valleys associated with misregulation of target genes should be avoided. In the following paragraphs, we describe the results of recent studies that have elucidated mutational pathways underlying the evolution of duplicated transcription networks. Together, these studies begin to shed light on how transcriptional regulation evolves.

Subfunctionalization of duplicated transcriptional networks

Many transcription factors interact with a multitude of other proteins and also with different DNA motifs. In case of subfunctionalization, loss of some of these ancestral interactions in the resulting paralogs can lead to competitive interference between the two paralogs, a situation also referred to as paralog interference [41,45••]. Imagine for example a transcription factor that needs to bind a specific cofactor as well as DNA. If, after duplication, one of the paralogs acquires mutations that impair cofactor binding but do not affect DNA binding, then this paralog will reduce transcriptional activity of the other copy by competing for DNA binding. Baker *et al.* demonstrated the negative effects of such paralog interference in the case of a fungal MADS-box transcriptional regulator [45••]. Duplication of the ancestral transcription factor resulted in two paralogs that each control expression of a specific subset of targets of the ancestral regulator [45••]. The two paralogs diverged by acquiring specific mutations that altered cofactor binding preference. In a clever set of ancestral gene reconstructions, the authors showed that closely after the duplication the regulatory network was indeed experiencing paralog interference. Several specific subsequent mutations that weakened the DNA-binding affinity of one of the paralogs were required to resolve paralog interference.

However, in some cases, paralog interference can also be an integral part of the emergence of new regulatory loops.

Figure 1



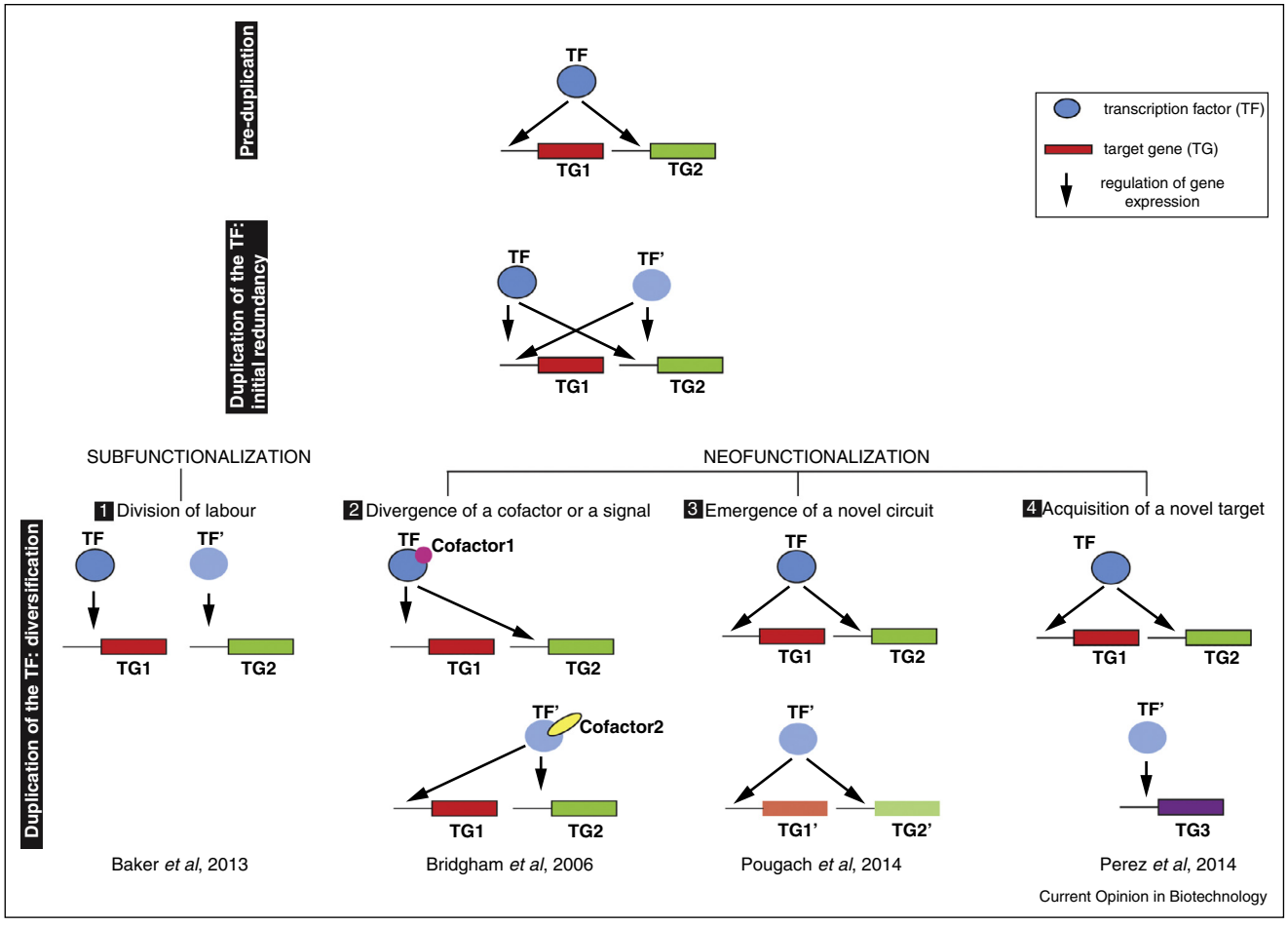
Duplication events are important drivers of regulatory network evolution. **(a)** Duplication of a transcription factor (TF) initially leads to both regulators (TF and TF′) controlling the same target gene (TG). Subsequent divergence can cause one of the paralogs (TF′) to acquire different target(s) (TG2) (see also Figure 2). **(b)** After duplication of a target gene (TG), both copies (TG and TG′) are initially regulated by the same transcription factor (TF). Divergence can cause the duplicated target to become regulated by a different transcription factor (TF2). It should be noted that both in scenario a and b, paralog divergence does not necessarily happen after duplication: the two copies of the regulator can regulate the same target genes (a); and the two copies of the target can be regulated by the same transcription factor (b) (not depicted). Alternatively, the duplicated copies can also be lost from the genome (not depicted). **(c)** Concerted duplication of a transcriptional regulator and its target (e.g., following whole-genome duplication events) can expand the regulatory network through divergence of target and/or regulator.

