Post-translational modifications influence transcription factor activity: a view from the ETS superfamily

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Summary

Transcription factors provide nodes of information integration by serving as nuclear effectors of multiple signaling cascades, and thus elaborate layers of regulation, often involving post-translational modifications, modulating and coordinate activities. Such modifications can rapidly and reversibly regulate virtually all transcription factor functions, including subcellular localization, stability, interactions with cofactors, other post-translational modifications and transcriptional activities. Aside from analyses of the effects of serine/threonine phosphorylation, studies on post-translational modifications of transcription factors are only in the initial stages. In particular, the regulatory possibilities afforded by combinatorial usage of and competition between distinct modifications on an individual protein are immense, and with respect to large families of closely related transcription factors, offer the potential of conferring critical specificity. Here we will review the post-translational modifications known to regulate ETS transcriptional effectors and will discuss specific examples of how such modifications influence their activities to highlight emerging paradigms in transcriptional regulation. ***BioEssays*** **27:285–298, 2005.**

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Abbreviations: CAMK II, Calmodulin-dependent protein kinase II; DBD, DNA-binding domain; EBS, ETS-binding site; ECM, extracellular matrix; ETS, E twenty-six; HDAC, histone deacetylase; HTH, helix-turn helix; K, Lysine; MAPK, Mitogen-activated protein kinase; MK2, MAPK-activated protein kinase 2; MLCK, myosin light chain kinase; Msk1, MAPK-stimulated kinase 1; O-GlcNAc, O-linked β-N-acetylglucosamine; OGT, O-GlcNAc transferase; PD, Pointed Domain; PEST, Proline Glutamic acid Serine Threonine; PKA, protein kinase A; PKC, protein kinase C; RRE, RAS responsive element; RTK, Receptor tyrosine kinase; S, Serine; SAM, Sterile Alpha Motif; SRE, serum response element; SRF, serum response factor; T, Threonine; TAD, transcriptional activation domain; TCF, ternary complex factor; TGF, Transforming growth factor; Y, Tyrosine.

Introduction

Site-specific DNA-binding transcription factors provide critical targets and effectors of signal transduction pathways that relay information from the cell surface to the nucleus. Many of these transcriptional regulators cluster into large families defined by highly homologous DNA-binding domains (DBD) that have the capacity to bind the same or highly similar DNA sequences. Yet, in practice, transcription factors must regulate distinct sets of target genes in temporally and spatially appropriate patterns and at correct levels to ensure normal development.

How then is specificity and accuracy of transcriptional output achieved? The answer to this question, while likely to be quite complex, is of paramount importance, as misregulation of the transcriptional response is a fundamental contributor to and consequence of many human diseases including cancer. In this review, using examples derived from recent studies of members of the ETS (E twenty-six) transcription factor superfamily, we will discuss how post-translational modifications, operating as dynamic and reversible sensors of upstream signaling events, may provide a cornerstone to the solution given their ability to modulate virtually all facets of transcription factor function.

ETS transcription factors are conserved in metazoans and play essential roles throughout development, functioning as downstream effectors of signal transduction cascades to regulate a broad spectrum of cellular processes. Reflecting their critical roles in regulating cell proliferation, differentiation, apoptosis, migration and epithelial–mesenchymal interactions during normal development, misregulated ETS proteins contribute, via a variety of mechanisms, to both the initiation and progression of many human cancers.[1–8] ETS transcription factors are defined by a highly conserved eighty-five amino acid motif called the ETS domain, which belongs to the

superfamily of winged helix-turn-helix (HTH) DNA-binding domains and binds a core recognition sequence, GGAA/T, referred to as the ETS-binding site (EBS).[9–11] Sequences flanking the core EBS are variable and contribute to the specificity of individual ETS transcription factors of which there are approximately thirty in mammals and eight in *Drosophila*.[11–13] The majority function as transcriptional activators, while some possess repressive activities, and others, in a context-dependent manner, act as both activators and repressors.[4,6,14]

One-third of ETS transcription factors also contain a conserved amino-terminal domain called the Pointed Domain (PD). PDs belong to the Sterile Alpha Motif (SAM) family, and mediate both homotypic and heterotypic protein–protein interactions.[15] Functions associated with PDs of ETS transcription factors include homooligomerization in the case of human Tel and its *Drosophila* homolog YAN,[16,17] heterodimerization, as exemplified by Tel-Fli-1 interactions,[18] and transrepression, documented for both Tel and YAN.[19,20] As will be discussed further below, PDs frequently provide the site of regulation by extracellular signaling pathways via MAPK-mediated phosphorylation.[4,21]

Numerous strategies have evolved to regulate transcription factor function and activity, providing the temporal and spatial specificity multicellular organisms require. This issue of specificity is particularly important for ETS transcription factors, due to the large number of family members, their overlapping expression patterns, and their similar or even identical DNA-binding preferences.[11] Because the same issue of specificity exists for other currently less well-understood multiprotein transcription factor superfamilies, principles elucidated from studies of the ETS family are likely to be broadly applicable.

The focus of this review will be to discuss how ETS transcription factor activity is regulated by phosphorylation and other post-translational modifications as a paradigm for how signaling cascades influence the transcriptional response. We will first overview the post-translational modifications that are known to affect ETS transcription factors: phosphorylation, glycosylation, sumoylation, acetylation and ubiquitination. Modifications that have not yet been implicated in regulating ETS transcription factors, such as methylation, prolyl isomerization, hydroxylation and ribosylation, are beyond the scope of this review.

We will then present several case studies of ETS transcription factors whose activities are regulated by post-translational modifications, primarily changes in phosphorylation state, although additional modifications will be discussed as appropriate. Rather than presenting an exhaustive list of all ETS proteins reported to be phosphorylated, we have selected our examples to direct attention to the pleiotropy of molecular mechanisms whereby phosphorylation contributes specificity to the transcriptional response. Importantly, multisite modification is emerging as a powerful mechanism for integrating information in the cell, as multiple signaling pathways can converge to regulate a particular transcription factor by differential phosphorylation, or other post-translational modification, at distinct or identical residues. Thus, combinatorial usage of multiple post-translational modifications provides the cell with a sophisticated language that is likely to be applied broadly to ETS and other transcriptional regulators.

A primer on post-translational modifications that target ETS family members

Given the comparatively small number of genes possessed by higher eukaryotes relative to the enormous number of functions that the encoded protein products must perform, post-translational modifications may have evolved to increase the effective protein complement. Indeed, the diverse spectrum of covalent modifications, either individually or in complex combinatorial patterns, dynamically and reversibly influence protein–protein interactions, protein–DNA interactions, subcellular localization, stability, activity and other post-translational modifications of the target protein, thereby significantly increasing the functional complexity of the proteome.

