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Review Article

How phosphorylation impacts intrinsically disordered proteins and their function

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Phosphorylation is the most common post-translational modification (PTM) in eukaryotes, occurring particularly frequently in intrinsically disordered proteins (IDPs). These proteins are highly flexible and dynamic by nature. Thus, it is intriguing that the addition of a single phosphoryl group to a disordered chain can impact its function so dramatically. Furthermore, as many IDPs carry multiple phosphorylation sites, the number of possible states increases, enabling larger complexities and novel mechanisms. Although a chemically simple and well-understood process, the impact of phosphorylation on the conformational ensemble and molecular function of IDPs, not to mention biological output, is highly complex and diverse. Since the discovery of the first phosphorylation site in proteins 75 years ago, we have come to a much better understanding of how this PTM works, but with the diversity of IDPs and their capacity for carrying multiple phosphoryl groups, the complexity grows. In this Essay, we highlight some of the basic effects of IDP phosphorylation, allowing it to serve as starting point when embarking on studies into this topic. We further describe how recent complex cases of multisite phosphorylation of IDPs have been instrumental in widening our view on the effect of protein phosphorylation. Finally, we put forward perspectives on the phosphorylation of IDPs, both in relation to disease and in context of other PTMs; areas where deep insight remains to be uncovered.

Introduction

Protein phosphorylation is a common and reversible post-translational modification (PTM) that results in the addition of a phosphate group to an amino acid side chain. The process is mediated by protein kinases, while the reverse process of dephosphorylation is promoted by phosphatases. Since the first evidence of protein phosphorylation in the late 1950s [1], protein phosphorylation has been widely studied and shown to be involved in a multitude of cellular processes and human diseases ranging from cancers to neurodegenerative disorders [2–4]. Phosphorylation is the most common PTM (between 60000 and 70000 sites in mammalian proteins) [5]; indeed, the human genome encodes more than 500 distinct kinases [6] and more than 200 phosphatases [7]. In eukaryotic cells, most protein phosphorylation occurs in the cytosol and nucleus, and accordingly, most protein kinases are localized to these compartments [6]. The most commonly phosphorylated amino acid residues are serine (Ser), threonine (Thr), and tyrosine (Tyr), with Ser phosphorylation accounting for more than 75% of all [5]. However, other amino acids can also be phosphorylated, albeit much less frequently, including histidine, arginine, lysine, aspartate, glutamate, and cysteine [8].

Intrinsically disordered proteins (IDPs) or intrinsically disordered regions (IDRs, longer than 30 residues) are characterized by their lack of well-defined structure and are found in more than 30% of all

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eukaryotic proteins [9]. Bioinformatics studies have shown that phosphorylation of IDPs or IDRs occurs more frequently than of folded proteins [10]. This could be due to their increased flexibility and therefore improved kinase accessibility. In addition, IDPs typically contain several short linear motifs (SLiMs), i.e., sequence stretches used for partner recognition making both kinase recognition and phosphorylation relevant at these or proximal positions [11]. Practically, prediction tools to identify phosphorylation sites in proteins exist, including, e.g., NetPhos3, which uses known kinase consensus recognition sequences, or GPS5.0 and PhosphoPredict, which use more complex algorithms that combine functional and sequence features [12–15]. A tool specific to IDPs, PhosIDP, allows not only for the prediction of phosphorylation sites but also how phosphorylation impact charge and folding propensity [16]. Experimentally, one can detect *in vitro* protein phosphorylation by radiolabeling with P-32 followed by purification and electrophoresis. Alternatively, phosphorylation may be detected using an SDS-PAGE system copolymerized with Phos-Tag™, which allows for direct separation of differently phosphorylated species in gels [17,18]. Mass spectrometry can be especially helpful not only in determining the number of phosphorylation sites and the level of phosphorylation [19] but to pinpoint site location as well [20,21]. Nuclear magnetic resonance (NMR) spectroscopy can also identify these sites by specific chemical shift perturbations and additionally allows for extraction of phosphorylation kinetics [20–22]. For structural characterization of phosphorylated IDPs, many techniques are available. Besides NMR [23,24] and small angle X-ray or neutron scattering (SAXS/SANS) [25], computational methods like molecular dynamics simulations [26] can help obtain ensemble descriptions of the conformations explored by the phosphorylated IDP [27], either alone or coupled with both low and atomic resolution techniques such as SAXS and NMR [28].