Bridgham *et al.* showed that in the evolution of steroid hormone receptors, mutations in one of the paralogs after duplication abolished its activation by a specific ligand [41]. Upon ligand binding, these receptors normally undergo a conformational change that allows them to recruit cofactors that facilitate transcription of their targets. Mutations affecting ligand binding caused one of the paralogs to evolve a new function: it acted as a repressor for the transcriptional activity of the other paralog by competing for the same DNA binding site (neofunctionalization through loss-of-function). This in turn allowed for fine-tuning of expression of the target genes of these transcriptional regulators.

Neofunctionalization of transcriptional networks

While subfunctionalization of duplicated networks clearly plays an important role in the evolution of gene regulation, other cases, such as the one described in the paragraph above, involve the emergence of novel functions (neofunctionalization). This is often the case when novel gene functions arise, since this likely requires the evolution of new regulatory networks to ensure proper regulation of the novel functions. A recent study of the evolutionary history of the *MAL* gene family in yeast reveals in detail how such 'neofunctionalization' of networks can occur. In this case, duplication and

Figure 2

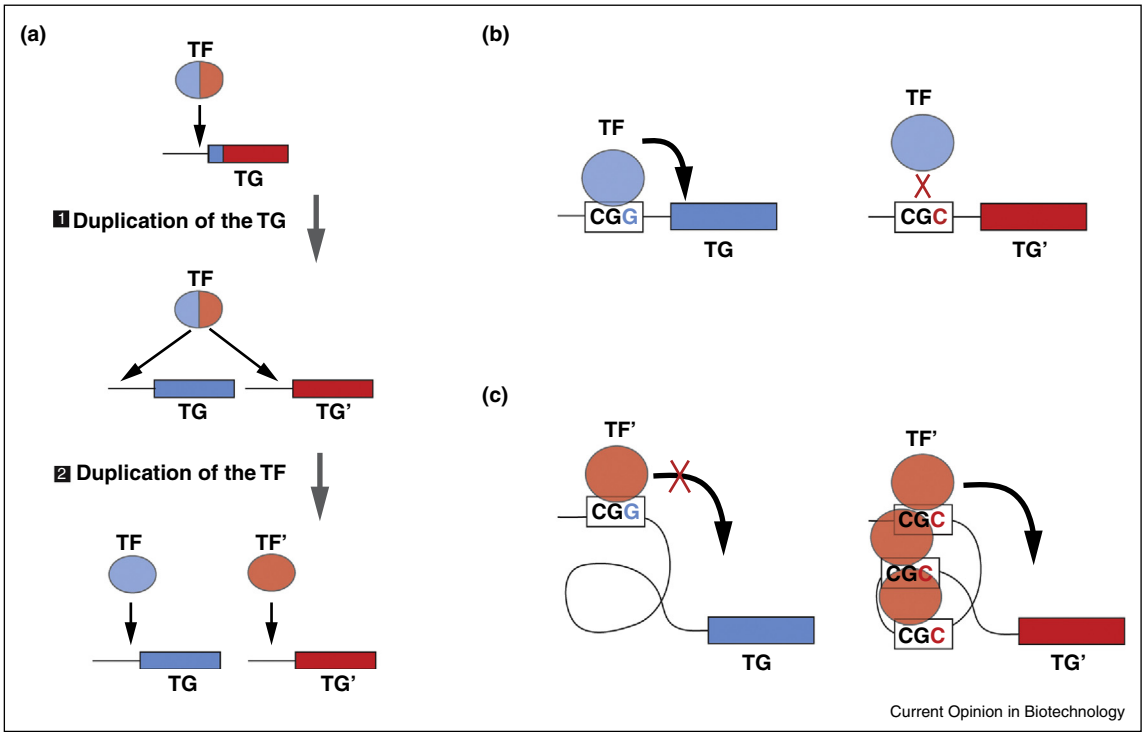


Transcription factor duplication can lead to expansion of the existing regulatory network or emergence of a novel network. Early after the duplication of the ancestral transcription factor (TF) the resulting paralogs are redundant and regulate the same set of target genes (TG1 and TG2). Subsequent diversification can occur through subfunctionalization (or division of labor, **1** [45]) or neofunctionalization (**2**–**4**). (2) Some transcription factor paralogs diversify at the level of signal or cofactor recognition, so that they still regulate the same set of target genes but are activated by different cofactors [46]. (3) Duplication of the target genes together with the regulator can lead to emergence of a novel network, where each transcription factor paralog activates its own set of target gene copies (TG1, TG2 and TG1′ and TG2′ are regulated by TF and TF′ respectively) [43]. (4) Some transcription factor paralogs can acquire regulatory control over new target genes that were not part of the ancestral network before duplication (TG3) [42].

neofunctionalization of a hydrolytic enzyme was followed by duplication of its transcription regulator gene, which eventually allowed the emergence of a novel regulatory circuit [43••]. The *MAL* gene family is involved in the uptake and metabolism of complex carbohydrates such as maltose, palatinose and other α-glycosides. It consists of three subfamilies: *MALS*, *MALT* and *MALR* [44,49]. The *MALT* subfamily encodes a set of different transporters that import a range of disaccharides, which are subsequently hydrolyzed by a set of specific MalS glycosidases. Some of the intracellular disaccharides likely bind to the so-called MalR regulator proteins, and this complex regulates expression of *MALS* and *MALT* genes [49].

The present-day MalS enzymes originated from a single-copy ancestral gene through multiple duplication events followed by functional diversification. These events created two main groups of MalS enzymes, each with its own specific substrate preference: one group can hydrolyze carbohydrates with an α 1-4 glycosidic bond, whereas the other group can break down disaccharides with an α 1-6 bond [44,49]. Two independent regulatory networks, each one dedicated to regulating one class of MalS enzymes, emerged through duplication and diversification of a single copy transcription factor [43••,44] (see Figure 3). This ancestral regulator displayed broad binding site specificity and was able to bind two different DNA motifs present in the promoters of the two substrate classes. After duplication, one paralog evolved increased binding specificity for one of the motifs, whereas the other paralog acquired mutations that reduced its affinity for specific sequences, so that it now needs multiple

