Chapter 12
Regulation of Gene Expression by the Ubiquitin–Proteasome System and Implications for Neurological Disease

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Abbreviations

APC	Adenomatous polyposis coli
ARD1	Arrest-defective 1 N-acetyltransferase
BARD-1	BRCA-associated RING domain protein
BMP	Bone morphogenetic protein
BRCA	Breast cancer susceptibility gene 1
CBP	CREB binding protein
CDK9	Cell cycle arrest associated protein 9
GSK-3β	Glycogen synthase kinase-3β
HAF	Hypoxia-associated factor
HAT	Histone acetyltransferase
HIF-1	Hypoxia-inducible factor-1
HOS	Homologue of slimb
MAD	Mothers against decapentaplegic
MDM2	Mouse double minute 2 protein
NES	Nuclear export signal
PHD	Prolyl hydroxylases
Pin1	Peptidylprolyl cis/trans isomerase
PML-NB	Promyelocytic leukemia nuclear bodies
PPIase	Peptidyl-prolyl cis–trans isomerase
pVHL	von Hippel Lindau tumor suppressor
RACK	Receptor of activated protein kinase
SAG	Sensitive to Apoptosis Gene
SCF	Skp-fbox-cullin
SUMO	Small ubiquitin related modifier
TGF	Transforming growth factor

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TRRAP	Transcriptional histone acetyltransferase cofactor
UCP	E2-EPF Ubiquitin carrier protein
UPS	Ubiquitin–proteasome system
VDU2	p-VHL-interacting deubiquitinating enzyme 2
VEGF	Vascular endothelial growth factor
βTrCP	Beta transduction repeat containing protein

12.1 Introduction

This chapter will focus on the regulation of transcription factor function by the ubiquitin–proteasome system and its implications in neurological diseases. Many transcription factors and transcription co-factors are regulated by their rapid degradation by the ubiquitin–proteasome system, to enhance or inhibit gene expression. Following their prior phosphorylation, proteins, such as transcription factors, bind E3-ligases resulting in their ubiquitination. As such, transcription factors are the convergence point of multiple intracellular signaling pathways enabling the complex regulation of gene expression to various biological stimuli.

Many transcription factors are ubiquitinated in the cytoplasm following their export from the nucleus. In addition, the enrichment of ubiquitin and proteasome subunits in the promyelocytic leukemia nuclear bodies (PML-NB) sub-compartment of the nucleus suggests some transcription factors are subject to local ubiquitination and degradation in the nucleus. PML-NBs are dynamic regions that are in intimate contact with the cellular chromatin (for more information the reader is referred to some recent reviews (Fedorova and Zink 2008; Krieghoff-Henning and Hofmann 2008; Zimber et al. 2004)). The PML-NBs contain other post-translational modification enzymes, such as acetylases and protein kinases. The PML-NBs have an organized structure consisting of a capsule enriched in PML protein, small ubiquitin related modifier1 (SUMO1) and CREB binding protein (CBP), whereas the central lattice contains the proteasome subunits. As such, there is a concentration of nuclear ubiquitin–proteasome system components in close proximity to sites of gene expression and regulation.

12.2 Overview of the Ubiquitin–Proteasome System

The following is a brief overview of the ubiquitin–proteasome system to address key details (for more details the reader is directed to the previous chapters in this book). The degradation of proteins by the ubiquitin–proteasome system is a two-stage process (Fig. 12.1). First proteins are conjugated to ubiquitin, an 8–9 kDa protein present in all cells, by the sequential action of E1-, E2- and E3-ligase proteins. Second, the ubiquitinated protein is enzymatically degraded by the 26S proteasome, a multi-catalytic protease complex. The biological importance of the proteasome is

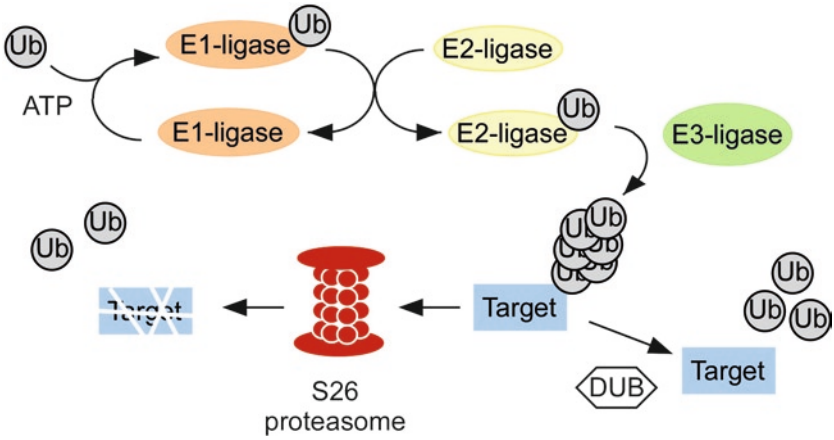

Fig. 12.1 Overview of protein ubiquitination and proteasomal degradation. The following is an overview of the common components of the ubiquitin–proteasome system. Ubiquitin (*gray*) is added to an E1-ligase protein (*orange*) in a reaction requiring ATP. The ubiquitin is transferred to the E2-ligase (*yellow*) and then transferred to the substrate protein (*blue box*) by an E3-ligase (*green*). Poly-ubiquitinated proteins are transferred to the S26 proteasome for degradation. De-ubiquitinating enzyme (DUB, *white hexagon*) removes ubiquitin modifications from substrate proteins. The colour and shapes of these components are consistent throughout Figs. 12.2–12.6

