Opinion

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Moving from transcriptional to phospho-evolution: generalizing regulatory evolution?

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Much of biological diversity is thought to arise from changes in regulatory networks. Although the role of transcriptional regulation has been well established, the contribution to evolution of changes at other levels of regulation has yet to be addressed. Using examples from the literature and recent studies on the evolution of protein phosphorylation, we argue that protein regulatory networks also play a prime role in generating diversity within and between species. Because there are several analogies between the regulation of protein functions by kinases and the regulation of gene expression by transcription factors, the principles that guide transcriptional regulatory evolution can also be explored in kinase–substrate networks. These comparisons will allow us to generalize existing models of evolution across the complex layers of the cell's regulatory links.

Regulatory evolution as the driver of biological complexity

The origins of biological diversity have puzzled scientists since ancient times [1]. As a result of the extraordinary success of molecular biology over the last 50 years we are now poised to answer a fundamental question: how is the genetic component of morphological and physiological differences between species generated from the underlying molecular differences? [2]. The general consensus that has emerged in recent years is that regulatory evolution at the transcriptional level plays a key role in generating this diversity. Although this might be largely true, the study of regulatory evolution should not be limited to transcriptional regulation and should be extended to other levels of regulation [3,4]. Here we argue that protein regulation by post-translational modifications should also be considered; we focus in particular on the study of protein phosphorylation because it is one of the most abundant forms of post-translational modifications and because recent developments in proteomics now allow the study of protein phosphorylation on a proteome-wide scale [5].

A major theory to explain the diversity of closely related organisms is the '*cis*-regulatory evolution hypothesis': molecular differences in the *cis*-regulatory sequences controlling transcriptional regulation lead to changes in gene expression of multifunctional developmental genes and thus to physiological and morphological differences between species [6–9]. *Cis*-regulatory sequences therefore disproportionally contribute to the evolution of organismic forms and functions. Comparative functional genomics studies indeed indicate that there is abundant molecular complexity and diversity in transcriptional regulatory networks, providing the needed raw material for *cis*-regulatory evolution [10–13].

Post-translational regulation is another level of regulation that could play a key role in generating phenotypic diversity. Here we use protein phosphorylation as a case study (Box 1). Recent technological developments now allow studies of phosphorylation and kinase–substrate interaction networks on a large scale and in a systematic manner, allowing researchers to address their modes of evolution directly. It is therefore timely to ask whether we can generalize previous findings on transcriptional regulation to other levels of regulation. We highlight lines of research that are of prime interest, and show that past research on transcriptional regulatory evolution can serve as a guide to test hypotheses regarding the evolution of phospho-regulation.

Life after transcription

Organismal development and physiology cannot be reduced to turning genes on and off. Transcriptional regulation is only one step in the regulation of protein activity. Once a transcript has been produced it has to be processed, transported and degraded. Between the birth and death of the transcript, translation initiation and elongation have to take place and, as for transcription, these processes are highly regulated. Examples of molecular differences at post-transcriptional levels clearly show how all levels of regulation might contribute to biological diversity. For instance, during translation, *cis*-regulatory elements in mRNAs affect the rate at which polypeptide chains are produced. The presence of reading frames upstream of the main ORF (uORFs) regulate protein abundance by interfering with translation of the main ORF. In humans, uORFs are present in half of the transcripts, and are often polymorphic and modulate disease risk [14], and therefore contribute significantly to phenotypic variation in our species [15]. Once the protein is produced, a whole battery of regulatory mechanisms comes into play: the protein is processed, transported and regulated by other proteins

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Box 1. Phosphorylation is a major mechanism of protein regulation in eukaryotes

The activity of proteins in the cell is tightly controlled after translation. This can be accomplished in four ways, namely through changes in enzymatic activity, subcellular localization, protein stability, and interaction ability. These alterations are reversible and can switch proteins between different functional states. Perhaps the most familiar of these mechanisms is the regulation of enzymatic activity through conformation changes induced by reversible phosphorylation [70]. Indeed, phosphorylation is a major post-translational modification employed by eukaryotic cells to regulate gene activity and current estimates indicate that ~30% of eukaryotic proteins are phosphorylated, predominantly on serine (S), threonine (T) and tyrosine (Y) residues [71,72].