Figure 3



Evolution of MAL gene regulatory network. **(a)** In case of the *MAL* genes, the ancestral transcription factor (TF) was regulating expression of the ancestral hydrolytic enzyme (target gene, TG). This ancestral transcription factor was promiscuous and could be activated in presence of two classes of disaccharides (each activity is depicted as either red or blue), while the ancestral enzyme could effectively hydrolyze only the red disaccharides (with α 1-4 glycosidic bond), and had only minor activity for the blue disaccharides (α 1-6 glycosidic bond). (**1**) Duplication of the target gene led to significant improvement of the blue hydrolytic activity and was followed by the duplication of the transcription factor gene (**2**). One of the emerging transcription factor paralogs took regulatory control over the target gene with the novel activity, thus leading to emergence of a novel regulatory network (blue). The effective disentanglement of the red and blue regulatory networks was possible because the blue regulator lost the ability to bind to the promoter regions of the red target genes and can only bind its own blue target genes. The opposite is true for the red regulator. The details of the mutations that lead to these changes are depicted in panels b and c. **(b)** The blue regulator acquired a specific mutation in its DNA binding domain that prevents it from binding CGC motifs present in the promoters of the red target genes, but allows binding CGG motifs from the promoters of the blue target genes. **(c)** The red regulator retained the ancestral ability to bind both types of motifs (CGG and CGC) but requires multiple binding sites to activate the expression. Blue target genes have only one binding site in their promoters and thus cannot be activated by the red regulator. For more information, see [43].

binding sites before it can bind (see also Figure 3). This example shows in detail how duplication and subsequent diversification of a promiscuous transcription factor allowed avoiding fitness valleys associated with paralog interference [43••]. In other words, the two networks were gradually disentangled so that in the end, each *MALS* gene is specifically activated by its own substrates. Interestingly, comparative genomics revealed that the changes in the amino acid sequence of the Mal target proteins preceded the changes on the regulatory level. Hence, in this case, functional divergence of the target genes probably acted as a driving force for the gradual disentanglement of the two regulatory networks. Moreover, the promiscuity of the ancestral, pre-duplication transcription factor, which was able to bind multiple binding motifs, was likely a key facilitator of the post-duplication divergence. Indeed, a gradual increase in the binding specificity of a duplicated promiscuous transcription factor may allow gradual disentanglement of the paralog networks.

A very recent study focused on the mechanism of binding site specificity changes in vertebrate steroid hormone receptor evolution [50••]. One of the duplicated transcription factors retained the ability to recognize the DNA motif preferred by the ancestral regulator, while the other acquired mutations and now specifically binds a different motif. In contrast to the *MAL* study discussed above, the ancestral regulator in this case was not promiscuous and could only bind one type of motif. Interestingly, negative protein–DNA interactions were intimately involved in the evolution of the new DNA binding specificity: after duplication, mutations in one copy compromised its binding to the original motif, whereas other mutations

caused a loss of unfavorable interactions with the new motif.

Another study of the divergence of a duplicated transcription factor elucidated the molecular details of how a duplicated transcription factor can acquire a set of completely novel target genes [42••]. By studying a group of *LYS* transcription factors in *Candida albicans* that arose through successive duplications, they demonstrated that these paralogs diverged through a combination of changes in DNA binding specificities (preferences for different DNA motifs, different spacing between motifs, preferences for direct versus inverted repeats), as well as different cofactor binding properties. Interestingly, *LYS14* of *Saccharomyces cerevisiae* is a key regulator of lysine biosynthesis, but in *C. albicans* none of the four *LYS14* homologs regulates the lysine pathway. Instead, they are involved in the white-opaque switching or proliferation in a mammalian host.

Interestingly, mutations that do not perturb the actual protein–DNA binding site can also affect binding affinity and specificity of transcriptional regulators. An elegant study on the transcriptional activator CAP demonstrated that mutations far away from the binding site altering protein internal dynamics can have a very strong effect on binding affinity [51]. Hence, many different types of mutations can lead to transcriptional regulator divergence.