Phosphorylation and glycosylation: reciprocal regulation of transcription factors

By far the best-studied post-translational modification, phosphorylation plays a pivotal role in modulating the activity of a broad spectrum of cellular proteins, including transcription factors.[22] Phosphorylation occurs by addition of a phosphate group to the hydroxyl group of serine (S), threonine (T), or tyrosine (Y) residues in an ATP-requiring reaction mediated by two broad families of kinases, S/T protein kinases and Y protein kinases.[23] Like most post-translational modifications, phosphorylation is reversible with dephosphorylation mediated by phosphatases, either S/T, Y or dual specificity.[24] S/T phosphorylation as a means of regulating transcription factors is better characterized than Y phosphorylation, appears more widespread, and will be the exclusive focus of our discussion. As presented in the specific examples in the second half of the review, and summarized more generally in Table 1, many ETS family members are subject to S/T phosphorylation in response to a variety of upstream signals, and these modifications exert a broad spectrum of effects on their activity.

In contrast to phosphorylation, glycosylation has only recently achieved prominence as a means of influencing transcription factor activity. Known targets include, in addition to the ETS transcription factor Elf-1,[25] nuclear pore proteins, chromatin-associated proteins, RNA polymerase II and its associated transcription factors, hormone receptors, proteasome components, phosphatases and kinases, suggesting roles in nuclear transport, chromatin structure, protein turnover, signaling and transcription.[26–30]

Glycosylation of nuclear and cytosolic proteins occurs by the addition of the simple monosaccharide O-linked β-N-

Table 1. Functional consequences of ETS transcription factor phosphorylation

ETS protein	Kinase	Effects of phosphorylation	Reference
Tel	MAPK	Loss of repression, nuclear export	(45,56)
YAN *Drosophila*	MAPK	Loss of repression, nuclear export, downregulation of in vivo activity, degradation?	(44,53,57,58) T.L.T. & I.R, unpub.
LIN-1 *C. elegans*	MAPK	Loss of repression	(119)
Ets1	MLCK CAMKII	Inhibits DNA binding, stabilizes autoinhibitory state, decreases protein stability, converts to repressor	(5,76–78)
	PKCα	Increases activation	(80)
	MAPK	Increases activation	(72,73)
Ets2	MAPK	Increases activation, increases protein stability	(120)
PNT-P2 *Drosophila*	ERK	Increases activation, delayed attenuation, required for in vivo function	(20,53,58,121)
Er81	PKA	Reduces DNA binding, increases activation	(85)
	MAPK Msk1/Rsk1	Increases activation	(83–86)
	Mk2	Blocks/decreases activation	(88)
Erm	PKA	Decreases DNA binding affinity, increases activation	(87,122)
	MAPK	Increases activation	(122)
Pea3	MAPK	Increases activation	(123)
Net (Sap2)	ERK	Switch from repressor to activator	(124,125) (126)
	JNK	Nuclear export, loss of repression	(126,127)
Sap1	MAPK	Increases activation, increases DNA binding, promotes ternary complex	(128,129)
PU.1 (Spi-1)	Casein kinase II	Potentiates protein–protein interactions, increases activation	(130)
	MAPK	Increases activation	(131)
Spi-B	Casein kinase II	Increases activation, reduces stability	(132,133)
	MAPK ERK/JNK	Alters protein–protein interactions	(132)
Erf	MAPK	Nuclear export, loss of repression	(134–136)
GABPα	MAPK (ERK and JNK)	Increases activation, increases stability of protein complex	(137–141)
Elk-1	MAPK	Increases DNA binding affinity and ternary complex formation, increases activation, inhibits sumoylation	(96–100,142) (101)
MEF	CyclinA/cdk2	Decreases DNA binding, decreases activation, restricts function to G1/S	(143)
Elf-1	PKC? other kinases?	Promotes dissociation from Rb, promotes nuclear translocation, enhances DNA binding, increases activation	(25,110,117)
ERG	PKC	Unknown	(144)

With the noted exceptions of LIN-1 from C. elegans and YAN and PNT-P2 from *Drosophila*, all examples refer to mammalian ETS factors.

acetylglucosamine (O-GlcNAc) to the hydroxyl group of either S or T residues.[26,28,30] Just as phosphorylation levels depend on the balance between the kinase and the phosphatase, O-GlcNAc levels depend on the balance between O-GlcNAc transferase (OGT) and O-GlcNAcase. Although the regulation of OGT and GlcNAcase is not well understood, the rapid and dynamic changes in O-GlcNAc levels that have been observed in response to cell cycle progression, stress, glucose metabolism and insulin signaling suggest responsiveness to and possible coordination of upstream metabolic and signaling events.[29,31]

While there is no consensus motif for O-GlcNAc attachment, nor a known protein interaction motif that specifically recognizes glycosylated S/T residues, many of the sites are identical or immediately adjacent to those recognized by S/T protein kinases,[26,28,30] raising the possibility that glycosylation and phosphorylation play competing and antagonistic roles. Consistent with such a reciprocal relationship, phosphatase inhibitors decrease while kinase inhibitors increase the levels of O-GlcNAc modification.[29] Intriguingly, O-GlcNAc modification sites frequently occur within high-scoring PEST sequences,[28,30] motifs often associated with phosphorylation-induced proteasome-mediated degradation. Thus O-GlcNAc may neutralize the effect of PEST sequences by preventing phosphorylation and subsequent degradation. Given the potential for reciprocal and regulatory relationships between glycosylation and phosphorylation, further investigations into the extent, contexts and consequences of O-GlcNAc modification of transcription factors would seem an important priority.

Competing over lysines: acetylation, ubiquitination, and sumoylation

Acetylation, sumoylation and ubiquitination all modify lysine (K) residues.[32] The potential diversity afforded by different post-translational modifications targeting the same site is enormous, and increases exponentially if multiple residues are involved. Thus to truly grasp how fine-tuning of the transcriptional response is achieved, it will be critical to understand the combinatorial control and information integration that is likely achieved by context-specific multisite modifications of transcription factors.