Phosphorylation was initially envisioned as a simple *on/off* switch in signaling pathways and is amply described as such in biochemistry textbooks. With our current increased understanding of IDPs, many other mechanisms related to single or multisite phosphorylation have been observed. For example, multisite phosphorylation can act as a rheostat, inducing a gradual response [29–34] or by modifying the kinetic parameters of an interaction, thereby impacting the duration of the triggered effect [35]. It can also be used as a timer to start and stop a given biological response thanks to the different phosphorylation kinetics of different residues [36] or to barcode different responses depending on the combination of phosphorylation sites (and other PTMs) present [37–39]. Phosphorylation can trigger major structural rearrangement through local proline *cis/trans* isomerization [24,40], secondary structure change [41,42], folding/unfolding [43], or through long-range allosteric effects [44–46]. This variety of mechanisms demonstrates how phosphorylation of one or multiple sites can regulate function, ranging from coarse *on/off* switches to fine-tuned and highly balanced effects.

With the emergence of more in-depth descriptions of the effects of phosphorylation in IDPs, general principles start to appear. In this Essay, we aim to describe the impact of the three most frequently phosphorylated amino acids (Ser, Thr, and Tyr) on both the structure and function of IDPs, detailing the main mechanisms identified to date. We will discuss relevant biological systems using specific examples to illustrate such mechanisms and address disease relevant effects related to IDP phosphorylation. The short format does not allow for more elaborate descriptions within specific biological systems; for that we refer the reader to these recent, excellent, and in-depth reviews [47–49].

Impacting the chemistry and the conformational ensembles of the IDP

The first and most obvious change induced by phosphorylation is the addition of a significant volume and two negative charges (Figure 1A). The volume of a phosphoryl group is 41 $Å^3$, thus increasing the volume of Ser and Thr side chains by 55% and 47%, respectively. Tyr has already a relatively large side chain and increases in size by only 27% (Figure 1A). Replacement of the alcohol group by a phosphoryl group also directly and drastically changes the pKa of the side chain—from 13 for Ser and Thr and from 10 for Tyr to values of 5.8–6.3 (Figure 1A). The phosphoryl group is therefore usually dianionic at physiological pH. In this form, the phosphoryl group can only act as a hydrogen bond acceptor, while the alcohol groups of Ser, Thr, and Tyr function as both hydrogen bond donor and acceptor, which can lead to significant changes in backbone interactions. The additional negative charges can introduce additional interactions with the positively charged side chains of lysine and arginine, the latter of which forms a stronger bond with the phosphoryl group [50]. Backbone effects are almost negligible for phosphotyrosine (pTyr) due to the large distance between the backbone and the phosphoryl group (Figure 1B); however, long-range effects have been described in which Tyr phosphorylation leads to weakened interactions distant to the site [51]. The influence on secondary structure by phosphoserine (pSer) and phosphothreonine (pThr) is much greater, as shown by the larger NMR secondary chemical shifts (SCS) of the backbone nuclei of pSer and pThr, compared with those of

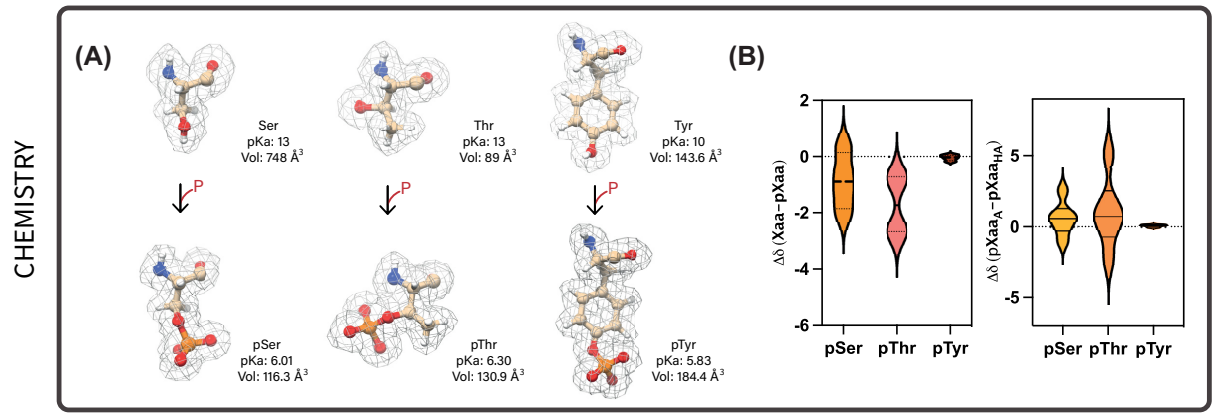

Figure 1. Effects of phosphorylation of IDPs on chemistry

(**A**) Chemical structure, volume, and pK_a for Ser, Thr, and Tyr as well as for pSer, pThr, and pTyr. (**B**) Differences in backbone secondary chemical shift distributions between the phosphorylated and non-phosphorylated amino acids (left) and between the protonated and non-protonated phosphorylated states (right) measured in QQXQQ peptides [52].

