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PROSPECTS

Ub on the Move

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Abstract Ubiquitination is an increasingly common post-translation modification that controls both the expression and activity of numerous proteins in the eukaryotic cell. One frequent target of the ubiquitin (Ub) modification machinery is transcription factors. Although ubiquitination generally modulates their function by inducing proteasome-dependent degradation, past and recent studies indicate that ubiquitination also regulates nuclear–cytoplasmic trafficking of transcriptional regulators. Ubiquitination is known to modulate transcription factor localization by destroying sequestering proteins and by directly promoting nuclear import and export of modified substrates. This review discusses old and new paradigms relating Ub modification and the control of transcription factor shuttling in and out of the nucleus. J. Cell. Biochem. 93: 11–19, 2004. © 2004 Wiley-Liss, Inc.

Key words: Ubiquitin; SUMO; proteasome; transcription factors; sub-cellular localization

Ubiquitination is a highly conserved post-translational protein modification process that results in the covalent attachment of a 76 amino acid ubiquitin (Ub) polypeptide to a Lys of the targeted substrate [reviewed in Hershko and Ciechanover, 1998; Pickart, 2001]. Ub modification is a multi-step process and requires the coordinated activity of at least three proteins. These are an Ub-activating enzyme (E1), an Ub-conjugating enzyme (E2), and a specificity factor or complex termed an Ub ligase (E3). Ub is first activated by an E1 in an ATP-dependent manner through the formation of a thiol ester bond between a reactive Cys in the E1 and the carboxy-terminal Gly of Ub. Ub is then transferred from the E1 to a catalytic Cys within an Ub conjugation domain of E2 enzymes. Attachment of an activated Ub moiety to a substrate is mediated by a specificity factor or complex that interacts with both the target and E2. This association generally facilitates the covalent attachment of Ub to the substrate via an amide linkage to the ε-amino group of an internal Lys residue. E3 enzymes promote the transfer of Ub directly from the E2 to the substrate or as in the case of HECT domain containing ligases, capture Ub via their carboxy-terminal Cys residue from the E2 prior to placement on the target. Repeated transfers of Ub to internal Lys residues of already attached Ub results in the formation of Ub chains. It is generally thought that Ub chain assembly is mediated by the same E2/E3 complexes. However, recent studies point to existence of E4 proteins that may catalyze chain formation on particular substrates [Koegl et al., 1999; Grossman et al., 2003]. In most cases, poly-Ub chains result from Lys 48 linkages. Interestingly, other types of linkages occur, including those on Lys 29 and Lys 63, and the type of Ub polymer formed on the substrate is critical in dictating the fate of the modified protein. For example, Lys 48-Gly 76 polymers target proteins for ATP-dependent proteolysis by the 26S proteasome [Chau et al., 1989] while Lys 63-Gly 76 chains modulate protein function in the absence of inducing degradation [Hofmann and Pickart, 1999; Deng et al., 2000] or label proteins for destruction via non-proteasome dependent mechanisms [Galan and Haguenauer-Tsapis, 1997]. Although proteins are usually poly-ubiquitinated, some are modified by a single Ub moiety on one or multiple Lys. This mono-ubiquitination can serve as a signal for lysosomal or peroxisomal degradation of plasma membrane proteins or modulate

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protein function in the absence of promoting proteolysis [reviewed in Hicke, 2001]. It should be noted that although ubiquitination is a covalent protein modification, this process is reversible due to activity of isopeptidases or deubiquitinating enzymes [Hochstrasser, 1996]. These proteins remove Ub from itself or the substrate.

Several other polypeptides that share similarities with Ub as a post-translational protein modifier have been identified [recently reviewed in Schwartz and Hochstrasser, 2003; Seeler and Dejean, 2003]. One member of this Ub-like family is a 101 amino acid long polypeptide, called SUMO (Small Ubiquitin-like MOdifier). SUMO-1 (*Saccharomyces cerevisiae* contains only one *SUMO* gene while vertebrates harbor three family members) shares approximatey 18% sequence identity with Ub and is conjugated to proteins via a similar mechanism as Ub. SUMO is covalently attached to the Lys residues of substrates via a "three-enzyme-step" mechanism involving a heterodimeric (Aos1/Uba2) SUMO activating enzyme (E1), the Ubc9 SUMO conjugating enzyme (E2), and E3-like proteins (e.g., Siz1p and Siz2p in yeast and PIAS in higher eukaryotes). In contrast to Ub modification, SUMO-1 attachment generally occurs within a consensus site (i.e., ψ-Lys-X-Gly where ψ is a large hydrophobic amino acid and X is any amino acid) and does not form chains on substrates. Interestingly, SUMO-2 and SUMO-3 contain a consensus SUMOylation site and can form chains with themselves or other SUMO proteins. The function of these SUMO polymers in signaling remains unclear.