Although best known for its involvement in regulating histones and thus the state of chromatin, acetylation also

directly regulates multiple aspects of transcription factor activity including protein stability, protein–protein and protein–DNA interactions.[33–35] Acetyltransferases, a diverse family of enzymes with the most prominent being p300, transfer an acetyl group to the specific K on the target protein with the reverse reaction mediated by histone deacetylases (HDACs). HDACs recruit a variety of corepressor proteins, and thus are frequently found associated with transcriptional repressors. However, it is important to note that, in contrast to histones, deacetylation of transcription factors is not intrinsically inhibitory for transcription, nor is acetylation always stimulatory.[35]

Sumoylation and ubiquitination are also reversible modifications of K residues that affect the stability, activity and localization of a broad spectrum of transcription factors,[36–39] including those of the ETS family. Ubiquitin and SUMO are both small polypeptides, 9 and 11 kDa, respectively, that are added to a protein through a multistep process catalyzed by three different enzymes: E1 activating enzymes, E2 conjugating enzymes and E3 ligases.[39,40] In both cases, the E3 ligases constitute a diverse collection of enzymes and are thought to confer specificity to the reaction.

Ubiquitin and sumoylation-mediated processes have extremely pleiotropic functions with respect to transcriptional regulation. For example, ubiquitination plays critical roles in regulating transcription factor activity, both indirectly by inducing proteasome-mediated degradation of the protein and directly by altering its transcriptional properties.[40–42] Sumoylation also affects the stability and activity of transcription factors, although its most-widespread role appears to be in regulating their subcellular localization, which depending on the particular target, increases or decreases transcriptional activity.[36,43]

Although numerous examples of transcription factors regulated by acetylation, sumoylation and ubiquitination have recently emerged, we are likely only in the initial stages of uncovering the full extent and significance of such regulation. Furthermore, because all three modifications target lysine residues, the possibility for both competition at a single site and cooperativity or antagonism between multiple sites is immense and provides a critical area for future investigations.[32]

Regulation of ETS transcription factors by post-translational modifications

Below we present several examples of how ETS transcription factors are regulated by post-translational modifications, focusing on phosphorylation but taking into account other modifications and their effects, to highlight the complex mechanisms that couple integration of upstream signals to specificity of transcriptional output. It is not our intent to discuss all known post-translational modifications of ETS transcription factors. Rather the examples have been selected to illustrate general principles that are likely to be broadly applicable to understanding the the complex combinatorial code of post-translational modifications as applied to transcriptional regulation.

Conserved mechanisms of repressor downregulation: YAN and Tel

Drosophila YAN, and its mammalian ortholog Tel, represent the best-characterized transcriptional repressors within the ETS superfamily and function as downstream effectors of the receptor tyrosine kinase (RTK)/Ras/MAPK signaling pathway.[44,45] Functionally, YAN prevents undifferentiated cells from responding inappropriately to mitogenic or inductive signals, while Tel is required for the development and maintenance of complex vasculature and for adult hemotopoiesis and is frequently rearranged or deleted in human leukemias and solid tumors.[46–52] Structurally, YAN and Tel have an amino-terminal Pointed Domain (PD) that mediates both homotypic and heterotypic protein–protein interactions, and a carboxy-terminal ETS DNA-binding domain that recognizes the classic GGAA/T core sequence in target gene promoters.[18,19,53–55] Homo-oligomerization via PD–PD interactions is essential for transcriptional repression, and mechanistically, it has been proposed that the DNA may be wrapped around the oligomer, resulting in repression.[16,17] As will be discussed below, the two repressors appear to be regulated by similar, but not identical, mechanisms involving complex patterns of multisite post-translational modifications that influence DNA-binding, protein–protein interactions, subcellular localization, stability and transcriptional repression (Fig. 1).

Both YAN and Tel are regulated by specific MAPK-mediated phosphorylation events that lead to removal of their transcriptional repressive activities and induction of their nuclear export (Fig. 1B–C, F–G).[20,45,46] Once in the cytoplasm, YAN, which possesses multiple high-scoring PEST sequences, many of which are associated with a MAPK phosphorylation site, is degraded, whereas Tel is stable. ERK MAPK phosphorylates Tel at S113 and S257, removing Tel's transcriptional repression by decreasing its DNA-binding ability.[45] In addition to ERK, p38, but not JNK phosphorylates Tel, reducing its transcriptional repression.[56]

In YAN, while the first of nine MAPK consensus phosphorylation sites, S127, is required for RAS/ERK pathway responsiveness, phosphorylation at the other sites appears important for amplifying and modulating the response, although the precise coordination and timing remain unknown.[44] Adding further complexity, multiple MAPK pathways appear to converge on YAN. Specifically JNK, targeting the same consensus sites used by ERK, similarly downregulates YAN activity in certain developmental contexts.[57] In addition, the p38 stress-responsive MAPKs are capable of phosphorylating YAN in vitro (F. Hsiao and I. Rebay, unpublished observation) although the in vivo significance remains to be determined.