pTyr [52] (Figure 1B). The dianionic form increases the propensity of pThr for ordered structure, including formation of an intraresidue phosphate-amide hydrogen bond, explaining the greater backbone effect observed for pThr compared with pSer [53], as directly reflected in the chemical shifts (Figure 1B). The effect of phosphorylation on local structure, however, depends also on several other factors, including the presence of residual secondary structure and the relative position of the phosphorylation site [54,55]. In addition, the potential of the neighboring sequence for hydrogen bonding and electrostatic interactions (e.g., through lysines and arginines at i,i+3 [42,56]) plays a role in the extent of phosphorylation-induced effect, along with the local environment, including the presence of aromatic residues [45,54] and the number of phosphoryl groups [57]. So far only few studies have investigated the role of phosphoryl groups as helical caps in IDPs. One study showed phosphorylation to induce an order-to-disorder transition disrupting a helix-capping motif [58], and mapping of positional effects within a model peptide found that phosphorylation at the N-cap position was the least favored [59]. The effects on helix populations from phosphorylation of an IDP are typically dual. Either the helix structure is stabilized when phosphorylation occurs in N-terminus facilitated by favorable electrostatic interactions and destabilized when positioned in the C-terminus, likely due to interference with the helix dipole (Figure 2A) [55,60–62]. Although the underlying structural mechanism is not clear, the presence of a phosphoryl group close to a proline can impact the relative populations of *cis* and *trans* isomers [24,63], leading to switched binding capacities and therefore function. An example of this is the preferential binding of the apoptotic effector protein BAX to the *cis* isomer of the tumor suppressor p53 [40], while the preferred binding of the *trans* isomer of p53 is to the oncogenic protein Mdm2 [64]. The conformational change induced by phosphorylation is drastic in some cases, leading to partial or complete folding or unfolding (Figure 2A). One example is the eukaryotic translation initiation factor 4E-binding protein 2 (4E-BP2), which is an IDP that binds eukaryotic translation initiation factor 4E (eIF4E) to suppress cap-dependent translation initiation. 4E-BP2 forms a helix upon binding, and then folds into a four-stranded β-domain due to phosphorylation of residues T37 and T46. In this folded form, the motif binding to eIF4E is sequestered, reducing the affinity of the interaction by 400- to 4000-fold depending on the exact phosphorylation state of 4E-BP2 [65,66]. Overall, the concerted contributions of all described factors determine the net modulation of the local and global structure by the presence of phosphoryl groups, but the origins and relative importance of these effects are not fully understood, making changes in structure upon phosphorylation difficult to predict.

More generally, phosphorylation can lead to remodeling of the conformational landscape explored by IDPs [67]. A common way of characterizing the conformational ensemble is using the radius of gyration (R_g), with an increase or decrease corresponding to an expansion or a compaction of the phosphorylated protein chain, respectively (Figure 2B). Several studies have demonstrated such behaviors, with typical observations that phosphorylation expands neutral or negatively charged IDPs, and shrinks positively charged IDPs [28,61,68]. For example, a recent study used NMR to demonstrate that hyperphosphorylated osteopontin becomes extended, thereby preventing correct biomineralization of the bone mineral hydroxyapatite and causing dystrophic calcification [69]. However, a general principle remains difficult to extract due to the number of other parameters at play. R_g can be influenced by the presence of salt