Recent estimates point to the existence of more than 40 different Ub conjugating enzymes and 500 different Ub ligases. The later group of proteins has been divided into specific subclasses, including single subunit RING finger proteins, multi-subunit RING finger proteins, HECT domain containing proteins and more recently, U-box containing E3's [reviewed in Hershko and Ciechanover, 1998; Pickart, 2001; Hatakeyama and Nakayama, 2003]. The sheer number of these proteins indicates that ubiquitination plays an important role within the vast majority of signaling pathways. In support of this statement, Ub modified proteins are known to perform functions in cell cycle progression, organelle biogenesis, antigen presentation, stress responses, signal transduction, DNA repair, apoptosis, and transcriptional regulation [reviewed in Hochstrasser, 1996; Hershko and Ciechanover, 1998; Pickart, 2001].

Ub modification impinges on various aspects of transcriptional regulation, including chromatin remodeling, direct regulation of transcription factor function and transcription-coupled repair [reviewed in Desterro et al., 2000; Muratani and Tansey, 2003]. Although most of the known examples involve proteasome-dependent degradation of transcriptional regulators or in the case of transcription-coupled repair, RNA polymerase II, some do not involve degradation of modified proteins. For example, mono-ubiquitination of H2B histone stimulates methylation of H3 histone and subsequent gene silencing without affecting levels of H2B [Sun and Allis, 2002].

Transcription factor function is controlled at multiple levels and processes modulating nuclear import and export are emerging as a key regulatory mechanism for many transcription factors. Recent studies suggest that Ub and SUMO modification modulates intracellular localization of transcription factors via multiple mechanisms. This review discusses old and new paradigms relating to Ub and SUMO modification and the control of transcription factor shuttling.

Ub AND NUCLEAR IMPORT

There are currently very few examples of Ub mediated control of transcription factor import into the nucleus and these are for the most part restricted to the NF-kB transcriptional regulatory complex in vertebrates and the endoplasmic reticulum localized transcription factors Mga2p and Spt23p in *S. cerevisiae* (see Fig. 1). NF-κB is the best-studied example of how the Ub-proteasome pathway modulates transcription factor localization. Latent NF-κB, comprised of p50 and p65 subunits, is sequestered in the cytoplasm via an interaction with the inhibitors, including IκBα, IκBβ, IκBε and related proteins [reviewed in Baldwin, 1996; Karin, 1999]. Stimuli (such as the proinflammatory cytokines interleukin-1 and tumor necrosis-factor α, lipopolysacharide, and viral proteins) that activate NF-κB function do so by initiating a cell surface receptor mediated signaling cascade that results in phosphorylation of IκBs by IκB kinase (IKK) complexes. In the case of IκBα, the phosphorylated protein is recognized by the $SCF^{\beta TrCp}$ multi-RING finger