defined by the observation that deletion of 13/14 genes which make up the core 20S complex results in lethal phenotypes (Heinemeyer et al. 1991; Orlowski 1999). Protein degradation by 26S proteasome requires ATP. A chain of at least five ubiquitin molecules attached to the substrate is required for 26S proteasomal degradation (Thrower et al. 2000).

The specificity of the ubiquitination reaction is regulated by the E3-ligase which catalyses the transfer of the ubiquitin from the E2-ligase to either a Lys residue on the substrate protein or the N-terminus of the protein (Orlowski 1999; Pickart 2001). Two classes of E3-ligases have been defined; those containing either a HECT or a RING domain. RING E3-ligases can be single proteins, containing the substrate recognition site and the catalytic ligase domain in the single protein, or a multi-protein complex, whereby the recognition/binding protein is distinct from the catalytic protein (i.e. the SKIP/cullin/Fbox family; SCF). In addition, the E4-ligase regulates the poly-ubiquitination of a previously mono-ubiquitinated target substrate (Grossman et al. 2003). The different mechanisms of ubiquitin transfer to a target protein have been considered in the following review (Hochstrasser 2006).

The existence of six lysine residues within the ubiquitin amino acid sequence enables the formation of complex poly-ubiquitin chains with different topologies and functions (Varadan et al. 2002, 2004). The Lys48 poly-ubiquitin chain modification is associated with the proteasomal targeting of substrate proteins. In contrast, mono-ubiquitination regulates protein–protein interactions and signaling. The Lys63 poly-ubiquitin modification is associated with lysosomal degradation of proteins and the formation of signaling complexes (Ikeda and Dikic 2008). Because of the multiple biological roles of ubiquitination, when considering the evidence for a role

of the ubiquitin–proteasome system in regulating gene expression, one must consider the evidence for the role of the proteasome (usually via proteasome inhibitors) as well as the type of modification added to the target protein. This is important for neurological studies because high levels or prolonged exposure to proteasome inhibitors induces neuronal cell death (Qiu et al. 2000).

12.3 Regulation of Gene Expression by the Ubiquitination of Histones

Eukaryotic DNA is tightly packaged into chromatin. The DNA is wound around nucleosomes, which consist of octamers of four histones (H2A, H2B, H3, H4). In addition to providing an efficient mechanism to store DNA, the structure of the nucleosomes also represses transcription by blocking promoter regions and preventing the binding of RNA polymerases. Ubiquitination of histones, which affects packing efficiency, has a significant impact on transcriptional regulation. Since the subject of ubiquitination modifications of histones has been reviewed elsewhere in this volume, we will give only a brief review of the subject.

Histones are modified through both mono-ubiquitination and poly-ubiquitination which regulate chromatin winding and integrate other post-translational modification events. The E3-ligases, Bre1, mouse double minute 2 (MDM2) and breast cancer suppressor gene 1 (BRCA1), have all been implicated in the ubiquitination of histones. First identified in *S. cerevisiae,* the E2-ligase Rad6 works in conjunction with Bre1 and MDM2 to ubiquitinate histones. Two potential mammalian homologues of Rad6, HR6A and HR6B, have also been identified (Baarends et al. 1999; Koken et al. 1991).

The most studied of the histone E3-ligases is the *S. cerevisiae* Bre1 (named Brl1 in *S. Pombe* and RNF20/40 in humans), a RING-finger type E3-ligase for H2B. Mono-ubiquitination of H2B by Bre/Rad1 is required for disassembly of nucleosomes during the RNA polymerase II-mediated elongation of transcripts. Bre/Rad1 is recruited to histones through a multi-step process. The histone chaperone FACT, a factor required for displacement of the H2A/H2B dimer from the core nucleosomes, recruits the transcription initiation complex Paf1 to chromatin. Chromatin bound Paf1 recruits Rad6 and Bre1 to promote ubiquitination of H2B on Lys120, which is required for efficient elongation by RNA polymerase II. Other studies have found that RNA polymerase II is required for the recruitment of Rad5 and Bre1 to histones promoting transcriptional elongation (Henry et al. 2003; Hwang et al. 2003; Kao et al. 2004; Robzyk et al. 2000; Wood et al. 2003; Xiao et al. 2005).