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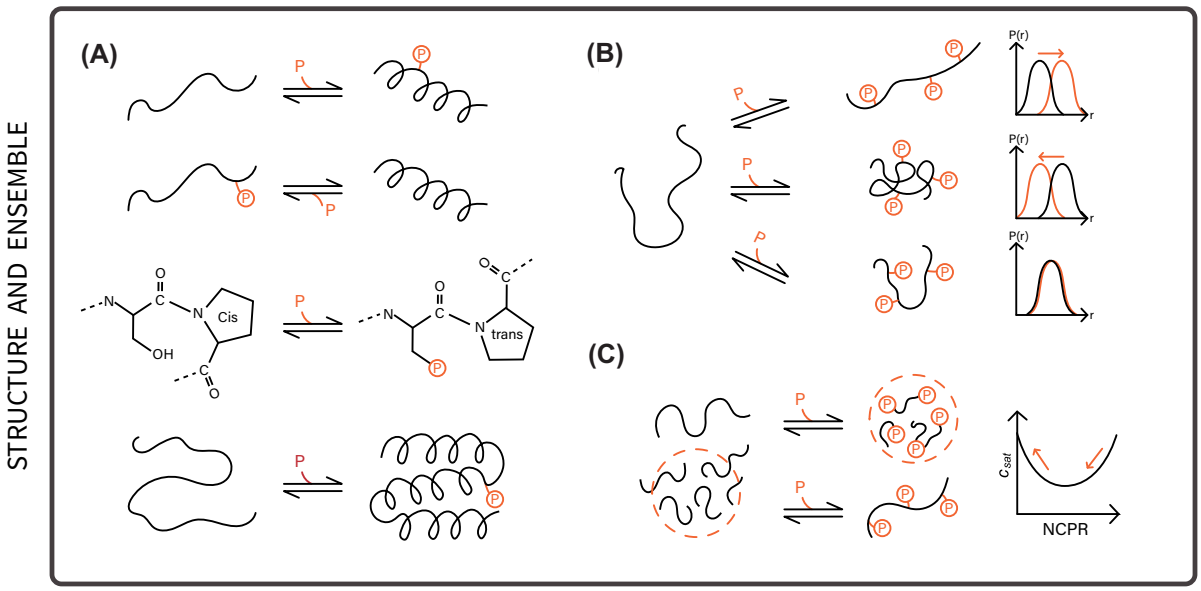

Figure 2. Effects of phosphorylation of IDPs on the structure and ensembles

(**A**) Structural effects upon phosphorylation of IDPs. Secondary structure stabilization (top), destabilization (second row), proline *cis-trans* isomerization (third row) and global folding (bottom). (**B**) Global conformational ensemble distribution changes upon multisite phosphorylation of IDPs leading to expansion (top), compaction (middle), or no change (bottom) dependent on the chain's original properties as well as other PTMs (Adapted from [28]). (**C**) Supramolecular assemblies of IDPs depend on phosphorylation, leading to coacervates or aggregation or fibrils (top) or the dissolution of the coacervates (bottom), where c_{sat} is the saturation concentration and NCPR is the net charge per residue [77].

bridges, repulsion, or attraction between charges, as well as by the primary structure [26,28,70], but the experimental conditions (ionic strength, temperature, pH, etc.) are also important. Furthermore, one must consider charge distribution, the position of charge relative to the phosphorylated site, and the number of phosphoryl groups, as well as the presence of salt bridges and possibility for competition from the introduced negative charges [26]. Lastly, the presence of prolines can alter the extent of compaction [71], as in the case of the yeast transcription factor Ash1, in which R_g is dependent upon a synergistic relationship between prolines and charged residues, resulting in an extended conformation that is independent of the number of phosphoryl groups [70]. Finally, the presence of other PTMs, such as acetylation can modulate sequence properties and hence compaction of the disordered chain [72]. Thus, depending on the sequence properties as well as the number and diversity of PTMs, the effects of phosphorylation on chain compaction may not yet be accurately predicted (Figure 2B).

Phosphorylation can also trigger the assembly of IDPs into supramolecular structures, occurring both in normal cellular processes and as aberrant effects linked with cellular stress or disease. In normal cellular processes, several cases of phosphorylation triggering the formation or dissolution of membrane-less compartments (often termed liquid–liquid phase separation (LLPS), coacervates, or percolation) have been reported [47,73,74]. Although the exact molecular mechanisms regarding PTMs and phase separation are still under investigation [75], up-to-date, comprehensive reviews on this topic have been published recently [47,76]. However, new work on prion low-complexity domains and charge properties including the net charge per residue (NCPR) suggests that, depending on intermolecular interaction propensities of the IDP, phosphorylation may either reduce or increase the saturation concentration (c_{sat}) for phase separation leading to both formation and dissolution of condensates [77] (Figure 2C). Aberrant effects are particularly well exemplified by the mechanisms behind several neurodegenerative diseases. For example, in the case of Parkinson's disease, a single phosphorylation of the neuronal protein α-synuclein at position S129 is believed to cause α-synuclein aggregation, leading to its accumulation in neurons and the formation of Lewy bodies, a hallmark of the disease [78]. Another well-known example is the tubulin-associated protein tau, which becomes hyper-phosphorylated with up to 45 phosphoryl groups, leading to tau's supramolecular assembly into neurofibrillary tangles as observed in Alzheimer's disease [79]. Furthermore, tau can undergo LLPS on the surface of the microtubules

