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Review

Minireview

Post-translational protein modification as a tool for transcription reprogramming

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Summary

Precise modulation of transcription plays a vital role in both development and the response of all higher organisms to their environment. Temporal activation or repression of specific genes is accomplished via a plethora of transcriptional regulators. However, relatively little is known about how the activities of these proteins are controlled. Recent findings indicate that post-translational modifications fine-tune the function of transcription regulators by affecting their localization, conformation or stability. Here, we discuss these regulatory mechanisms in the context of the plant immune response. This system lends itself particularly well to studies of transcriptional regulators as activation of plant immunity is associated with rapid and dramatic reprogramming of the transcriptome. A case study of the plant immune coactivator NPR1 (nonexpressor of pathogenesis-related (*PR*) genes 1) illustrates that transcription regulator activity may be controlled by redox-based modifications of cysteine thiols (e.g. disulphide bonding and *S*-nitrosylation), phosphorylation, and ubiquitinylation coupled to protein degradation. Importantly, cross-talk between distinct protein modifications may determine the spatial and temporal activity of transcription regulators that in turn profile the cellular transcriptome.

Introduction

The eukaryotic transcriptome is highly dynamic and changes considerably through time due to developmental progression and circadian rhythms, and in response to the environment. Changes in gene expression are controlled by a large array of transcriptional regulators, some of which are repressors while others are activators. Although many such proteins have now been discovered in a variety of organisms, the mechanisms by which their activity is controlled remains largely elusive. In this mini-review we will focus on recent advances in our understanding of transcription control in plant immunity. Here, the reprogramming of gene expression is largely orchestrated by the signalling molecules salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). These molecules modulate the activity of downstream transcription regulators that control a large set of defence genes. We will focus particularly on the SA-responsive

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coactivator NPR1 (nonexpressor of pathogenesis-related (*PR*) genes 1) (Dong, 2004), one of the most intensively studied factors in plant immunity, as the modulation of its activity may be a paradigm for the control of transcription regulators in general. Interestingly, the activity of NPR1 is regulated by several post-translational modifications that occur at distinct cellular locations, yet communicate through changes in protein homeostasis.

Redox-based control of transcription regulators

Regulatory thiol-disulphide bonding controls transcription regulator conformation

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can cause serious injury to cells, including DNA damage, lipid oxidation and malfunction of protein activity. Nevertheless, cells have adapted to cope with the highly reactive nature of ROS and RNS and even utilize these small molecules as potent cues to control cellular protein homeostasis, development, and immune responses. While these signal molecules often cause detrimental protein oxidation, the reversible oxidative modifications of cysteine residues play pivotal roles in redox-based signal transduction (Hess *et al.*, 2005). Accumulating evidence indicates that ROS and RNS largely signal through post-translational cysteine thiol modifications, providing robust spatial and temporal control of protein conformation to fine-tune activity. Indeed, the bacterial transcription factor oxygen stress regulator (OxyR) was shown to be modified by distinct, reversible thiol modifications, including disulphide bonding, *S*-nitrosylation (covalent attachment of nitric oxide (NO)), *S*-glutathionylation (disulphide attachment of glutathione) and *S*-hydroxylation, all of which conferred different DNA-binding affinities and transcription activator activities (Kim *et al.*, 2002). Thus, regulatory thiol oxidation allows transcription activators to directly sense changes in redox conditions and respond rapidly according to cellular and physiological needs.