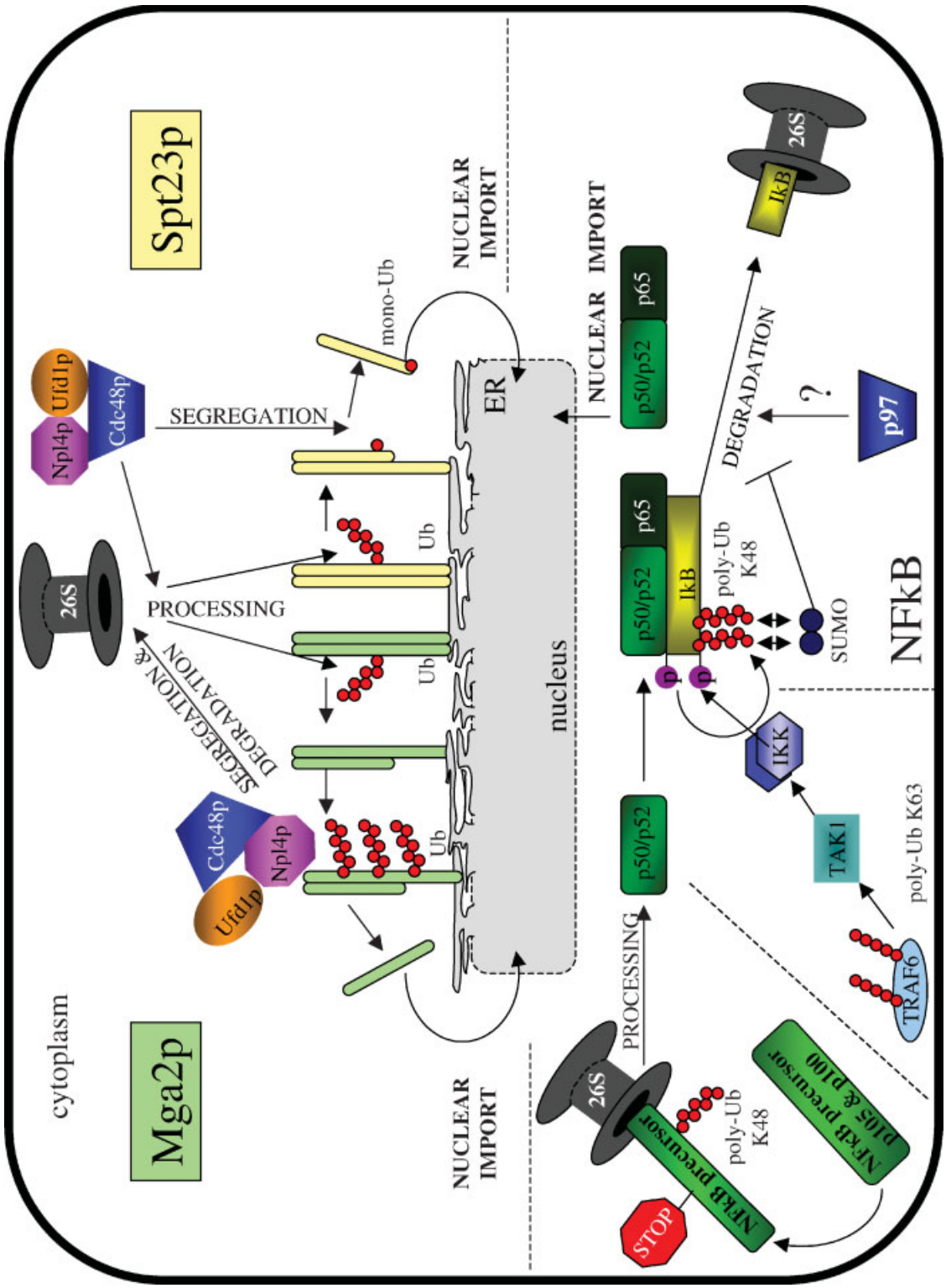

Fig. 1. Mechanisms of Ub-mediated nuclear important of transcription factors.

E3 complex and in association with Ub conjugating enzyme Cdc34 or Ubc5 is poly-ubiquitinated and subsequently degraded by the 26S proteasome [reviewed in Desterro et al., 2000]. Degradation of IκBα unmasks the nuclear localization signal of p50 and p65 and the NF-κB complex migrates to the nucleus where it regulates the expression of genes harboring NF-κB response elements [Baldwin, 1996]. IκBα is also a NF-κB responsive gene, establishing an autoregulatory feedback loop between the proteins [Sun et al., 1993]. Upon accumulation in the nuclear compartment, IκBα binds NF-κB and the complex is exported to the cytoplasm via the nuclear export signal (NES) present in the carboxy-terminal region of IκBα [Arenzana-Seisdedos et al., 1997; Johnson et al., 1999].