However, conformation changes and corresponding modulation of enzyme activity is only one of many major mechanisms by which phosphorylation can regulate protein activity [35,73]. For example, the p53 tumor-suppressor protein is thought to be phosphorylated at 17 sites by at least 10 different kinases, and these modifications can promote DNA-binding activity, increase or decrease protein stability, or modulate binding to other proteins (such as Mdm2) in response to specific signals such as DNA damage, UV light, IR stress or glucose deprivation [41]. More generally, reversible phosphorylation underlies several important mechanisms, including signaling by receptor tyrosine kinases [e.g. by the epidermal growth factor (EGF) receptor], MAP kinase cascades (such as pheromone sensing in yeast), and the activity of modular protein-binding domains (such as the 14-3-3 or src-homology SH2 domains) where protein interactions depend on phosphorylation state [74].

Regulation by phosphorylation seems particularly important when rapid cellular changes are necessary because transcriptional and translational responses are inherently limited by the time needed for transcribing RNA molecules and to fold and process proteins. Key cell decisions are therefore taken before transcription can occur. For instance, yeast cells decide to undertake the morphological transformation necessary for mating at a given concentration of pheromone (switch-like response). The shape of the dose–response is encoded in the interaction and the competition between a protein kinase and a protein phosphatase for specific phosphorylation sites on another protein kinase [75]. As more and more studies on the genetic variation affecting dynamic traits are performed, the importance of signaling networks in generating phenotypic diversity will grow.

that will greatly influence its activity, localization, stability, and ability to interact. For instance, evolutionary changes in protein localization can allow the protein to take up new functions. On the lineage leading to humans, duplication of the cell division-control gene *CDC14B* gave birth to *CDC14Bretro*, which underwent intense positive selection in the African ape ancestor and shifted its cellular localization, going from an association with microtubules to an association with the endoplasmic reticulum, and probably acquiring new functions [16].

Phospho-regulation has great potential to contribute to the evolution of phenotypic diversity but has not yet received the attention it deserves. Mutations in phospho-sites can have strong phenotypic effects by affecting the regulation of protein localization and degradation, by creating new crosstalk in signaling networks, and regulating the activity of proteins that were once constitutively active. Examples for each of these types of mutations have been documented [17–20]. One spectacular example comes from the phospho-polymorphism associated with familial advanced sleep phase syndrome caused by loss of a phosphorylation site on the hPer2 protein, thereby affecting its degradation rate and localization [19,20]. An example of the evolution of new links among signaling cascades and effector proteins comes from the common K to T polymorphism (K897T) in *ERG1* [17], which creates a phosphorylation site in ERG1 protein for the Akt protein kinase. The normal activation of the channel is prevented by Akt-mediated phosphorylation of the K897T mutant channel, and profoundly affects hormonal signaling and responses. Finally, an example of a constitutive protein function that becomes regulated by phosphorylation is the interaction between the proteins activation-induced cytidine deaminase (AID) and the replication protein A (RPA) during immunoglobin class-switch recombination [21]. In mammals this interaction is modulated by the phosphorylation of S38 on AID by protein kinase A, whereas in zebrafish the absence of the S38 is compensated by a negatively charged aspartate at position 44 that provides similar *in vitro* and *in vivo* functionality as the mouse proteins but with a constitutive rather than a regulated interaction.

Evolution of phospho-sites and kinase–substrate relationships

One powerful approach to address the evolution of phospho-regulatory sites directly is to compare the phosphoproteomes of individuals within or between closely related species and the organization of their kinase–phosphatase substrate networks. Most studies so far have compared the set of phosphorylated residues of one reference species to the proteomes of other species and have revealed that, whereas phosphorylation sites are on average more conserved than non-phosphorylated but otherwise equivalent residues, their turnover rate is high; a large fraction of the phospho-sites identified by these methods appear to evolve at rates similar to non-phosphorylatable sites [22–26]. Phospho-regulation is therefore evolutionarily labile and is a source of diversity from which natural selection can draw. A single study has directly addressed the evolution of eukaryotic phosphoproteomes by characterizing the phospho-proteomes of three species of fungi and comparing the patterns of genetic interactions involving protein kinases [27]. The conclusion of this study confirms our expectations. First, the relative levels of phoshorylation per functional group are relatively well-conserved between species, such that proteins regulated by phosphorylation in one species are also regulated by this mechanism in other species. Second, whereas indirect and rough measurements of evolutionary rates show that the turnover of kinase–substrate interactions is slower than that of transcription factor–binding-site interactions, the direct comparison of the interaction network shows that genetic interactions involving kinases and transcription factors are less conserved than those involving other proteins, suggesting that, if these changes do not simply reflect neutral changes (see below) kinase–substrate interactions are likely to make a significant contribution to the phenotypic divergence of these three fungi.