In plants, oxidative thiol modifications were shown to play an important role in modulating activity of the immune coactivator NPR1 (Dong, 2004). In unchallenged cells, conserved cysteines in NPR1 form intermolecular disulphide bonds, resulting in the formation of a cytosolic NPR1 oligomer (Mou *et al.*, 2003). Importantly, this renders NPR1 transcriptionally inactive as it is excluded from the nucleus. Upon pathogen attack, however, SA-mediated redox changes reduce the NPR1 oligomer to a monomer, allowing it to translocate to the nucleus and activate target gene expression. Intriguingly, SA-mediated redox changes also regulate the interaction of NPR1 with DNA-binding TGA transcription factors. Intramolecular disulphide bonds in TGA1 and TGA4 that preclude interaction with NPR1 are disrupted upon SA-induced cellular reduction, allowing

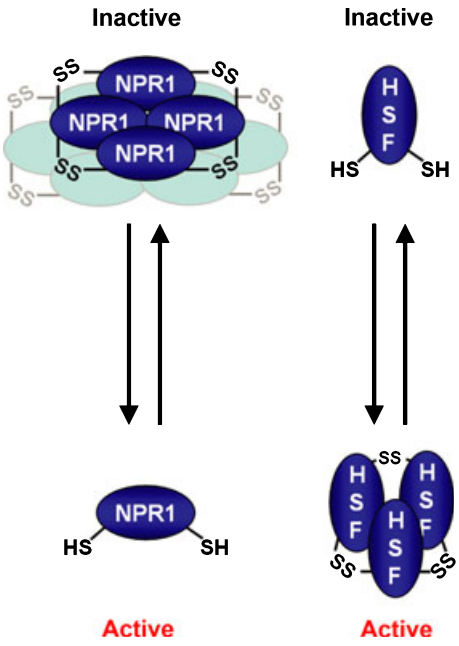

Fig. 1 Differential effects of cysteine disulphide bridges on transcription (co)activator activity. In resting cells, the NPR1 (nonexpressor of pathogenesis-related (*PR*) genes 1) coactivator forms a disulphide-mediated oligomer in the cytoplasm, which suppresses its activity. Upon pathogen infection, cellular reduction promotes the formation of a transcriptionally active monomer that translocates to the nucleus. By contrast, the HSF1 (heat shock factor 1) activator exists as a cytoplasmic monomer in yeast and mammalian cells. Upon stress induction, cellular oxidation facilitates the formation of a disulphide-mediated trimer, allowing this transcriptionally active form of HSF1 to enter the nucleus and activate gene expression.

these TGAs to form a transcriptionally active complex with NPR1 in the nucleus (Després *et al.*, 2003). By contrast, the mammalian transcription factor heat shock factor 1 (HSF1), which plays a critical role in protecting cells from diverse environmental and physiological stresses, is activated by oxidation rather than reduction of two redox-sensitive cysteines within its DNA-binding domain. This leads to HSF1 trimerization, nuclear translocation, and transcription activation of its target genes (Ahn & Thiele, 2003). Taken together, these studies indicate that redox-sensitive transcription (co)activators can form transcriptionally active complexes in response to thiol oxidation or reduction to coordinately translate cellular redox information into gene expression (Fig. 1).

Thiol-disulphides are controlled by opposing action of oxidative and reductive systems

Unregulated disulphide linkage could result in the aggregation and misfolding of proteins, producing potentially fatal physiological conditions. So how is the formation of disulphide bonds controlled? Oxidation and reduction of thiols are regulated by both enzymatic and nonenzymatic systems.

For example, the yeast Yes-associated protein 1 (Yap1) transcription factor is activated by hydrogen peroxide (H_2O_2), leading to the formation of an intramolecular disulphide bond between Cys303 and Cys598. Interestingly, these cysteines are not directly oxidized by H_2O_2, but through glutathione peroxidase-like 3 (Gpx3). H_2O_2 perception by Gpx3 generates a mixed disulphide bond between Gpx3 and Yap1. Subsequently, the mixed disulphide is resolved into a Yap1 intramolecular disulphide bond that masks the nuclear export signal, allowing Yap1 to exert its function in the nucleus (Delaunay *et al.*, 2002).