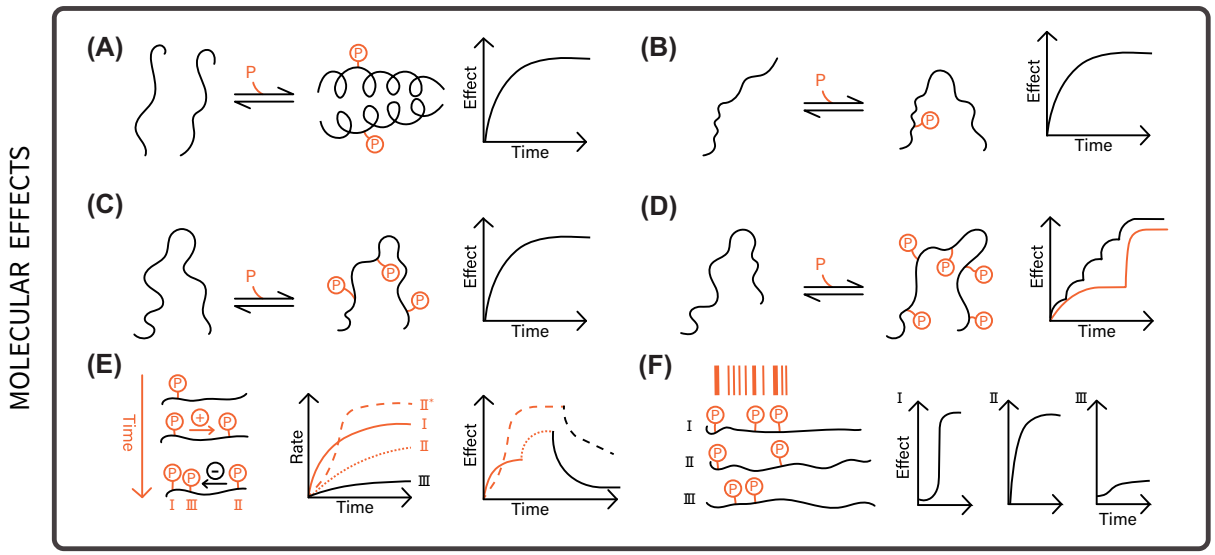

Figure 3. Effects of phosphorylation of IDPs on function

Functional effects of IDP phosphorylation (left) with graphical schematics of the resulting biological output (right). (**A**) *On/Off* switch by single phosphorylation resulting in helix unfolding [35,60] or (**B**) compaction [42]. (**C**) Multisite phosphorylation leading to a single observed effect achieved through the combination of multiple phosphoryl groups attached [68]. (**D**) A complex system with multiple phosphoryl groups added progressively and hierarchical at different sites all adding to the same effect, either stepwise (black) or by switch-like behavior (red) [87,128]. (**E**) Progressive addition of phosphoryl groups at different sites where some of these cause protein activations (red-solid and red-dashed lines) while further phosphorylation causes attenuation of the protein function (black) [89,90]. Co-operativity can exist between sites, in which case sigmoidal behavior is observed illustrated by the dashed lines for positive co-operativity between sites I and II (red-dashed lines, II*) and negative co-operativity between sites II and III (black-dashed lines). In the case they are not co-operative, but still hierarchical this results in kinetics illustrated by the solid and doted lines. (**F**) Barcoding where several phosphorylation sites exist in the IDP, which may be phosphorylated at different times by different kinases to cause different effects [91,92]. In the case of I, there is a priming phosphorylation required for an activating phosphorylation, leading to a lag in the effect, and in this case also co-operativity.

to nucleate microtubules bundles. Upon phosphorylation at disease-related positions, tau still undergoes LLPS, but the associated tubulin is no longer able to form microtubules [80].