The Ub modification process plays at least two other atypical roles within the NF-κB pathway that deserve attention. First, the unique poly-Ub assembly activity (i.e., promotion of Ub chain assembly on Lys 63) of the RING finger ligase TRAF6 has been linked to activation of TAK1 kinase (an up-stream activator of IKK) via a yet to be described mechanism that does not involved proteasome-dependent degradation of participating proteins [Deng et al., 2000]. Second, it has been shown that the p50 and p52 subunits of the NF-κB complexes are generated from larger molecular weight precursors p105 and p100, respectively, by Ub modification and limited proteasome dependent degradation of the proteins [reviewed in Rape and Jentsch, 2002]. In the case of p105, the degradation signal is generated by a similar mechanism as IκBα. Phosphorylation of the carboxy-terminus by IκB kinase complex leads to recruitment of Ub ligase $SCF^{\beta TrCp}$ [Lang et al., 2003]. Although it is clear that highly stable regions within the amino-terminal Rel homology domain suppress complete proteasome-dependent degradation of the precursors and allow for survival of p50 and p52, there is still some debate of whether proteolysis initiates through an endoproteolytic cleavage event or occurs from a free polypeptide terminus [Lee et al., 2001; Lin and Kobayashi, 2003].

Two distantly related p100 and p105 proteins from *S. cerevisiae*, the endoplasmic reticulum localized transcriptional regulators Mga2p and Spt23p, have also recently been documented to undergo Ub-proteasome-dependent processing and Ub-regulated localization [Hoppe et al., 2000; Rape et al., 2001; Shcherbik et al., 2003]. Mga2p and Spt23p play overlapping roles in transactivating the essential yeast gene *OLE1*. *OLE1* encodes Δ9 fatty acid desaturase, an enzyme required for the synthesis of unsaturated fatty acids and maintenance of membrane integrity [Zhang et al., 1999]. It is thought that the localization of these proteins at the ER membrane relates to control mechanisms that sense changes in membrane fluidity and modulate their processing, release and subsequent biological activity. Mga2p and Spt23p are initially synthesized as p120 kDa precursor molecules and each harbor a transmembrane domain, which anchors the proteins to the ER membrane [Hoppe et al., 2000]. The precursors are capable of forming homodimers via their IPT domains (this domain is also present within the Rel homology region of NF-κB precursors p100 and p105) at the ER and homodimerization is essential for proteasome-dependent processing of one of the monomers [Rape et al., 2001]. For Spt23p, the HECT domain-containing ligase Rsp5p is required for supplying the processing signal while in the case of Mga2p, it can occur by an Rsp5p-independent mechanism [Hoppe et al., 2000; Shcherbik et al., 2003]. Nevertheless, only one of the dimerized polypeptides undergoes limited degradation and the resulting processed p90 kDa product remains tethered to the ER membrane via an interaction with the unprocessed ER-bound anchor [Rape et al., 2001].

In addition to providing the processing signal, ubiquitination also appears to be required for nuclear mobilization of the transcriptionally active p90 forms of Mga2p and Spt23p. Two processes, although not necessarily exclusive of one another, have been proposed relating to the release of these transcription factors from the ER membrane [Rape et al., 2001; Shcherbik et al., 2003], both of which appear to require the activities of the highly conserved HECT domain containing ligase Rsp5p and the Cdc48p containing Ufd1p-Npl4p protein complex. Cdc48p (valosin/p97 in mammalian cells) is an AAA-ATPase, which can dissemble macro-molecular complexes and unfolded proteins while Npl4p and Ufd1 function as a dimer in binding to both mono and poly-ubiquitinated proteins [reviewed in Woodman, 2003]. This $Cdc48p^{Ufd1p/Npl4p}$ complex has been shown to have numerous activities, most notably binding poly-ubiquitinated ER proteins, extracting them from the ER and presenting them to the proteasome for degradation [reviewed in Bays and Hampton,