In plants, it was shown that *S*-nitrosylation may also have a profound impact on disulphide bond formation. SA induces *S*-nitrosylation of the NPR1 coactivator at Cys156, which is located at a predicted multimerization interface. Whereas NO donors strongly promoted the formation of intermolecular disulphide linkages between wild-type NPR1 monomers, mutation of Cys156 abolished NO-facilitated oligomerization. Surprisingly, in response to SA the Cys156-mutated protein completely failed to accumulate and thus could not activate immunity upon subsequent pathogen challenge. Importantly, this indicates that, although NO-induced NPR1 oligomer formation antagonizes the function of the transcriptionally active monomer, it does not suppress immunity in the long term. This apparent discrepancy may be explained by the requirement for *S*-nitrosylation in maintaining NPR1 protein homeostasis in order to guarantee a steady supply of transcriptionally active monomer upon future pathogen challenge (Tada *et al.*, 2008).

Enzymatic pathways for the removal of oxidative thiol modifications also exist. One reducing system, consisting of thioredoxin (TRX) and thioredoxin reductase, has attracted particular attention because of its involvement in many disorders and diseases. In yeast, nuclear TRX was shown to inactivate the Yap1 transcription factor by reducing its intramolecular disulphide bond, resulting in nuclear export (Izawa *et al.*, 1999). By contrast, cytosolic TRXs in plants were shown to be required for the SA-induced reduction of the NPR1 coactivator from oligomer to active monomer (Tada *et al.*, 2008). Thus, as is the case for thiol oxidation, TRX may positively or negatively impact gene transcription depending on cell type, compartment, and redox-modified transcription regulator. Interestingly, in addition to the reduction of disulphide bonds, human TRX was identified as a denitrosylase that specifically removes NO from *S*-nitrosylated proteins, providing a powerful mechanism to regulate *S*-nitrosylation (Benhar *et al.*, 2008).

Because thiol oxidation and reduction often have opposing effects on the activity of a transcription regulator, the question seems to be how these modifications are temporally regulated. This was in part addressed for the opposing actions of thiol *S*-nitrosylation and TRX-mediated thiol reduction on NPR1, because both of these modifications are required for full-scale SA-induced plant immunity. Crucially, it was shown that SA transiently triggers nonoverlapping oxidative and reductive phases that are translated into changes in NPR1 oligomer/monomer conformation. Accordingly, NPR1 appears to activate target genes only during the reductive phases (Mou *et al.*, 2003; Tada *et al.*, 2008; Spoel *et al.*, 2009). Thus, regulated redox fluctuations may define specific windows for coactivator (in)activity. It is unclear how defence proteins (such as NPR1) but not unrelated household proteins are specifically targeted by cellular redox fluctuations. It is plausible that regulated recruitment of TRXs can locally protect certain proteins from the changing redox environment. Moreover, several other reductive systems (e.g. glutaredoxins and protein disulphide isomerases) are present in a variety of cellular compartments and may all contribute to shaping the redox status of the proteome.

Utilizing ubiquitin-mediated proteasome activity to control transcription

Proteasome-mediated suppression of transcription

While transcription activation has been intensely studied, the ability to shut down transcription also needs to be investigated, as both are equally important for cell survival. Suppression of transcription was initially thought to be regulated solely by repressors. However, an alternative way of controlling transcription could involve the destruction of factors that promote transcription. Transcriptome analysis of yeast treated with a proteasome inhibitor indicated rapid up-regulation of a large set of genes involved in a variety of processes, including protein degradation, cell cycle and stress responses (Fleming *et al.*, 2002; Bhaumik & Malik, 2008). Subsequently, using chromatin immunoprecipitation in conjunction with DNA microarray technology, it was shown that the proteasome associates with many gene loci (Auld *et al.*, 2006). These and other studies imply that the 26S proteasome plays an important role in suppressing the activity of a large set of genes in eukaryotic genomes (Bhaumik & Malik, 2008).