Analogies between phospho-regulatory evolution and transcriptional regulatory evolution

Because transcriptional regulation and phospho-regulation both involve an interaction between regulatory proteins

Table 1. Analogies between transcriptional and phospho-regulatory evolution

Concept	Transcriptional regulation	Phospho-regulation
Cis-element	Transcription factor binding site	Phosphorylation site
Trans-regulator	Transcription factor	Protein kinase
Cis-regulatory module	Enhancer	Cluster of phosphorylation sites[a] Unstructured domains[a] Kinase recruitment motifs or domains[a]
Non-functional interactions	Non-functional DNA binding	Non-functional phosphorylation
Evidence for stabilizing selection	Compensatory binding site turnover	Compensatory phosphorylation site turnover
Cis-regulatory evolution	Binding site or enhancer creation/loss	Phosphorylation site, docking sites or recognition sites creation/loss
Trans-regulatory evolution	Transcription factor duplication and divergence; change in specificity	Kinase duplication and divergence; change in specificity

[a]Some analogies are more speculative.

(transcription factors, protein kinases and phosphatases) and their targets (regulatory sequences in non-coding DNA and proteins), questions that are central to the evolution of transcriptional networks are also highly relevant to phospho-regulatory networks and represent avenues of research that have yet to be explored. We outline several of these below and summarize them in Table 1.

Regulatory information is modular and combinatorial

An attractive feature of the '*cis*-regulatory evolution hypothesis' is its consistency with the observed modularity of transcriptional regulatory information in non-coding DNA. Enhancers (or *cis*-regulatory modules) are made up of short degenerate binding sites for sequence-specific transcription factors [28]. These binding sites allow enhancers to encode a so-called *cis*-regulatory logic by integrating signals from several *trans*-regulators [9]. During development the concentrations of combinations of several transcription factors determine when and where each enhancer will be active. Complex patterns of transcriptional regulation for multifunctional genes are constructed by combining several of these enhancers. In general, enhancers operate largely independently, and therefore evolutionary changes in non-coding DNA can create new enhancers [29,30] or modify pre-existing ones [31,32] without disrupting the myriad of other functions that the gene might perform.

If changes in phospho-regulation are to play an important part in regulatory evolution, a key question is whether similar forms of modularity and combinatorial logic exist in post-translational regulation. Such a model has been developed for so-called 'modular signaling domains' which often mediate signaling interactions. A protein with complex functions can contain many such domains that independently specify interactions with multiple pathways and functions [33]. These modular domains often contain phosphorylation sites that regulate the modular signaling domains, thus encoding a regulatory logic. Synthetic biology capitalizes heavily on this architecture to create new signaling pathways by swapping domains from one protein to another [34]. To what extent evolution also exploits this feature remains to be investigated. Similarly, in many cases phosphorylation sites tend to appear in clusters [35–37] in unstructured regions of the target proteins [38] or adjacent to other post-translational regulatory motifs [39,40]. These regions can also encode post-translational regulatory logic because they often contain sites for other protein modifications or interactions, or motifs that specify regulation such as localization or degradation. The interactions between the regulatory signals in one protein and the modulation of those signals by phosphorylation can lead to complex control of protein function. For example, the N-terminus of p53 contains a cluster of phosphorylation sites for multiple kinases that regulate binding of Mdm2, an E3 ubiquitin ligase that negatively regulates p53. The p53 N-terminus therefore integrates multiple signals to determine the stability of the protein [41]. Much like enhancers in non-coding DNA, the regions of proteins containing these regulatory signals can often be fused to reporter genes and impart the endogenous pattern of phospho-regulated localization, degradation or interaction to the reporter. Such 'regulatory modules' within proteins represent good candidates to allow modification of complex regulation of multi-functional proteins. One example where this type of modification seems to have taken place is in the regulation of the subcellular localization of the mini-chromosome maintenance (MCM) complex in yeast. Although the enzymatic functions of the MCM complex appear to be preserved, new phosphorylation sites and a localization signal arose in the C terminus of the Mcm3 subunit, leading to the phospho-regulated localization of this complex in the lineage leading to *Saccharomyces cerevisiae* [42].