2002]. Intriguingly, Cdc48p has also been linked to Ub proteasome-dependent degradation of IκBα [Dai et al., 1998]. In the case of Spt23p, it has been proposed that the Cdc48p$^{Ufd1p/Npl4p}$ protein complex binds mono-ubiquitinated Spt23p90 and separates it from the Spt23p120 membrane anchor, allowing nuclear import of the NLS containing protein [Rape et al., 2001]. Although it remains unclear how the Ub signal is placed on Spt23p90, it has been suggested that it is a remnant of the Rsp5p-induced processing signal. As for Mga2p, Mga2p90 release may occur through a different mechanism, one that involves Rsp5p-dependent poly-ubiquitination and Cdc48p$^{Ufd1p/Npl4p}$/Proteasome mediated degradation of the membrane-bound anchor [Shcherbik et al., 2003]. Many questions remain unanswered about both of these potential mechanisms. Considering that Rsp5p is dispensable for Mga2p90 generation, what is the identity of other ligases that may be involved in proteasome-dependent processing of Mga2p90? Similarly, is mono-ubiquitinated Spt23p90 a left over from the Rsp5p-induced processing reaction or placed there by another ligase or in a separate Rsp5p-dependent reaction? What are the domains of these proteins responsible for ligase interactions and Ub conjugation and what processes govern poly-ubiquitination versus mono-ubiquitination? Adding further complexity to this story is that the Cdc48p$^{Ufd1p/Npl4p}$ complex also appears to play an uncharacterized role in proteasome-dependent processing of Mga2p and Spt23p [Hitchcock et al., 2001]. Is Cdc48p$^{Ufd1p/Npl4p}$ activity required for extraction of the carboxy-terminus of one of the Ub-containing monomers from the membrane so it can be degraded processively by the proteasome? If processing occurs via an endoproteolytic cleavage mechanism, does this complex recruit proteasome binding and facilitate processing by a mechanism that does not involve "pulling" the proteins out of the membrane? In addition to answering these questions, future studies are needed to elucidate how these Ub proteasome dependent processes intersect with maintenance pathways ensuring proper amounts of unsaturated fatty acids in the cell and membrane fluidity.

Ub AND NUCLEAR EXPORT

In addition to modulating transcription factor import, ubiquitination also promotes export of transcription factor from the nucleus (see Fig. 2). Of particular relevance to this review is a recent report linking mono-ubiquitination of the tumor suppressor and transcription regulator p53 to nuclear export [Li et al., 2003]. The mammalian tumor suppressor p53 controls the transcription of a plethora of genes encoding proteins involved in regulating cell cycle arrest, DNA repair, and apoptosis [reviewed in Vogelstein et al., 2000; Balint and Vousden, 2001]. p53 has a short half-life and is maintained at low levels in unstressed cells. Stress-inducing signals such as DNA damage or inappropriate activation of oncogene function induces p53 accumulation by blocking ubiquitin-proteasome-mediated degradation. It is widely accepted that the single subunit RING finger ligase MDM2 promotes p53 poly-ubiquitination and proteasome-dependent degradation and this activity is suppressed by DNA damage or oncogene activation [reviewed in Michael and Oren, 2003]. Also, it has been known for some time that MDM2 promotes nuclear export of p53 and this activity is dependent on the RING finger domain of MDM2 and Lys residues present within the carboxy-terminus of p53. Because the carboxy-terminal region of p53 contains a NES, it has been postulated that Ub modification induces a conformation change in the protein, leading to unmasking of the NES and nuclear export. Curiously, p53 is degraded by both nuclear and cytoplasmic proteasomes [Xirodimas et al., 2001], raising questions regarding the significance of nuclear export in MDM2-mediated ubiquitination and degradation processes. A series of recent studies suggest that the consequence of MDM2-mediated ubiquitination of p53 may be more complex that initially appreciated and may play multiple regulatory functions. MDM2 has recently been shown to promote mono-ubiquitination of p53 at low ratios and poly-ubiquitination at high ratios [Li et al., 2003]. Unlike poly-ubiquitinated p53, mono-ubiquitinated p53 appears to escape degradation by nuclear proteasomes and is exported to the cytoplasm. Although the physiological significance of these observations remain unclear, it is possible that this regulation is important under conditions where a cytoplasmic, but not nuclear function of p53 is desired. Interestingly, cytoplasmic p53 has been linked to the promotion of apoptosis by a mechanism that does not involve transcriptional regulation [reviewed in Baptiste and Prives,