How does the proteasome restrict gene transcription? In embryonic stem cells, the proteasome is specifically recruited to intergenic sequences to suppress permissive gene transcription. Suppression is accomplished by the targeted turnover of RNA polymerase II (PolII) and general transcription factors of the transcription pre-initiation complex (PIC) (Fig. 2a) (Szutorisz *et al.*, 2006). In plants, it was previously shown that the proteasome targets not only general but also gene-specific transcription activators. The DNA-binding transcription factor ethylene-insensitive 3 (EIN3) is a potent activator of ET-responsive genes. In the absence of ET, however, EIN3 is continuously targeted by the F-box proteins EBF1/EBF2 (EIN3-binding F-box

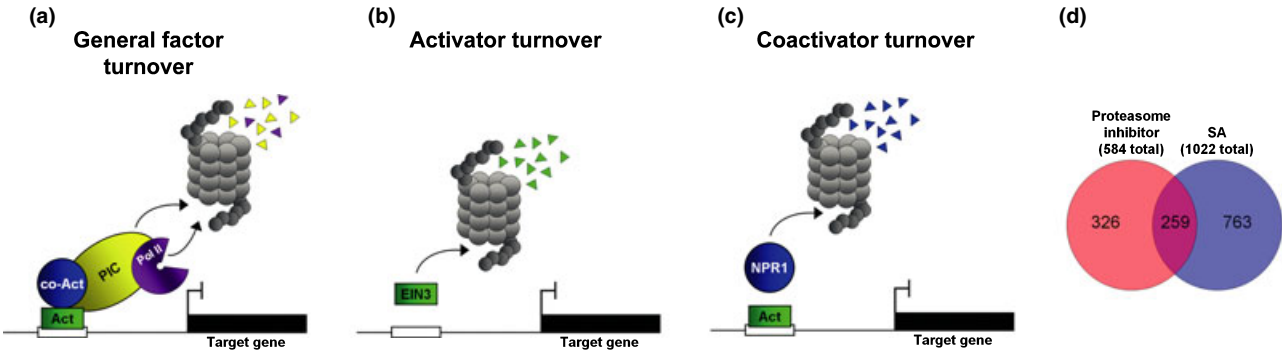

Fig. 2 Proteasome-mediated suppression of gene transcription. The proteasome may suppress untimely gene transcription by a variety of different mechanisms. Gene transcription is silenced by (a) targeting general regulators such as the pre-initiation complex (PIC) and RNA polymerase II (PolII) for degradation, or (b, c) turning over (b) gene-specific transcription activators (Act) [e.g. ethylene-insensitive 3 (EIN3)] and (c) coactivators (e.g. NPR1 (nonexpressor of pathogenesis-related (*PR*) genes 1)). (d) Venn diagram of Arabidopsis genes induced by a proteasome inhibitor and salicylic acid (SA). Only genes induced ≥ 2-fold compared with the respective controls were considered (ANOVA, *P*-value < 0.05).

proteins 1 and 2) (Guo & Ecker, 2003; Potuschak *et al.*, 2003). EBF1/EBF2 are thought to recruit EIN3 to the Cullin1-based ubiquitin ligase $SCF^{EBF1/EBF2}$ (Skp1-Cullin-F box$^{EBF1/2}$), resulting in its ubiquitinylation and subsequent degradation by the proteasome. Genetic knock-out of EBF1/EBF2 results in accumulation of EIN3 and activation of ET-responsive genes. These findings indicate that proteasome-mediated degradation of gene-specific transcription activators may also silence untimely transcription (Fig. 2b).