Impacting the molecular functions of IDPs by phosphorylation

With the many effects on the chemistry, structure, and conformational ensemble of IDPs, it is not surprising that phosphorylation can also have a multitude of effects on IDP function. With respect to single-site phosphorylation, many systems are known in which one phosphoryl group can induce binding [35,81]. For example, the phosphorylation of the kinase inducible domain (KID) of cyclic-AMP response element-binding protein (CREB) modulates signaling by increasing its binding affinity for the kinase inducible domain interacting (KIX) domain of the co-activator CREB-binding protein [35]. Similarly, phosphorylation increases the internal interaction of the IDR with the DNA-binding domain of p53, regulating DNA binding [82] (Figure 3A). Phosphorylation can also turn binding off, as in the case of the phosphorylation of the cell cycle regulator p21, which inhibits its binding to the replication protein proliferating cell nuclear antigen (PCNA) [83]. In addition, a single phosphorylation site can alter both structure and function, as exemplified by a designed phosphorylation-dependent molecular switch where phosphorylation caused a heterodimeric protein to unfold and dissociate [60] (reverse of Figure 3A). This folding and unfolding reversibly linked the protein to its membrane-bound binding-partner, thereby acting as a reversible protein-anchoring system. Whether the effect of a single phosphorylation is folding, unfolding, binding, or dissociation, the effect follows single exponentials according to rate-laws, as illustrated in both Figure 3A,B.

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Although a single phosphorylation site may allow for new functions and structures, there are many instances of multisite phosphorylation in IDPs where complex functional effects are beginning to emerge and where new mechanisms are appearing (Figure 3C–F). The degree of complexity depends on several factors, including the number of phosphorylation sites necessary for triggering an effect, the order in which the sites are phosphorylated as well as their kinetics [49]. Multiple phosphorylation sites can either be dependent or independent. Sic1 is an example of an IDP where phosphorylation happens independently. It contains nine suboptimal phosphodegrons and will only bind with high affinity to its partner Cdc4 when any six sites are phosphorylated, leading to its degradation and progression of the yeast cell cycle from G1 to S phase (Figure 3C) [84]. When dependent, phosphorylation can follow a hierarchy, so that certain sites need to be phosphorylated before others, leading to stepwise changes in activity one site at the time or switch-like behavior once all sites are phosphorylated, or even a combination of the two (Figure 3D) [49]. Multisite phosphorylations can also be co-operative, so that once a site is phosphorylated, it increases or decreases the kinetics and amplitude of the following event, typically through conformational changes [49], resulting in either positive or negative co-operative responses (Figure 3E) [85,86]. Thus, multiple phosphorylation events that are dependent can result in different timing and amplitudes of a biological outcome.

Here, we present four different cases for the functional effects of multisite phosphorylation (Figure 3C–F). In the first example, we explore how phosphorylation can lead to a single observed outcome only achieved through the combination of multiple phosphoryl groups attached to the IDP. An interesting example of such multisite phosphorylation can be seen for the IDR of the inner centromere protein (INCEP), which is phosphorylated at up to 78 positions [68]. INCEP is necessary for chromosome segregation and must interact with the Aurora B kinase to fulfill this role. Phosphorylation of the IDR within INCEP has been suggested to lead to structural changes that enable the protein to correctly condense and retract to deliver Aurora B at the correct location. Thus, both structural and functional effects depend on the phosphorylation pattern. Although the extent of this mechanism is currently unknown, the general principle is illustrated in Figure 3C. The effect is functionally and mechanistically distinct from the stepwise multisite phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (eIF4-BP1) [87] (Figure 3E). Here, hierarchical phosphorylation is initiated by an early phosphorylation priming the protein and initiating binding, followed by phosphorylation events that subsequently reduce the affinity of eIF4-BP1 for eIF4 [88]. This stepwise reduction in affinity lets translation ramp down, leading to a more gradual reduction in protein production. This multisite phosphorylation event might be useful in contexts where the full effect of the protein is not necessary or desired, and the effect regulated in a more nuanced way than a simple *on/off* switch.

Another hierarchical system also involves the addition of phosphoryl groups at different sites, initially causing protein activation, but where further phosphorylation causes attenuation of protein function (Figure 3E). An example of such a system is the transcriptional regulatory protein prostate-associated gene 4 (PAGE4). This protein contains a large IDR that is phosphorylated by multiple kinases including homeodomain-interacting protein kinase 1 (HIPK1) and CDC-like kinase 2 (CLK2) [89]. Phosphorylation by HIPK1 at two sites leads to increased expansion of the chain and facilitates activity of PAGE4 via interaction with activator protein-1 (AP-1), while CLK2 phosphorylates an additional six sites, which causes compaction and attenuation of PAGE4:AP-1 interaction [90]. Thus, in this case, phosphorylation is acting as both the *on*- and *off*-switch depending on the position and the hierarchical timing of the phosphorylation.