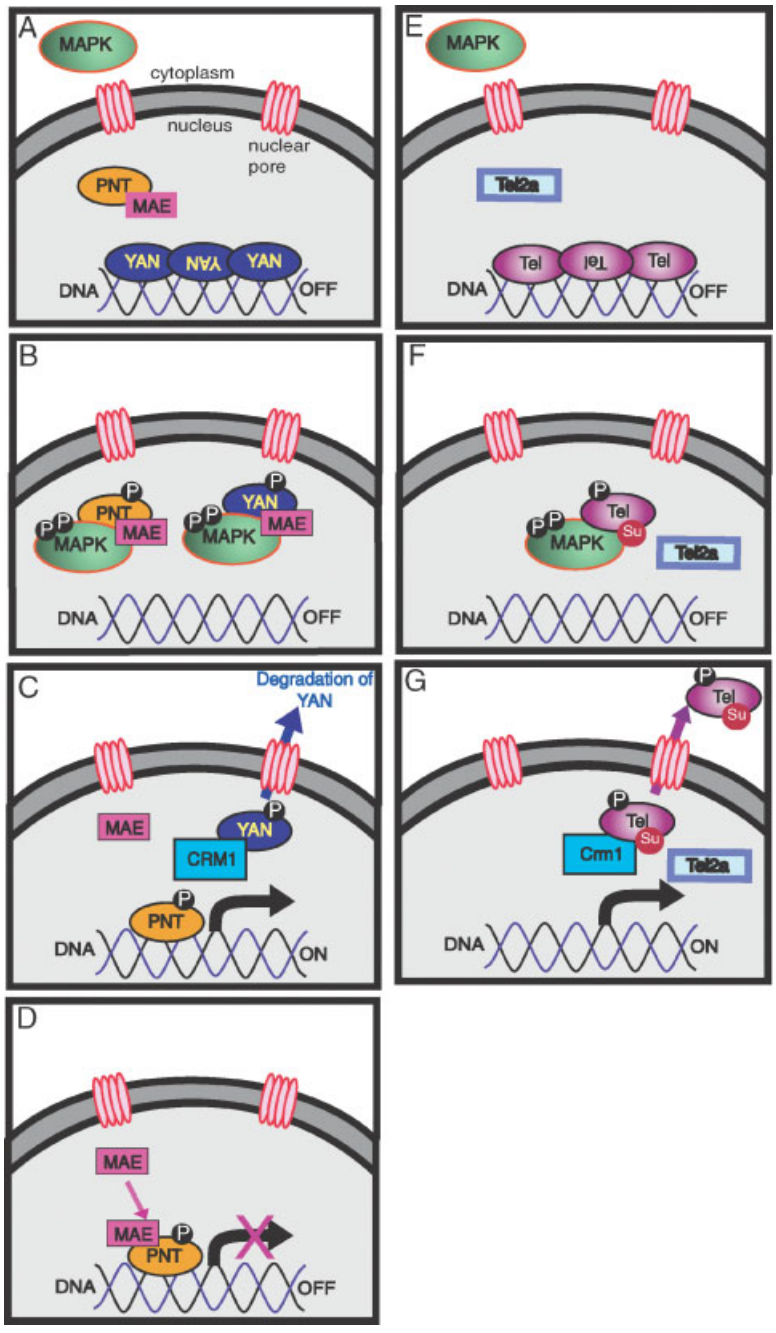

Figure 1. Phosphorylation triggers the downregulation of YAN and Tel. **A–D:** Series of events whereby MAPK-mediated phosphorylation downregulates YAN and activates PNT-P2. **A:** In the absence of MAPK activation, unphosphorylated YAN oligomers outcompete PNT-P2 for access to ETS-binding Sites (EBSs) to repress transcription of target genes. **B:** In response to RTK signaling, activated di-phospho-ERK MAPK enters the nucleus and phosphorylates YAN and its antagonist PNT, in a process likely mediated by MAE. This breaks up the YAN polymer and removes it from the DNA, although the relative order in which these two events occur is not yet clear. **C:** The exportin CRM1 interacts with and exports YAN into the cytoplasm where it is ultimately degraded. Removal of YAN allows PNT-P2 to bind the EBSs and activate transcription of target genes. **D:** In a negative feedback loop, MAE binds PNT-P2 and attenuates transcriptional activation by an unknown mechanism. **E–G:** Series of events whereby MAPK-mediated phosphorylation and sumoylation downregulate Tel. **E:** Tel oligomers repress transcription in the absence of signaling; whether other ETS factors are specifically outcompeted is not known. **F:** Upon pathway activation, MAPK-mediated phosphorylation and sumoylation of Tel removes it from the DNA, thus abrogating transcriptional repression. It is unclear which modification occurs first. **G:** Tel then interacts with CRM1 and is exported to the cytoplasm, but is not degraded. It is possible that Tel2a plays a role analogous to that of MAE in these events. Abbreviations: PNT, PNT-P2; P, denotes phosphorylation; Su, denotes sumoylation.

While in vitro kinase assays have shown the ERK can directly phosphorylate YAN and Tel,[45,58] other studies have revealed that phosphorylation of YAN by ERK at S127 is mediated by MAE (Modulator of Activity of ETS), which interacts with YAN via a PD–PD interaction.[59] Thus according to the current model, YAN-MAE interactions depolymerize YAN, exposing the critical S127 phosphorylation site and facilitating ERK-mediated phosphorylation and subsequent abrogation of transcriptional repression.[17,20,59] While no mammalian orthologs of *mae* have been identified yet, a second *Tel*-like gene, referred to as *Tel2* or *TelB*, encodes a splice variant, *Tel2a*, that yields a PD-containing protein with 39% identity to MAE.[60–62] Thus Tel2a could potentially modulate Tel phosphorylation and activity analogously to how MAE regulates YAN.

In addition to being regulated by phosphorylation, Tel is also sumoylated (Fig. 1F, G). The E2 SUMO-conjugating enzyme

UBC9 interacts with the PD of Tel, with K99 providing the predominant SUMO-1 modification site.[63,64] SUMO-modified Tel localizes to nuclear bodies termed Tel-bodies, which are transient structures formed during S phase.[65] Tel K99R, which cannot be sumoylated, cannot be exported from the nucleus or localize to Tel-bodies, and functions as a better transcriptional repressor than wild-type Tel.[64] These results suggest that SUMO modification contributes to the abrogation of transcriptional repression and nuclear export of Tel and that Tel bodies may be the loading docks for nuclear export.

While both phosphorylation and sumoylation appear to be required for nuclear export of Tel,[45,64] the order in which Tel is phosphorylated and sumoylated is unclear, as is whether the two types of modifications function cooperatively or independently. Intriguingly, the other ETS members known to be regulated by phosphorylation-mediated nuclear export, NET and YAN, also contain putative SUMO acceptor sites,[64] suggesting that phosphorylation and sumoylation may generally work in concert to mediate the downregulatory nuclear export of transcriptional repressors.

Conserved mechanisms of activation: Ets1 and PNT-P2

The mammalian transcriptional activator Ets1 and its *Drosophila* ortholog PNT-P2 provide prime examples, backed by extensive in vivo validation, of how post-translational modifications can exert distinct context specific effects on transcription factor activity. Like YAN and Tel, PNT-P2 and Ets1 possess an amino-terminal PD and a carboxy-terminal ETS DNA-binding domain.[5] Ets1 is an oncoprotein implicated in mediating the invasiveness and angiogenesis of a variety of cancers and the differentiation of all lymphoid lineages during normal development.[5,66,67] PNT-P2 acts antagonistically to YAN (Fig. 1A–D), promoting differentiation and proliferation by competing for access to target gene promoters in multiple developmental contexts.[68–71] Whether Tel and Ets1 similarly compete for target sites is currently not known. As discussed below, Ets1 and PNT-P2 are regulated by both similar and distinct post-translational modifications that influence DNA binding, protein–protein interactions, and transcriptional activation (Figs. 1A–D, 2).