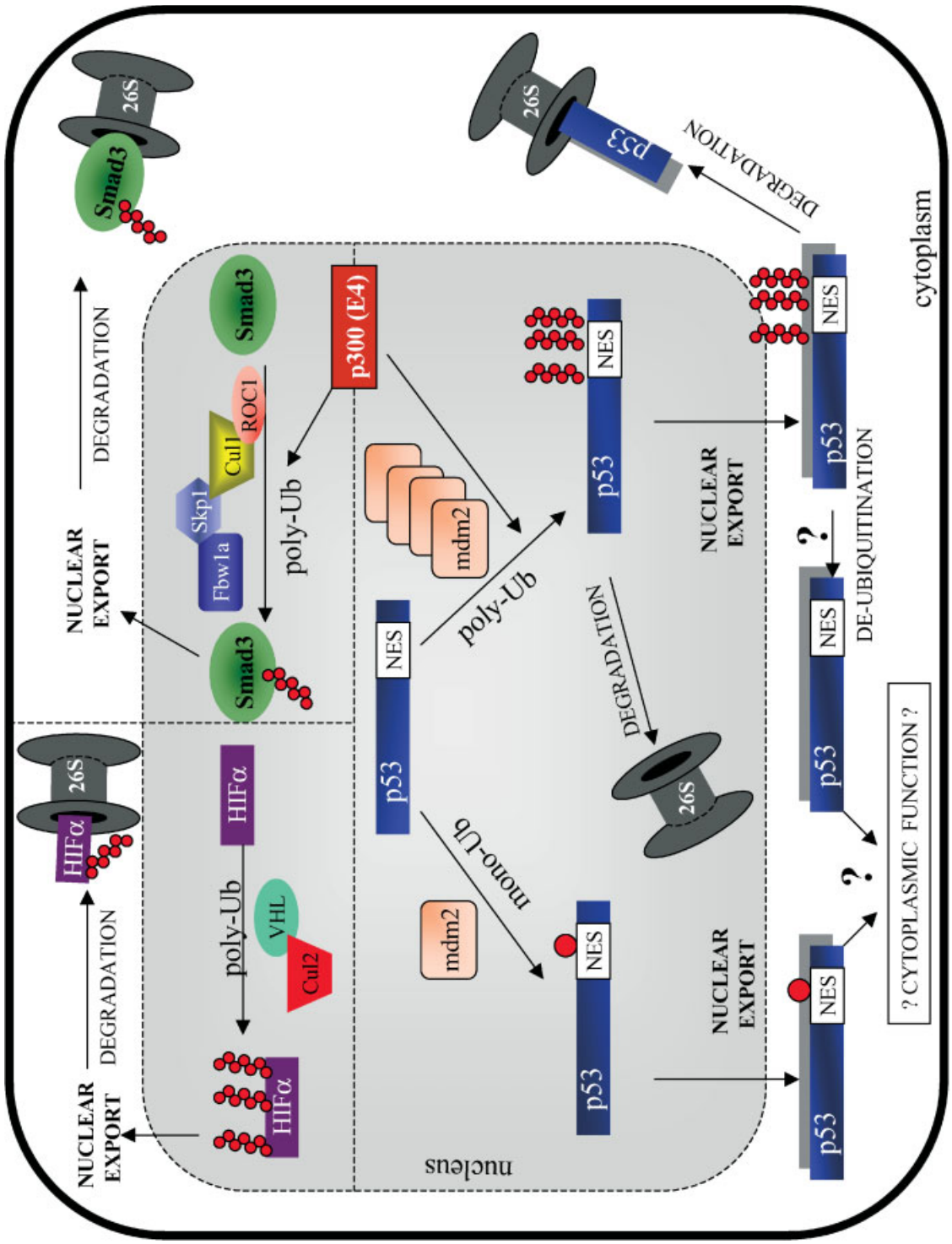

Fig. 2. Mechanisms of Ub-mediated nuclear export of transcription factors.

2004]. Considering that MDM2 expression is down-regulated at the RNA level under conditions of high DNA damage [Arriola et al., 1999] and the protein is a substrate of apoptotic proteases [Chen et al., 1997], it is possible that MDM2 mediated mono-ubiquitination under certain situations plays an important role in driving the cells towards an apoptotic rather than a cell cycle arrest response. Alternatively, MDM2-mediated export by certain signals such as those that induce transient cell cycle arrest may provide a rapid but reversible inactivation of p53 function. More experiments are obviously needed to address these issues as well as to determine the functional relationship between those proteins (e.g., p300 [Grossman et al., 2003]) that facilitate MDM2-mediated p53 poly-ubiquitination and p53 export, degradation, and modulation of the p53 response.