Barcoding via phosphorylation is also becoming an established mechanism by which phosphorylation alters the function of a protein [37]. In this case, several phosphorylation sites exist within a protein and may be phosphorylated at different times by different kinases to cause different effects, including acting as primers for other sites. Such priming sites do not cause a direct functional outcome [91] (Figure 3F). This mechanism is prevalent in G protein-coupled receptors (GPCRs), which often feature an intracellular C-terminal IDR [92], but it is also found in the RNA polymerase II, where phosphorylation barcoding is not limited to phosphorylation but involves other PTMs, e.g., acetylation [93,94]. A classic example of barcoding is the phosphorylation of C-terminal β(2)-adrenergic receptor, which is phosphorylated differentially by different GPCR kinases, resulting in different effects on β-arrestin [95]. The wide range of effects exerted by arrestins on GPCRs has led to the barcode hypothesis for receptor arrestin interactions, whereby without differential phosphorylation, GPCRs would be substantially less versatile [96,97]. In a recent study, the effects of different GPCR kinases on GPCR-arrestin complexes were measured and modeled, clearly demonstrating the complexity of these systems [98].

Impact on biological output and relevance for disease

The many effects of phosphorylation on the structure, conformational ensemble, and function join in to execute the biological output of the modifications. Thus, predicting the output of a phosphorylation event is not straightforward

and currently generally not possible. In the following, we will provide a few select examples of how phosphorylation of IDPs is critical for biological function and of relevance to disease.

A prominent and well-characterized group of SLiMs are the so-called phosphodegrons that regulate the turnover of the proteins in which they are found. Unlike other degrons, such as those that direct misfolded protein for degradation [99], phosphodegrons are often rich in acidic and proline residues [100] and found in IDRs [101] to allow access for protein kinases. Upon phosphorylation, the phosphodegron is activated and able to bind specific members of the SCF-family of E3 ubiquitin-protein ligases. In turn, this leads to ubiquitylation of the protein carrying the phosphorylated phosphodegron and ultimately its degradation by the 26S proteasome [102]. Some of the best characterized phosphodegrons are found in cyclins and cyclin-dependent kinase inhibitors, the timely proteasomal degradation of which is critical for cell cycle progression [103]. However, phosphodegrons also regulate many other important cellular events, as shown for the regulation of the protein period 2 (PER2). PER2 is a transcriptional repressor that forms a core component of the circadian clock [104,105], a cell-autonomous time-keeping system, which generates 24-h oscillating rhythms in gene expression that ultimately regulate physiological processes including rhythms in metabolism and behavior. Like most transcriptional regulators, PER2 contains a long IDR, and casein kinase 1 (CK1) phosphorylates PER2 at S478 within this. In turn, this recruits the E3 ubiquitin-protein ligase $SCF^{\beta TrCP}$, leading to PER2 ubiquitylation and degradation [106–108]. Accordingly, transgenic *Per2* S478Ala mice showed lengthened circadian behavioral rhythms [108].

CK1 does not phosphorylate PER proteins exclusively at their phosphodegrons, but also at several other sites in their long IDRs and, importantly, with different kinetics [109–111] as well as outcomes (Figure 3E). The IDR functions as a phosphoswitch with different phosphorylation sites counteracting each other in their effects on PER stability [107,112]. Phosphorylation of the familial-advanced sleep phase (FASP) SLiM SxxSxxSxxSxxS [113] stabilizes PER2 by preventing phosphorylation of the degron [107,114], and the relative rates of phosphorylation of the two sites determine the circadian period length. CK1 is highly active on primed substrates, which have an acidic or a phosphoryl group upstream of the target Ser or Thr [91]; the first Ser of the FASP motif is one example of a priming site that empowers high phosphorylation rates, acting co-operatively. However, and of importance to the circadian function, CK1 may also phosphorylate an unprimed site with slower kinetics [110,115]. In those cases, phosphorylation depends on anchoring of CK1 to a binding domain within the PER protein to increase its local concentration and to access substrate IDRs via dynamic looping [90,97], which has been proposed to allow temporal precision in the clock [109]. Furthermore, the CK1 tail ensures an additional layer of regulation of the clock. It was thus suggested that binding of differently auto-phosphorylated CK1 tail to CK1 itself regulates activation loop dynamics and substrate specificity, including the relative FASP and phosphodegron specificity [112,115,116]. Together, the molecular studies of the circadian clock show an intricate interplay between structure, conformational ensemble, and kinetics in fine regulation of a complex physiological system.