MAPK-mediated phosphorylation positively regulates the transcriptional activation functions of both Ets1 and PNT-P2 and in further contrast to its effects on YAN and Tel, does not alter DNA binding, subcellular localization or protein stability.[72,73] In response to RTK pathway activation, the MAPK ERK phosphorylates PNT-P2 amino-terminally to its PD at T151 and Ets1 at the analogous residue T38.[58,72] This phosphorylation event is required for PNT-P2 mediated transcriptional activation (Fig. 1C) and for Ets1 to function in ternary complexes with AP-1 to activate RAS-responsive elements (RREs) (Fig. 2A).[53,72,73] Revealing the physiological relevance of ERK-mediated phosphorylation, transgenic mice carrying the analogous alanine substitution mutation (T72A) in the paralogous Ets-2 protein exhibit defects consistent with a hypomorphic loss-of-function allele.[74] Similarly,

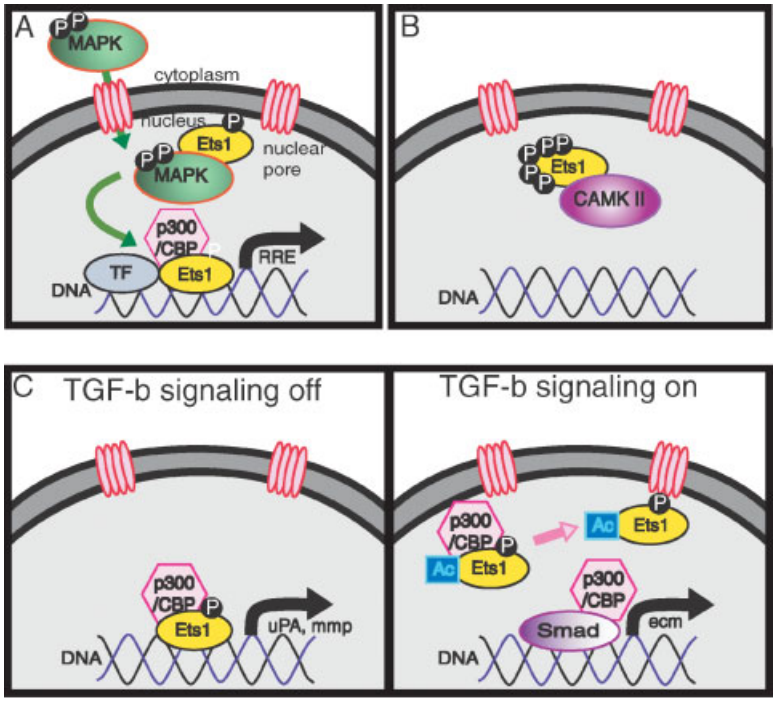

Figure 2. Ets1 is regulated by multiple post-translational modifications. **A:** MAPK-mediated phosphorylation of Ets1 leads to recruitment of the coactivator p300/CBP and transcriptional activation. **B:** CAMK II phosphorylation of Ets1 inhibits DNA binding and transcriptional activation. **C:** Sequence of events illustrating the antagonism between TGFβ signaling and Ets1 activity. In the absence of signaling, TGFβ OFF, Ets1 and its coactivator p300/CBP activate transcription of uPA and MMP to promote ECM breakdown. Pathway activation, TGFβ ON, results in acetylation of Ets1 and subsequent dissociation of p300/CBP, thus removing Ets1-mediated transcriptional activation and allowing p300/CBP to interact with SMADs and activate transcription of ECM maintenance proteins. Abbreviations: uPA, urokinase plasminogen activator (serine protease); MMP, matrix metalloproteinases; ECM, extracellular matrix; TF, transcription factor such as AP-1 that forms ternary complex with Ets-1; RRE, Ras-responsive element; P, denotes phosphorylation; Ac, denotes acetylation.

the T151A mutation in *Drosophila* PNT-P2 impairs in vivo function.[58]

Mechanistically, how might ERK-mediated phosphorylation of this critical residue potentiate transcriptional activation? Structural studies of the PD-containing N terminus of Ets1 revealed that T38 resides in a flexible unstructured region that is not altered upon phosphorylation, raising the possibility that phosphorylation influences interactions with specific binding partners, rather than intrinsic activity of Ets1.[75] In fact, recent results suggest phosphorylation of Ets1 at T38 promotes binding to the coactivators p300/CBP, leading to enhanced transcriptional activation (B. Graves, personal communication).

In contrast to the stimulatory effects of ERK-mediated phosphorylation, phosphorylation of Ets1 by calcium calmodulin-dependent protein kinase II (CAMK II) or by myosin light chain kinase (MLCK) on multiple sites near the ETS DNA-binding domain inhibits DNA binding by promoting or stabilizing an autoinhibitory structural conformation and by decreasing protein stability, and has even been postulated to convert Ets-1 from an activator to a repressor[5,76–78] (Fig. 2B). PNT-P2 lacks these consensus sites[79] and therefore is unlikely to be identically regulated although it possible that other phosphorylation-mediated events might similarly negatively regulate its activity. Adding further complexity, phosphorylation of Ets-1 by protein kinase C alpha (PKCα) at unknown sites likely in or near the autoinhibitory domain, may potentiate transcriptional activation in a calcium-independent process, although further investigations will be required to assess the in vivo significance of such regulation.[80] Thus fine-tuning of Ets1 activity by distinct but antagonistic phosphorylation events illustrates how post-translational modifications in response to different signaling pathways may be used as a means of information integration.

In addition to the versatile regulation provided by multisite phosphorylation, studies investigating the antagonism between Ets1 and TGFβ signaling in the context of regulation of extracellular matrix (ECM) proteins have revealed an important role for acetylation in modulating Ets1 activity (Fig. 2C). TGFβ stimulation leads to rapid and prolonged acetylation of Ets1, but has no effect on its phosphorylation.[81] Acetylation of Ets1 results in dissociation of the p300/CBP-ETS1 complex, releasing p300/CBP to interact with and potentiate the activity of transcription factors downstream of TGFβ signaling, or SMADs (Fig. 2D). The competition for limiting amounts of the coactivator and acetyltransferase p300/CBP exhibited by Ets1 and TGFβ signaling components will likely prove to be a broadly used mechanism of transcriptional regulation.

In conclusion, Ets1 is differentially regulated by both multisite phosphorylation and acetylation (Fig. 2), although it does not appear to be regulated by both modifications at the same time, at least in the specific contexts that have been investigated. *Drosophila* PNT-P2 is also regulated by phosphorylation (Fig. 1A–D), and it has not been determined whether it is otherwise modified. It will be critical to our understanding of the regulation of this subfamily of ETS transcription factors to elucidate all the post-translational modifications that occur and the interplay, or lack thereof, between them.

Signaling via Her2/Neu regulates Er81 by multiple post-translational modifications

The transcriptional activator Er81 provides another example of how coordinated and/or antagonistic phosphorylation, acetylation and ubiquitin-mediated degradation modulates protein–protein interactions, protein–DNA interactions, transcriptional activity, and protein stability (Fig. 3). Er81, along with the closely related Pea3 and Erm proteins, has been implicated in mammary tumor development in *Her2/Neu* transgenic mice and belongs to the subfamily of ETS transcriptional activators which lack PDs.[4,82]

Er81 is phosphorylated at multiple sites in response to signaling downstream of the HER2/Neu RTK by ERK and p38 MAPKs[83] (Fig. 3A). Transcriptional activation is enhanced by ERK- or p38-mediated phosphorylation of Er81 at three sites (T139, T143, S146) and by a MAPK-stimulated protein kinase, Msk1 (or Rsk1)-mediated phosphorylation at two additional sites (S191, S216) (Fig. 3B).[83–86] Mutation of all five phosphoacceptor sites to alanine severely compromises, but does not abolish, Her2/Neu signaling induced transcriptional activation, suggesting additional modifications at other sites may be involved as discussed below.