Poly-ubiquitination of transcription factors Smad3 [Fukuchi et al., 2001] and hypoxia inducible factor (HIF-α) [Groulx and Lee, 2002] has also been linked to their export from the nucleus. Termination of Smad3-induced gene regulatory function occurs via ROC1-SCF^{Fbw1a} ligase mediated poly-ubiquitination of Smad3 in the nucleus in a p300 dependent manner [Fukuchi et al., 2001]. After ubiquitination, Smad-3 appears to be exported to the cytoplasm for degradation by 26S proteasomes. In the case of HIF-α, it has been proposed that the protein shuttles constitutively with its ligase complex (i.e., VBC/Cul-2) between the nuclear and cytoplasmic compartments [Groulx and Lee, 2002]. Under hypoxic conditions, HIF-α disassociates from the ligase complex, accumulates in the nucleus and induces the expression of hypoxia-inducible genes. Upon a return to normoxia, the ligase complex binds HIF-α and poly-ubiquitinates the substrate, leading to nuclear export and degradation. Because it is sometimes difficult to discern between mono- and poly-ubiquitination, it will be of interest of future studies to determine if regulation of Smad3 and/or HIF-α occurs by a similar or distinct mechanism as p53 and if the ligases are differently co-transported with transcription factors during export.

SUMOYLATION AND TRANSCRIPTION FACTOR SUB-CELLULAR SHUTTLING

Considering the topic of this review, it is difficult not to mention the role of the Ub-like protein SUMO in transcription factor movement. The best-studied consequence of transcription factor SUMOylation is modulating intra-nuclear localization, which can result in both the inactivation and activation of transcription factor function [reviewed in Seeler and Dejean, 2003]. The SUMO E3 ligase PIASγ dampens the transactivation function of the Wnt-responsive transcription factor Lef1 by promoting its localization to sub-nuclear POD (for PML oncogenic domain) structures, although SUMO conjugation to Lef1 by the ligase does not appear to be required for sequesteration [Sachdev et al., 2001]. In contrast, SUMO modification of the transcription factor Sp3 on specific Lys residues appears critical for inactivation of its transactivation function and translocation to nuclear periphery and nuclear speckles [Ross et al., 2002; Sapetschnig et al., 2002]. Interestingly, there are also examples (i.e., HSF1 and HSF2) where SUMO mediated modification leads to redistribution of the proteins in the nucleus and activation of transcription factor function [Goodson et al., 2001; Hong et al., 2001].

Besides directly affecting transcription factor localization within the nucleus, SUMOylation can modulate transcription factor localization between nuclear and cytoplasmic compartments by suppressing Ub-mediated signaling. It has been shown that IκBα is SUMOylated at the same Lys utilized for Ub conjugation [Desterro et al., 1998]. SUMO-1 modified IκBα is resistant to signal-induced ubiquitination and proteasome-mediated degradation, leading to retention of NF-κB in the cytoplasm. Similarly, both SUMO-1 and Ub can be conjugated to the same Lys of Smad4 [Lin et al., 2003] and Smad4 SUMOylation in the nucleus suppresses export to the cytosplasm and leads to stabilization of the protein.

SUMMARY AND CONCLUSIONS

Old and new studies point to an important role for Ub modification in controlling subcellular shuttling of transcriptional regulators. Considering the large number of transcription factors and Ub ligases present in the cell, it is possible that Ub or Ub-like mediated regulation of transcription factor localization will turn out to be a more common process than currently appreciated. Besides identifying ligases responsible for such regulation and working out mechanisms of specificity, there will be other

aspects of this regulation that will need elucidation. For example, although we are getting a handle on components required for transcription factor ubiquitination, we have very little information on processes involved in transcription factor mobilization down-stream of Ub conjugation. For example, does mono-ubiquitination simply induce a conformation change in proteins that unmasks localization signals or is mobilization dependent on Ub binding proteins that directly facilitate migration from one cellular compartment to the other? In addition, considering that transcriptional regulators are likely present within protein complexes (e.g., such as NF-κB with IκBs), how does the biologically active molecule escape degradation when their closely associated partners do not? Is this dependent on proteasome-mediated segregase activity or separate protein complexes such as the Ub binding $Cdc48p^{Ufd1p/Npl4p}$ complex? Finally, what role does de-ubiquitination play in this process and is this activity also an important regulator of transcription factor localization? In addition to satisfying intellectual curiosity, it of hope that these studies will lead to the design of specific therapeutics that beneficially modulate the activity of these proteins in disease processes where their functions are de-regulated or counteract current therapies.

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Due to reference limitations, we apologize for not citing many of the original research articles on the discussed topics.

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