Evolutionary turnover and stabilizing selection

One of the most surprising observations that resulted from evolutionary analysis of well-characterized *cis*-regulatory modules is that the function of the enhancer can be preserved even when the transcription factor binding sites are not conserved [43]. For example, despite high sequence diversity in the transcription factor binding sites in the *eve* stripe 2 enhancer, orthologous enhancers from closely related species drive nearly indistinguishable expression patterns in *Drosophila melanogaster* [44]. However, chimeric enhancers (half *D. pseudoobscura* and half *D. melanogaster*) no longer showed the normal expression patterns [43], supporting the model of compensatory binding-site turnover due to stabilizing selection on the overall enhancer output. A related phenomenon has been described at the level of transcriptional regulatory networks, where the identity of a transcriptional regulator changes over evolution, but the regulatory logic encoded is preserved [45]. For example, the expression of the different protein subunits of the ribosome is tightly coregulated in all fungal species examined, but the specific transcription factors that are responsible seem to change during evolution [46].

Although compensatory turnover in phosphorylation sites has not been demonstrated through analysis of chimeric proteins, several reports suggest that similar patterns of evolution can be observed in regions of proteins with multiple phosphorylation sites. For example, the cyclin-dependent kinase (CDK) consensus sites in the linker region of ORC1 (the largest subunit of the origin recognition complex) have not been conserved in position or number even amongst closely related species such as the mammals. Nevertheless, this region of ORC1 is a target of CDK in *Drosophila* and in mammals, strongly suggesting that the changes in CDK consensus sites have not altered their regulatory function [42]. Evolutionary turnover of phosphorylation sites has also been observed in analyses of high-throughput data [47,48], in some cases when the kinase–substrate relationships are preserved [47]. However, specific experiments that interrogate whether phosphorylation sites in one species can perform the functions of those in another are needed to establish conclusively whether an analogous process of turnover occurs at this level of regulation.

'Compensatory network turnover' is even less understood than 'compensatory site turnover', but there is some evidence that similar processes take place at the level of phospho-regulation. For example, the Cdc6 protein is degraded by multiple mechanisms during the cell cycle to ensure faithful once-per-cell-cycle replication of DNA. Recent functional comparison of Cdc6 proteins from *S. cerevisiae* and related yeasts revealed that not all the specific mechanisms are conserved, although the regulatory logic for the integrity of DNA replication is conserved [49]. Regulatory network turnover could also take place if a phosphorylation site is preserved but the flanking sequences diverge, leading to a change in the identity of the kinase that phosphorylates the site, but preserving regulation.

Gene duplication and regulatory divergence

Gene duplication followed by functional divergence is a major source of evolutionary innovation. Transcriptional evolution has clearly been shown to contribute to the diversification of gene function following gene duplication [50]. Gains of transcription factor binding sites in one paralog can lead to new expression specificities and thus to new functions, whereas loss of binding sites by the paralogs could lead to their subfunctionalization [51]. For instance, in multicellular organisms, regulatory changes following duplication often result in divergence of the tissue specificity of expression [52]. Similar regulatory evolution is expected to take place at other levels of regulation. It has recently been shown that post-translational modifications also impact upon the fate of gene duplicates. Paralogous proteins from the yeast whole-genome duplication are more likely to have been maintained if they were highly regulated by these modifications [53]. A large number of phosphorylation sites (2.5–7% of sites) appear to follow the paths of subfunctionalization. For instance, the two yeast paralogous Boi1 and Boi2 proteins involved in actin cytoskeleton reorganization and the establishment of cell polarity diverge at many of their phospho-sites, and these appear to have either been lost in one or the other of the paralogs (when compared to their probable ancestral sequence), thus contributing to their functional divergence [53]. Clearly, more investigation is needed to determine what evolutionary forces lead to the regulatory differentiation of these paralogs, but results have shown so far that divergence of phospho-sites can contribute to the divergence of molecular functions among gene duplicates.

Cis versus trans evolution

An important aspect of phospho-regulatory evolution is whether changes that affect the phosphorylation status of proteins *in vivo* depend on the sequence of the protein itself (mutations in the phospho-site or flanking sequences) or are upstream in the network (abundance, localization, sequence of the protein kinases and their regulatory subunits). This question has occupied much of the research on the evolution of gene expression levels, whereby the relative contribution of *cis*-acting and *trans*-acting mutations was estimated. *Cis*-regulatory divergence appears to play a larger role than *trans*-regulatory divergence, but both contribute a significant fraction to within- [54] and between-species gene expression diversity and divergence [55,56]. This issue will also need to be investigated for protein kinases, phosphatases and their substrates. Proteins clearly evolve slower than DNA *cis*-regulatory sequences, but phospho-evolution in *cis* is also likely to play a major role because the vast majority of phosphorylation sites are in disordered regions of proteins [22,38] – these are rich in serines and threonines and evolve rapidly due to their lack of structural constraints. The molecular bases of substrate proteins recognition by kinases are not completely understood, but *in vitro* studies on yeast paralogous kinases suggest that substrate specificity can change rapidly: the set of substrates recognized by paralogous kinases can overlap by less than 10% after 100 My of evolution [57].