Protein kinase A (PKA) recognizes similar sequences to Msk1 and indeed phosphorylates ER81 at S191 and S216, although S334 appears to be the preferred site in vivo (Fig. 3C).[85] Phosphorylation at S334 reduces DNA binding but enhances transcriptional activation by Er81.[85] As phosphorylation of the highly related ETS transcription factor Erm by PKA causes a conformational change resulting in decreased DNA binding and increased transcriptional activation, it is likely that PKA phosphorylation also structurally alters Er81.[87] While the two outcomes of phosphorylation at S334 seem counterintuitive, decreased DNA binding may prevent activation of low-affinity promoters, but have no effect on those with high affinity. Thus changing DNA affinity may be a fundamental strategy for determining target specificity of transcription factors, including Er81.

While the phosphorylation events discussed above all positively regulate Er81 activity, Er81 is also negatively regulated by phosphorylation (Fig. 3F). MAPK-activated protein kinase 2 (Mk2), which functions downstream of p38, phosphorylates Er81 at S191 and S216, the latter site being in the inhibitory domain of Er81, and suppresses basal transcriptional activity.[88] By competing with activating kinases for access to S191, Mk2 passively blocks transcriptional activity of Er81, and by targeting a distinct residue, S216, Mk2 actively blocks transcriptional activation.[88] Thus Mk2 may both inhibit Er81

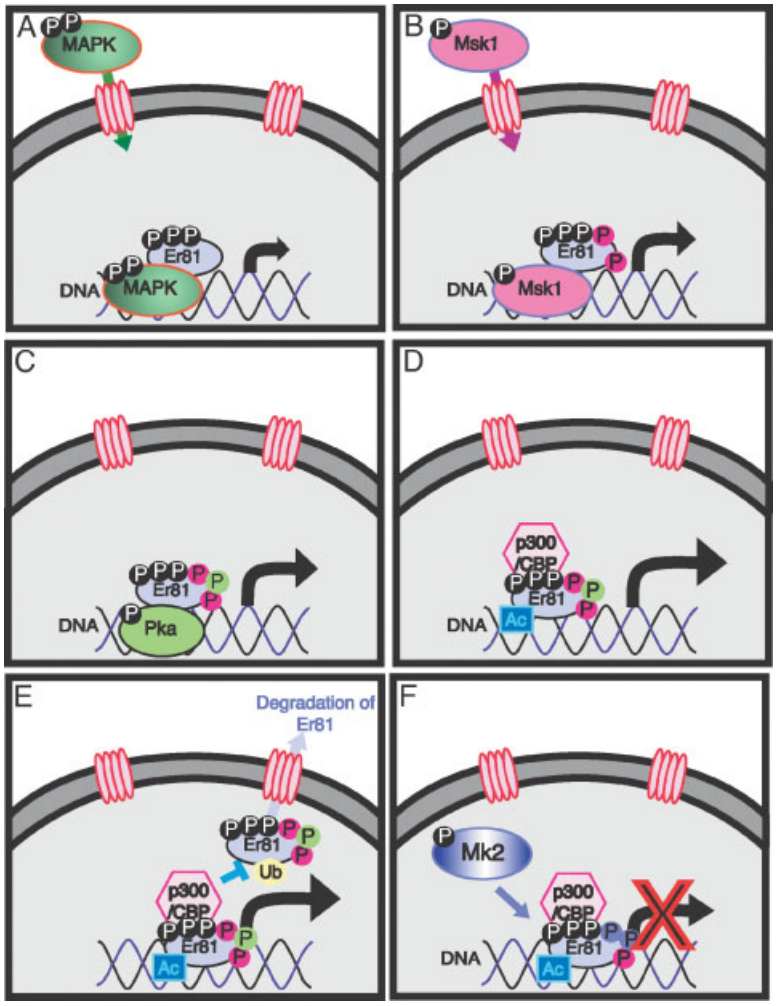

Figure 3. Her2/Neu signaling initiates a series of post-translational modifications both positively and negatively regulating Er81. **A:** Her2/Neu RTK activates MAPK, which phosphorylates Er81 on multiple residues (black circled P's), turning on transcription. **B:** Msk1 is also activated by the signaling event, and phosphorylates Er81 at two additional sites (pink circled Ps), increasing the transcriptional activity of Er81. **C:** Msk1 also activates Pka, which can phosphorylate Er81 at the same sites as Msk1, plus an additional site (green circled P). **D:** These phosphorylation events, and MSK-1 mediated activation of p300/CBP, results in the acetylation of Er81. All of the modifications are required for maximal transcriptional activation by Er81. **E:** Acetylation of Er81 inhibits ubiquitin mediated degradation. **F:** By a negative feedback loop, Her2/Neu signaling leads to another phosphorylation event by Mk2 (blue circled Ps), resulting in removal of transcriptional activation and converting Er81 into a repressor. Abbreviations: P, denotes phosphorylation; Ac, denotes acetylation.

transcriptional activation in the absence of signal and attenuate activation in response to signal.

A second consequence of Her2/Neu signaling is acetylation of Er81 at two lysine residues in its TAD, K33 and K116 (Fig. 3D).[89] Acetylation at K116 by either p300 or P/CAF enhances Er81's affinity for DNA, most likely due to a conformational change allowing the ETS domain to bind DNA better, and increases the potency of Er81's amino-terminal TAD, likely by recruiting coactivators or chromatin-remodeling complexes[89,90] (Fig. 3E). Additionally, acetylation of either K33 or K116 increases the in vivo half-life of Er81.[89] While acetylation often increases protein stability by masking the Ks that are to be ubiquitinated, thereby blocking proteasome-mediated degradation,[32] this is not the case for Er81, suggesting that acetylation at K33 and K116 prevents the ubiquitination of other Ks by inducing a conformational change and/or altering interactions with proteins that shield Er81 from or target it to ubiquitin ligases.[89]

Interestingly CBP/p300 potentiation of Er81 transcriptional activation leads to phosphorylation at S191 and S216,[91] the sites targeted by the inhibitory Mk2.[88] This suggests that Her2/Neu activation first leads to MAPK phosphorylation of Er81, then phosphorylation by Msk1 and acetylation by CBP/p300/P/CAF, and lastly Mk2 phosphorylation of Er81. Thus Her2/Neu signaling activates Er81 to multiple levels, which presumably results in context-specific differential expression of target genes, and then attenuates this activation. Adding further complexity, Er81, in a complex with CBP/p300, activates the *Her2/Neu* promoter, creating a positive feedback loop that likely modulates the level and duration of signaling.[83]

In conclusion, multiple post-translation modifications on a transcription factor provide extraordinary possibilities for combinatorial integration of information. In this light, the ETS transcription factor Er81, which is post-translationally modified on at least nine residues, seven S/Ts and two Ks, by at least five kinases and two acetyltransferases provides an ideal focus for

future investigations into the complexity of the language of post-translational modifications and the enormous potential that it conveys for generating transcriptional specificity.