In addition to DNA-binding factors, it was recently shown that gene-specific coactivators, which do not associate directly with DNA, may also be targets of the proteasome. In the absence of pathogen threat, the SA-responsive NPR1 coactivator was constitutively cleared from the nucleus in a proteasome-dependent manner (Spoel *et al.*, 2009). Failure to remove NPR1 from the nucleus resulted in activation of its target genes and constitutive disease resistance, indicating an important role for coactivator turnover in suppression of transcription (Fig. 2c). To estimate the genome-wide impact of proteasome activity on SA/NPR1 signalling, we analysed publicly available microarray data sets for seedlings that were pulse-treated for 3 h with SA or a proteasome inhibitor (http://affymetrix.arabidopsis.info). Strikingly, up to 44% of the 584 genes that were rapidly induced by proteasome inhibitor treatment also showed early responsiveness to SA (Fig. 2d). Such a dramatic overlap suggests that a large share of proteasome activity is devoted to genome-wide suppression of SA-dependent defence signalling. As constitutive SA-dependent defences are associated with detrimental fitness costs (Dong, 2004), the proteasome thus plays a crucial role in avoiding inappropriately high cellular energy demands. Taken together, the accumulating evidence indicates that the ubiquitin-mediated proteasome suppresses untimely transcription by targeted turnover of distinct activators at different stages during the assembly of a transcriptionally active complex (Fig. 2).

Promoting transcription by proteasome-mediated activator turnover

In animals, the ubiquitin-mediated proteasome has also been implicated in the activation, elongation, and termination of transcription (Collins & Tansey, 2006). Research in plants has also shown a dominant role for the proteasome in the control of transcription (Smalle & Vierstra, 2004). Gene activation through proteasomal degradation of repressors and corepressors appears to be a recurring theme in plant hormone signalling pathways, including JA, auxin and gibberellin signal transduction, and is reviewed excellently elsewhere (Santner & Estelle, 2009). In addition to (co)repressor proteolysis, turnover of transcription activators plays an important role in controlling the rate of transcription. Proteasome-dependent destruction of certain activators has long been recognized to limit or shut down the expression of target genes (Collins & Tansey, 2006; Kodadek *et al.*, 2006). More recently, however, emerging evidence indicates that (co)activator instability is also required for activation of transcription. Early indications of this discrepancy came from a study that found a striking overlap between transcription factor domains that regulate gene activation and domains that regulate ubiquitin-mediated degradation. Subsequently, the turnover of several important animal and yeast transcription activators was shown to activate gene expression (Collins & Tansey, 2006; Kodadek *et al.*, 2006; Bhaumik & Malik, 2008). In plants, the coactivator NPR1 was reported to activate target gene transcription in a similar manner. Upon induction of plant defence, SA-induced NPR1 is translocated to the nucleus where it activates transcription while becoming increasingly unstable. Pharmacological inhibition of the proteasome and genetic knockdown of Cullin 3 (CUL3), the ubiquitin ligase responsible for poly-ubiquitinylation of NPR1, both dramatically reduced NPR1 target gene transcription (Spoel *et al.*, 2009). This suggests that SA-

induced NPR1 turnover is necessary for full-scale activation of transcription.

How does activator turnover stimulate target gene transcription? To initiate transcription, gene-specific activators recruit general transcription factors of the PIC and finally PolII. The highly ordered recruitment of these transcriptional regulators is critical and probably thermodynamically favourable. Once transcription is successfully initiated, re-initiation of transcription is necessary to maintain a high rate of gene expression. Re-initiation may require promoter clearance to allow another round of ordered recruitment of transcription regulators orchestrated by (co)activators. Indeed, temporal analysis of a human oestrogen-responsive gene promoter indicated rapid cyclical association of the activator oestrogen receptor α (ERα) (Metivier *et al.*, 2003; Reid *et al.*, 2003). In each cycle, ERα co-ordinated chromatin remodelling and the recruitment of general transcription factors to initiate transcription. Importantly, at the end of each cycle, the proteasome was required to clear the promoter of ERα. Thus, cyclical proteasomal degradation of this activator allowed new transcriptional cycles to recur.