With the many sites available in IDPs for phosphorylation, it is not surprising that mutations of Ser, Thr, or Tyr, which under some conditions are phosphorylated, may lead to aberrant functions, as well as diseases, just at introducing a Ser, Thr or Tyr by mutation also will. There are several examples of this including Tau (Alzheimer's disease) [117], the prion protein (prion disease) [118], and PAGE4 (cancer) [90]. Mutations in the kinase recognition sites are also possible, indirectly altering the phosphorylation status of the IDP. One example comes from the growth hormone receptor, whose intracellular domain is highly disordered [119,120]. A proline to Thr mutation, part of a general phosphodegron (TPxxxS), leads to stabilization of the receptor in the membrane. The mutation impairs binding of the negative regulator suppressor of cytokine signaling 2 (SOCS2) and with continued signaling, this leads to severe cancer progression [121]. Thus, removing the proline important for kinase recognition impacts phosphorylation and becomes relevant in disease progression. Another example was recently reported for the tau 'master site,' which can disrupt tau hyperphosphorylation associated with neurofibrillary tangles in the pathogenesis of Alzheimer's disease [122]. Ablation of a single phosphorylation site (T205) was enough to attenuate p38α kinase activity normally associated with hyperphosphorylation. Although many different kinases phosphorylate tau, this is an interesting example of multistep phosphorylation and the ways in which it can be altered to modify disease progression.

Perspectives and outlook

With the many and diverse effects that can be triggered by the addition of one of more phosphoryl groups to an IDP, it is intriguing how elegantly biology explores and exploits these in so many ways. In particular, the interplay between phosphoryl groups and other PTMs has become an interesting emerging topic within the IDP field [94], and reveals how context and interplay between PTMs orchestrate function. In terms of studying phosphorylation experimentally, the use of phosphomimetic mutations, i.e., aspartic acid and glutamic acid, makes biological sense in

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some instances as it increases the volume of the amino acid compared with Ser and Thr and adds a negative charge. However, aspartic acid and glutamic acid are considerably less electronegative compared with a phosphoryl group and have a smaller hydration sphere and volume. Therefore, the impact on local and global structure including compaction is not completely captured by these amino acid substitutions and should be used with care [123,124]. Going forward, there is a need to use nonremovable modifications to mimic phosphorylated IDPs, and the use of unnatural amino acids may be one way to achieve this, with similar strategies as used for the incorporation of a fluorophore at specific positions in proteins in live cells [125–127]. With new technical advances and the improved understanding of intrinsic disorder, we expect many of the intricate mechanisms related to IDP phosphorylation—alone and combined with other modifications—to be addressed in the future, revealing novel, even more complex mechanisms, and expanding our view on protein function.

Summary

- Phosphorylation of IDPs has diverse effects on local and global structure, conformational ensemble, and molecular function.
- Multisite phosphorylation widens the functional potential of IDPs and makes possible a variety of complex regulatory mechanisms, including hierarchical and co-operative events.
- Context, including sequence properties and interplay with other types of PTMs, can be expected to expand regulatory possibilities even further.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

E.A.N., E.D., K.S., R.H.P., and B.B.K. conceived and wrote the article.

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Abbreviations

4E-BP2, 4E-binding protein 2; AP-1, activator protein-1; CK1, casein kinase 1; CLK2, CDC-like kinase 2; CREB, cyclic-AMP response element-binding protein; c_{sat}, saturation concentration; eIF4E , eukaryotic translation initiation factor 4E; FASP, familial-advanced sleep phase; GPCR, G-protein coupled receptor; HIPK1, homeodomain-interacting protein kinase 1; IDP, intrinsically disordered protein; IDR, intrinsically disordered region; INCEP, inner centromere protein; KID, kinase inducible domain; KIX, kinase inducible domain interacting; LLPS, liquid–liquid phase separation; NCPR, net charge per residue; NMR, nuclear magnetic resonance; PAGE4, prostate-associated gene 4; PCNA, protein proliferating cell nuclear antigen; PER2, protein period 2; pSer, phosphoserine; pThr, phosphothreonine; PTM, post-translational modification; pTyr, phosphotyrosine; Rg, radius of gyration; SANS, small-angle nuclear scattering; SAXS, small-angle X-ray scattering; SCS, secondary chemical shift; Ser, serine; SLiM, short linear motif; SOCS2, suppressor of cytokine signaling 2; Thr, threonine; Tyr, tyrosine.

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