Antagonism between phosphorylation and sumoylation: Elk-1

Elk-1 belongs to the ternary complex factor (TCF) subfamily of the ETS transcription factors.[92–94] TCFs act through a nucleoprotein complex composed of a TCF, a serum-response factor (SRF), and a serum-response element (SRE), which is composed of adjacent DNA-binding sites for the two transcription factors. In response to growth signals and cellular stress, MAPK signaling leads to the phosphorylation of the TADs of TCFs and induction of their activities as transcriptional activators.[92–94]

Although its membership within the TCF subgroup implies an important role in mediating the rapid transcriptional response to extracellular signals and hence a likely involvement in the pathogenesis of human cancer, the physiological role of Elk-1 during development and adult life remains poorly understood as mouse knockouts appear viable and lack obvious defects.[95] Studies in vitro and in cultured cell systems, where issues of functional redundancy are less problematic, have demonstrated that Elk-1 functions as both a transcriptional activator and a repressor with the former activity stimulated by phosphorylation and the latter by sumoylation (Fig. 4A–D).

Members of all three MAPK subgroups, ERK, JNK and p38, phosphorylate Elk-1 at multiple residues within the TAD, with S383 being the first site targeted.[96–99] Multiple phosphorylation events on Elk-1 cause a conformational change that alters intramolecular interactions between the ETS domain and the TAD, resulting in increased DNA binding and transcriptional activation (Fig. 4C).[100,101] Contributing to enhanced transcriptional activation, phosphorylation of Elk-1 is necessary for protein–protein interactions with the Mediator complex.[102] Interestingly, phosphorylation is not required for Elk-1 binding to the co-activator CBP, but is required to make the complex transcriptionally productive.[103] Elk-1 interactions with the related coactivator p300 are also affected by phosphorylation, via altered protein–protein interactions that result in increased acetyltransferase activity and transcriptional output.[104] These data imply that Elk-1 is in a protein complex with a coactivator, either CBP or p300, prior to MAPK-mediated phosphorylation and activation (Fig. 4A), allowing for faster response to extracellular signaling.

In the absence of MAPK signaling, both the ETS domain and an inhibitory domain, called the R motif, recruit corepressors and suppress the activity of the Elk-1 TAD, maintaining the TCF in an inactive state.[105,106] Alanine scanning mutagenesis of the R motif revealed that the conserved residues K249 and E251 are important for repressive activity.[107] Subsequent sequence analysis identified two SUMO consensus sites within the R motif, K230 and K249, leading to the hypothesis that sumoylation may regulate R motif mediated repression.

Blocking sumoylation by mutating the SUMO modification sites (K230R/K249R), expressing dominant negative UBC9, or expressing the SUMO-specific protease SSP3, increases Elk-1 transcriptional activity in the absence of MAPK activation. This suggests that sumoylation plays a role in repressing the basal level the Elk-1 transcriptional activity, likely in part via recruitment of the histone deacetylases 1 and 2 (HDAC-1 and HDAC-2) (Fig. 4B).[107,108] Simultaneous activation of the ERK MAPK pathway and inhibition of sumoylation produce a synergistic increase in transcriptional response, indicating that the ERK and SUMO pathways function antagonistically to control Elk-1 transactivation potential.[107] Furthermore, these two post-translational modifications appear to directly antagonize each other as activation of the ERK MAPK pathway leads to both an increase in the level of phosphorylation and a decrease in the level of sumoylation (Fig. 4C).[107] Thus MAPK-mediated phosphorylation of Elk-1 both directly and indirectly enhances transcriptional activation, by potentiating activity of the TAD and by inhibiting sumoylation of the R motif, respectively. Adding an additional layer of complexity, sumoylation at three sites (K230, K249, K254) has recently been shown to influence the nucleocytoplasmic shuttling of Elk-1, thereby regulating its nuclear retention and potentially affecting transcriptional output.[109] Whether this influences access to MAPK and phosphorylation of Elk-1 remains to be investigated.

Finally, MAPK-mediated phosphorylation of Elk-1 at S383 not only leads to transcriptional activation but also initiates a temporally delayed negative feedback loop that involves recruitment of a corepressor mSIN3A-HDAC1 complex to Elk-1 occupied promoters, thereby limiting the duration of response by reverting Elk-1 to a repressive state (Fig. 4D).[105] This situation is highly reminiscent of the case of *Drosophila* PNT-P2, where MAPK-mediated phosphorylation initially stimulates transcriptional output, but eventually attenuates the response in a process likely to involve interactions with MAE (Fig. 2A–D). How the temporal delay is achieved is not yet understood in either case, but the two examples highlight how a single post-translational modification can regulate both the initiation and duration of a transcriptional response.

Cooperation between phosphorylation and glycosylation: Elf-1

Elf-1 is the only ETS transcription factor known to be glycosylated and is one of the few proteins known to be phosphorylated and glycosylated at the same time (Fig. 4E–F).[110] Elf-1 is the defining member of a subfamily of ETS transcription factors that lack a PD and is expressed in a broad range of tissues including those of the hematopoietic system.[111–114] Generally associated with regulating cell growth and differentiation, upregulation of Elf-1 has been observed in a variety