Does *coactivator* turnover stimulate transcription by similar mechanisms as *activator* turnover? Upon activation of plant immunity NPR1 interacts with TGA transcription factors, which bind to DNA motifs that have been shown to be necessary for defence gene induction (Dong, 2004). Association of TGA transcription factors with target genes is SA-inducible and dependent on NPR1 (Johnson *et al.*, 2003; Butterbrodt *et al.*, 2006). Intriguingly, recent studies suggest that NPR1 preferentially interacts with non-DNA-bound rather than DNA-associated TGA factors (Johnson *et al.*, 2008) and may play a role in their transactivation (Rochon *et al.*, 2006). Thus, NPR1 coactivator turnover may ensure continuous delivery of fresh TGA activators to target promoters (Fig. 3). However, many NPR1 target genes that are crucial for the development of plant immunity do not have TGA binding sites, suggesting that NPR1 interacts with other as yet unidentified transcription factors. It is plausible that these proteins constitutively bind defence promoters and function as genomic landmarks for NPR1 to commence cyclical ordered recruitment of the PIC.

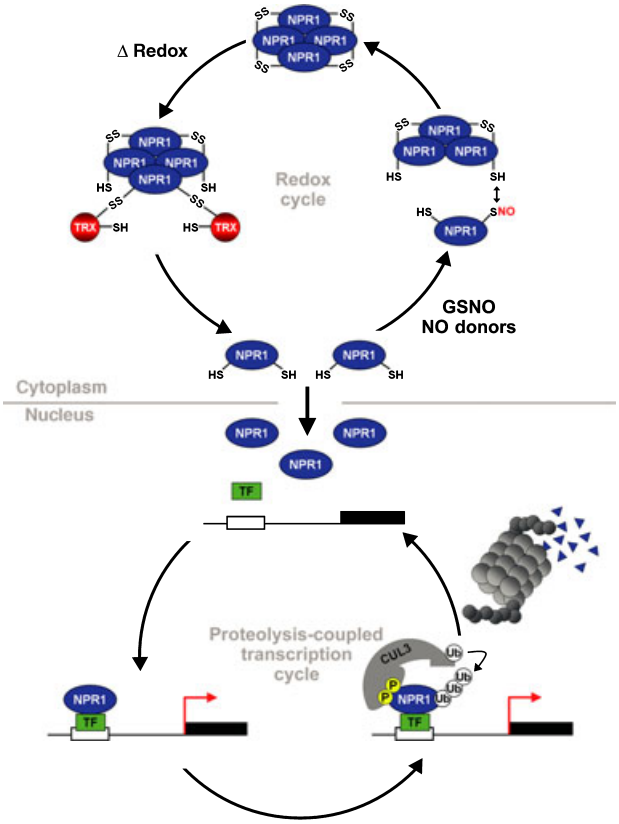

Fig. 3 The redox cycle and proteolysis-coupled transcription cycle regulate NPR1 (nonexpressor of pathogenesis-related (*PR*) genes 1) coactivator activity. Upon pathogen attack, cycles of thioredoxin (TRX)-mediated reduction followed by *S*-nitrosoglutathione (GSNO)-mediated oxidation regulate NPR1 oligomer/monomer conformations. Monomeric NPR1 translocates to the nucleus where it interacts with transcription factors (TFs) and activates target gene transcription. As a consequence NPR1 is phosphorylated (P), ubiquitinylated (Ub) by a Cullin 3 (CUL3) ligase, and degraded by the proteasome. Degradation of NPR1 clears the target gene promoter, allowing the transcription cycle to reinitiate. In this model, the redox cycle regulates the amount of transcriptionally active monomer that enters the nucleus and is intimately connected to the proteolysis-coupled transcription cycle that controls the rate of gene transcription.