Non-functional regulatory interactions

Another issue that arises in the study of *cis*-regulatory evolution, and which needs to be considered in the context of phospho-regulation, is the presence of non-functional interactions in kinase–phosphatase interaction networks. For example, a large fraction of transcription factor binding sites across genomes have been shown to have little or no function [58,59]. These are specific sequence elements in non-coding DNA where binding actually occurs but probably does not represent a functional interaction. The presence of such elements can be explained by the fact that transcription factor binding sites fall into families of short and degenerate sequences that can easily emerge through mutation alone. Similar neutral interactions are expected to accumulate in protein interactomes [60], and particularly in kinase–substrate interaction networks, because the selectivity of protein kinases appears to be limited [61,62]. There is little empirical evidence for this so far but the high rate of evolution of many phosphorylation sites has been suggested to result from the accumulation of these non-functional interactions [22]. The dynamic equilibrium of gains and losses of phospho-sites and the potential for the accumulation of non-functional sites has to be taken into account in evolutionary studies, especially in the large-scale analysis of phosphorylation sites and transcription factor binding sites.

Much of the evolutionary polymorphism and divergence might come from these non-functional sites.

Are there general principles of regulatory network evolution?

Given the analogies between transcriptional regulation and phospho-regulation, and the suggestion here that the '*cis*-regulatory evolution hypothesis' be extended to include regulation at other levels than transcription, an important question that arises is whether general principles can be found in regulatory evolution at all levels.

One way to look at the difference between regulatory evolution and protein evolution is that regulatory evolution concerns the interactions between the nodes in the network, whereas protein evolution concerns changes in the nodes themselves. Interestingly, at many levels of gene regulation that have been described so far, interactions in regulatory networks are often specified by short degenerate motifs: transcription factor binding sites, miRNA binding sites, splicing enhancers, short linear motifs in proteins and phosphorylation sites. Because these key regulatory sequences are all short and degenerate, arguments from information theory indicate that they should all be able to arise rapidly from random sequences [61,63,64]. This is in contrast to structural domains in proteins, which contain much more information. These domains rarely evolve from random sequence, and instead proliferate through gene duplication and divergence. Thus, regulatory interactions might in principle be easier to modify over evolution simply because they can be created by a few fortuitous point mutations or insertions and deletions. The evolutionary plasticity due to the low information content of regulatory motifs is expected at all levels of regulation, and therefore seems likely to be a general principle of regulatory evolution. We have focused here entirely on the post-translational modifications of proteins by protein kinases and phosphatases. However, many more linear-motif-dependent modifications (e.g. ubiquitination, acetylation and methylation) and interactions can affect the function of a protein [65]. The evolutionary importance of these modifications remains to be examined, but because they also often depend on short and degenerate linear motifs, they have the potential to evolve rapidly and contribute to phenotypic diversity.

Concluding remarks and future perspectives

Large collections of phosphorylation sites from high-throughput data [24] or curated from low-throughput experiments are now available (phosphoELM: http://phospho.elm.eu.org/, phospho-grid: http://www.phosphogrid.org/) and these are beginning to enable studies of the evolution of phosphorylation sites [22–26]. However, the comparative phosphoproteomic studies [27] have so far been performed mostly on distantly related species, and this limits our ability to study the rate at which phosphoproteomes evolve. More targeted studies on closely related species would provide better measurements of the rate of divergence and of how it compares to other regulatory levels. Furthermore, if phospho-regulatory evolution is to be as important as regulatory evolution at the level of transcription, several questions will need to be addressed. First, and perhaps most importantly, specific physiological and morphological changes that are the result of molecular changes at the level of phosphorylation will need to be identified. A second crucial issue will be to demonstrate that the changes observed are due to natural selection. This is a very difficult challenge in regulatory evolution [66] because models and methods for detecting selection developed for proteins are rarely powerful enough when applied to regulatory sequences. However, new statistical methods are being developed that could be applicable [67–69], and the large amounts of population genetic data that are becoming available should provide unprecedented power to detect the effects of selection.

Although the study of regulatory evolution is still relatively new it has already had a major impact on the way we think about the genetic component of organismal diversity. The generalization to levels of regulation beyond transcription, and particularly to phospho-regulation, is an important step in the completion of this theory. Evolutionary changes due to differences in phospho-regulation will probably represent an important piece in the puzzle of the diversity of life.

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