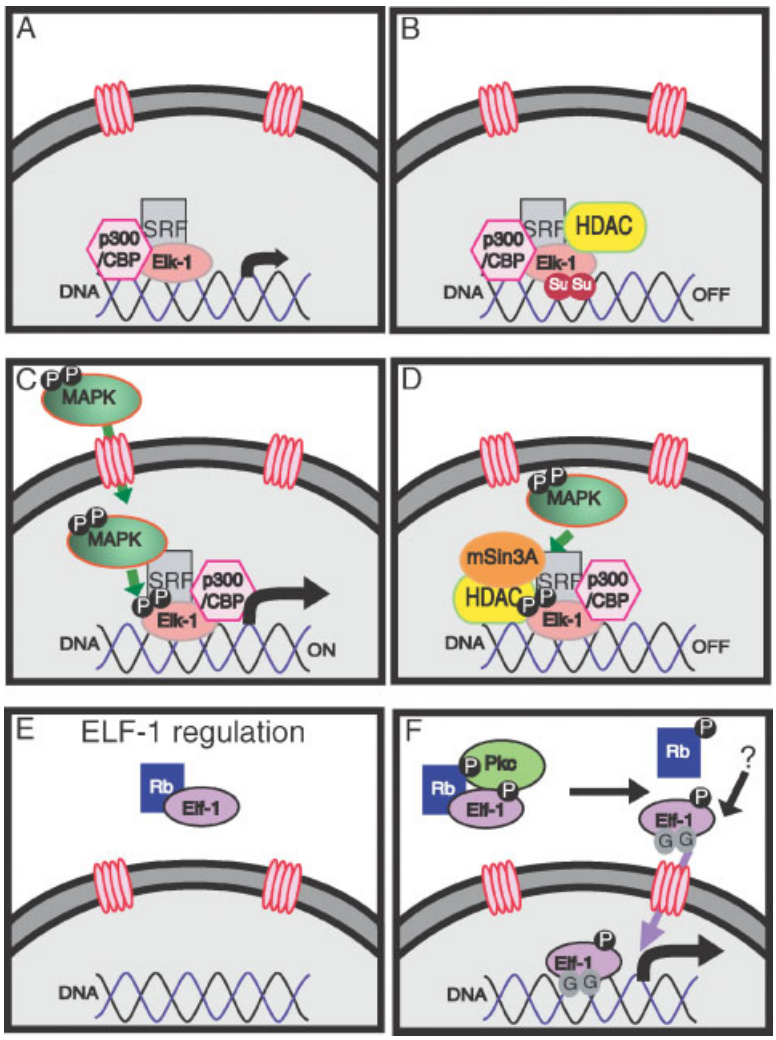

Figure 4. Elk-1 and Elf-1 are regulated by multiple post-translational modifications. **A–D:** Elk-1 is negatively regulated by sumoylation and both negatively and positively regulated by phosphorylation. **A:** In the absence of signaling, the ternary complex of Elk-1 and SRF is bound to the DNA in a complex with p300/CBP, resulting in a basal level of transcriptional induction. **B:** Complete repression of ternary complex-mediated transcription requires sumoylation of Elk-1, which leads to recruitment of HDACs. **C:** MAPK-mediated signaling results in phosphorylation of Elk-1, which alters its interaction with p300/CBP, resulting in transcriptional activation. **D:** By a negative feedback loop, MAPK phosphorylation of Elk-1 also leads to interactions with a corepressor complex, mSin3A/HDAC1, resulting in transcriptional repression and attenuating the response. **E–F:** Elf-1 is positively regulated by phosphorylation and glycosylation. **E:** Elf-1, which is both phosphorylated and glycosylated, is held in the cytoplasm by interactions with Rb. **F:** Both Rb and Elf-1 are further phosphorylated by PKC, resulting in dissociation of the complex and nuclear localization of Elf-1. Additional glycosylation also correlates with and contributes to nuclear localization of Elf-1, although as indicated by the arrow and question mark, the upstream signaling cues that regulate this event are unclear. Abbreviations: SRF, serum response factor; HDAC, histone deacetylase; Rb, retinoblastoma protein; P, denotes phosphorylation; G, denotes glycosylation; Su, denotes sumoylation.

of cancers, including prostate, ovarian, breast, osteosarcoma and leukemia/lymphoma.[115,116]

Studies of Elf-1 reveal that differential phosphorylation and glycosylation regulate subcellular localization, protein–protein interactions and protein–DNA interactions during T-cell activation (Fig. 4E,F).[25,110] Elf-1 is dynamically distributed between the cytoplasm and nucleus and migrates at two distinct mobilities, each larger than the predicted 68 kDa and each the result of complex patterns of phosphorylation, glycosylation and perhaps other modifications, that have not yet been mapped to individual residues. The 80 kDa form of ELF-1 is cytoplasmic, while the 98 kDa form is nuclear.[110]

Cytoplasmic sequestration of Elf-1 occurs via interactions with the retinoblastoma (Rb) protein[117] which preferentially binds the less extensively modified 80 kDa form (Fig. 4E).[110] Upon T-cell activation, an increase in both phosphorylation and glycosylation converts Elf-1 to the 98kDa form, resulting in dissociation of the Rb-Elf-1 complex and translocation of Elf-1 to the nucleus (Fig. 4F).[110,117]

In addition to promoting the nuclear localization of Elf-1, phosphorylation and glycosylation also modulate other aspects of Elf-1 function. For example, both modifications enhance Elf-1 DNA-binding activity with respect to at least one target promoter, that of the TCR ζ-chain gene.[25,110] Both modifications are required for maximal activation of this promoter, indicating that, in contrast to other transcriptional regulators such as Myc, where glycosylation and phosphorylation act antagonistically by competing for access to identical S/T residues,[118] in Elf-1, the two modifications target distinct residues and function cooperatively. Adding further complexity, conversion to the 98 kDa form decreases protein stability, although whether this is a consequence of increased phosphorylation, increased glycosylation or both remains to be determined (Fig. 4F).[110]

In conclusion, the contribution of O-GlcNAc modification to transcription factor activity remains in the initial stages of exploration. Studies of the ETS protein Elf-1 have expanded our view of how glycosylation and phosphorylation may either cooperatively or antagonistically target the same or distinct residues to influence transcription factor function. Given its potential role in coordinating the nutritional status of the animal with other developmental signaling cues, the extent and manner in which glycosylation is used to modulate transcription factor activity remains an important area for future investigations.

Conclusions

As illustrated by the examples discussed above, ETS family transcription factors provide an ideal context in which to investigate the multitude of strategies whereby post-translational modifications influence specificity of the transcriptional response under distinct signaling conditions. While it is impossible to deduce a priori how widespread different post-translational modifications will be within the ETS family, or within other collections of transcriptional regulators, given the enormous potential for exquisitely precise and dynamic regulation, it would seem logical that a broad variety of nuclear regulatory circuits will employ similar strategies for fine-tuning transcriptional output. Improved proteomic methodologies to identify sites of modification, and to follow changes in post-translational modification in response to different signaling conditions, should greatly enhance our ability to address this question.

Thus the model that is emerging, as exemplified from the studies of ETS transcription factors described here, is that the order, timing and combinations in which different post-translational modifications are added and removed provide the cell with an enormous repertoire of regulatory options. Specifically, the potential for multiple layers of co-operativity and/or competition among different modifications in response to distinct upstream signals yields immense regulatory opportunities that the cell almost certainly taking advantage of and that we are only beginning to appreciate.

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