Activator phosphorylation promotes proteolysis-coupled transcription

An inherent problem in the model for (co)activator proteolysis-coupled transcription is the necessity to signal (co)activator degradation *after* transcription has been initiated. A conspicuous feature of many unstable transcription (co)activators is the presence of a conserved phosphodegron motif, a sequence containing phosphorylatable serine residues that act as a signal to induce ubiquitin-mediated degradation (Wu *et al.*, 2007). In NPR1, residues Ser11 and Ser15 are part of a phosphodegron and are phosphorylated in response to SA (Spoel *et al.*, 2009). Phosphorylation occurs in the nucleus and has been shown to be required for the SA-induced ubiquitinylation of NPR1 by a CUL3 ubiquitin ligase. Phosphorylation-mediated turnover was required for full-scale expression of NPR1 target genes, implicating phosphorylation as a critical 'destruction label' on coactivators in proteolysis-coupled transcription. Coactivator phosphorylation is therefore expected to occur after transcription initiation. In yeast, compelling evidence for such a model was provided by studies of general control noninducible 4 (GCN4), an activator of amino acid biosynthetic genes. It was shown that gene activation by GCN4

turnover requires its phosphorylation (Lipford *et al.*, 2005). Strikingly, GCN4 is phosphorylated by SRB10 (suppressor of RNA polymerase B 10), a cyclin-dependent protein kinase that is associated with PolII, indicating that GCN4 is targeted to the proteasome after recruitment of PolII. Thus, after transcription initiation, PolII-mediated phosphorylation may mark (co)activators as 'spent' to facilitate their removal and promote binding of new 'fresh' (co)activator to re-initiate transcription (Fig. 3).

Perspectives

Post-translational control of transcription (co)activators has emerged as a crucial mechanism for reprogramming the transcriptome in eukaryotes. This robust regulatory mechanism provides the cell with an on/off switch for transcription as well as a system to control the rate of this process in response to changing cues. Research on NPR1 in plant immunity indicates that communication between distinct post-translational modifications also plays a role in transcriptional control. For example, phosphorylation of NPR1 occurs at a well-defined motif and is thought to provide a direct or indirect binding site for a specific adaptor protein that recruits it to a ubiquitin ligase. Thus, phosphorylation may provide the necessary specificity in proteasome-mediated (co)activator turnover. In addition to interplay between phosphorylation and ubiquitinylation, *S*-nitrosylation of NPR1 had a strong impact on the proteasomal degradation rate of NPR1. Mutation of NPR1 Cys156, which abolished NO-facilitated oligomerization, resulted in increased protein instability upon SA induction (Tada *et al.*, 2008; Spoel *et al.*, 2009). Further, TRX-mediated monomerization and *S*-nitrosylation-facilitated oligomerization act sequentially (Tada *et al.*, 2008), which may explain why NPR1 protein levels fluctuate in response to immune activation (Spoel *et al.*, 2009). Taken together, these reports suggest that the transcriptional activity of NPR1 is the net result of two interconnected cycles: a redox cycle in the cytoplasm and a proteolysis-coupled transcription cycle in the nucleus (Fig. 3). Although many questions still remain unanswered in this model, studies have now established NPR1 as a paradigm for post-translational control of (co)activator activity in eukaryotes. Similar regulatory mechanisms are expected to control other transcription regulators. In plants, NO-responsive promoters contain eight different overrepresented binding sites for transcription factors that are involved in stress and development (Palmieri *et al.*, 2008). This suggests that NO-mediated modification of transcription regulators is involved in a wide variety of signalling pathways. Moreover, proteolysis-coupled transcription cycles have now been found in different physiological processes across kingdoms, indicating the importance of this mechanism in transcription reprogramming in general (Collins & Tansey, 2006).

Major challenges for the future include deciphering further the interplay between different post-translational modifications of (co)activators. By appreciating how these interconnected modifications impose gene-specific patterns of coactivator recruitment to promoters, we may begin to understand the transcriptional behaviour of genes. Finally, as the discovery of new (co)activators continues, it has become clear that not all form unstable transcription complexes (Kodadek *et al.*, 2006). Understanding when stable (co)activators are preferred over unstable ones or *vice versa* may be the key to unlocking the mysteries of large-scale transcriptome reprogramming.

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