Importance of promiscuity for overcoming the negative effects of pleiotropy

Initially considered to be mostly unwanted side-activities, recent studies are now highlighting the importance of promiscuity as crucial factor for driving evolution. The evolution of the Mal network is a good example (see above). Promiscuity seems to be a feature inherent to most proteins and enzymes: a large fraction of them have been reported to possess multiple activities [52,53]. Many transcription factors also show variation in DNA binding abilities: they can recognize both a (preferred) motif as well as an additional motif(s) [54,55,56•].

Recent studies demonstrate the ability of duplicated transcription factors to diversify and bind a number of different DNA motifs [55,56•]. A new study focuses on the family of fungal C_2H_2 Zn-finger transcription factors [56•]. These proteins can be divided into several 'specificity' groups, each group encompassing proteins with identical canonical DNA-recognition amino acid residues. Since these residues are the same for all proteins of the same group, these regulators are expected to bind the same DNA sequences. However, other non-canonical binding sites specific for each protein are also present. This way, each transcription factor recognizes not only a motif common to all members of the group but also its own preferred motif, which is not bound by other regulators from the same group. Such modularity of DNA binding specificity enables a protein to bind different sites while not affecting binding to the core DNA motif. This allows the transcription factor to overcome negative effects of pleiotropy. Moreover, this modularity is conserved since the divergence of *C. albicans* and *S. cerevisiae* and thus may be functionally important.

Another beautiful example of the importance of promiscuity as a promoter of evolvability of transcription factors is the LEAFY plant regulatory protein [25••], and see also recent discussion in [67,68]. LEAFY is a major regulator of flower development and cell division in land plants. It binds different types of DNA motifs in different plant species, and this specificity is determined by only a handful crucial residues. The radical shifts in DNA binding specificity of this regulator observed in the evolution from algae to land plants were enabled by passing through an intermediate, highly promiscuous form that was able to bind all three types of motifs present in modern-day plant species. Interestingly, this promiscuous state is still preserved in hornwort, which is taxonomically situated somewhere between algae and higher land plants. Together, these results illustrate how a promiscuous intermediate can also provide an evolutionary route to new functions without passing through a fitness valley.

Small-scale versus whole-genome duplication: effect of origin of duplication on regulatory network evolution

While gene duplicates clearly contribute significantly to regulatory divergence, their exact contribution has been suggested to depend on their origin. In small-scale duplications, where only one or a few genes are duplicated, transcription factors and their target genes are usually not duplicated together, resulting in immediate expression divergence [57]. After whole-genome duplications, paralogs (might) take longer to diversify, and studies have shown that their contribution to regulatory divergence is more pronounced and more prolonged [32•,48]. The ancestor of the yeast *S. cerevisiae* underwent at least one whole-genome duplication; and several studies have shown that this event could have driven the evolution of novel regulatory patterns through divergence of paralog promoters. Key examples include the evolution of the *GAL* regulatory network and the emergence of fast anaerobic growth [10,58]. Examples of the effect of *trans* mutations also exist. The regulation of ribosomal gene expression offers a key example of how whole-genome duplication created two paralogs of an ancestral transcription factor that subsequently diverged into an activator and a repressor of ribosomal genes, respectively [34].

Conclusions

Rewiring and expansion of regulatory networks is crucial throughout evolution since these events generate new expression patterns and allow proper regulation of novel

gene functions. Increasing evidence points to transposable elements as an important source of DNA-binding domains that can be recruited as transcription factors and contribute to regulatory network evolution [59,60]. Additionally, transposable elements can provide new regulatory sequences, thus introducing new regulatory interactions [61–65].

Apart from this, the examples discussed in this review clearly show that transcription factor duplication and subsequent divergence can generate new patterns of gene expression while avoiding negative pleiotropic effects inevitably associated with changes in regulatory proteins [43••,45••]. However, studies have shown that duplication is not a prerequisite for a regulator to acquire a novel function [25••,66]. Promiscuity (or bifunctionality) of the transcription factor seems to play a major role in regulatory network evolution; with or without gene duplication.

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- •• of outstanding interest

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