Murine double minute 2 (MDM2), an E3-ligase best known for its role in the ubiquitination of transcription factor p53, also represses transcription of p53-responsive genes through its modification of histones. MDM2 promotes ubiquitination of H2A and H2B in vitro and H2B in vivo in close proximity to p53 promoters, thereby inhibiting transcription of p53-responsive genes. This transcriptional repression was demonstrated to be independent of the role of MDM2 in ubiquitin-mediated degradation of p53 (Minsky and Oren 2004). In addition, BRCA1 promotes ubiquitination of H2A,

H2AX, and H2B in vitro (Chen et al. 2002; Mallery et al. 2002). However, the effects these modifications have on histone assembly and transcription are not clear.

Ubiquitination also regulates histone methylation to modify histone function. Ubiquitination of H2B by Rad6/Bre1 promotes H3 methylation on Lys4 and Lys79 (Cerretti et al. 1992; Weake and Workman 2008). H2B ubiquitination regulates di- and tri-methylation on these Lys residues on H3 and not mono-methylation (Dehe et al. 2005; Schneider et al. 2005; Shahbazian et al. 2005). Recent evidence has demonstrated that ubiquitination of H2A regulates the binding of the Cps35 subunit of a methylation complex, COMPASS (Lee et al. 2007). Recruitment of Cps35 to chromatin was impaired in Rad-deficient *S. cerevisiae* strains and methylation of Lys4 on H3 was reduced (Lee et al. 2007).

Deubiquitination of histones by the deubiquitinating enzyme (DUB) Ubp8 has also been shown to be important for regulating methylation of H2B (Wyce et al. 2007). De-ubiquitination of H2B promotes the recruitment of the kinase Ctk1 resulting in phosphorylation of RNA polymerase II (Wyce et al. 2007). This phosphorylation promotes the recruitment of the methyltransferase, Set 2 (Wyce et al. 2007). The regulation of histones is clearly a complex system, whereby multiple post translational modification events regulate access of transcription factors to the chromatin, thereby affecting gene expression patterns and rates.

12.4 Regulation of Transcription Machinery by the Ubiquitin–Proteasome System

12.4.1 Regulation of RNA Polymerase by the Ubiquitin–Proteasome System

The recruitment of RNA polymerase to the chromatin is a critical step in RNA synthesis. RNA polymerase is a large multi-subunit complex (labeled rpb 1–8 in yeast), which is regulated by additional protein co-factors. Specifically, RNA polymerase II is ubiquitinated by multiple E3-ligases, although not all ubiquitination events result in proteasomal degradation. This may be a result of different poly-ubiquitin linkage topologies, for example Lys48 and Lys63 (Varadan et al. 2004). Some common features of RNA polymerase II ubiquitination have been identified. The ubiquitination and proteasomal degradation of RNA polymerase II results in a reduction in RNA synthesis. First, ubiquitin is predominantly added to the largest polymerase subunit, rpb1, on the C-terminal domain. Up to six lysine residues in this region are substrates for ubiquitination (Li et al. 2007a). Second, phosphorylation of the Ser5 residue in the C-terminal domain is a prerequisite for ubiquitination (Daulny et al. 2008; Mikhaylova et al. 2008; Starita et al. 2005; Yasukawa et al. 2008). In addition, the ubiquitination of RNA polymerase II subunits, rpb1 and rpb 2, result in rpd4/7 dissociating from the RNA polymerase complex, further decreasing transcription (Daulny et al. 2008).

The E3-ligase Wwp2 may ubiquitinate rpb1 in pluri-potent stem cells, but in a non-phospho-Ser5 dependent manner (Li et al. 2007a). Wwp2 is a member of the

Nedd-4 family of HECT E3-ligases and interacts with the C-terminus domain of rpb1 similar to other E3-ligases that regulate damage-induced rpb1 ubiquitination (Li et al. 2007a). Nedd 4 E3-ligases either bind their substrates directly via a WW-domain interaction with a PPxY motif, or via linker proteins for WW-domain independent interactions (Foot et al. 2008). Wwp2 is also associated with the regulation of membrane ion channels (Foot et al. 2008). This suggests that a single E3-ligase, Wwp2, has multiple substrates with diverging cellular location and functions.

RNA polymerase II is a point of convergence of multiple signaling pathways following UV radiation-induced DNA damage and transcription arrest. BRCA-1 associates with BRCA1-associated RING domain protein (BARD-1) to ubiquitinate a number of proteins associated with DNA damage and repair. Mutations in this gene are associated with a high susceptibility to develop breast cancer. Following DNA damage BRCA1 ubiquitinates the major RNA polymerase subunit rpb1 (Starita et al. 2005). Ubiquitination of rpb1 is dependent on Ser5 phosphorylation (Starita et al. 2005). In addition, BRCA1 also ubiquitinates rpb8 to promote cell survival following DNA damage (Wu et al. 2007).

Additional E3-ligases have been associated with rpb1 ubiquitination following UV-light induced DNA damage and transcription arrest. Nedd4 was identified as a direct acting Rpb1 E3-ligase (Anindya et al. 2007), however, in the same assay BRCA1 did not induce Rpb ubiquitination. The mammalian Elongin complex has also been shown to regulate Rpb1 ubiquitination following UV exposure. Following damage, Elongin associates with the cul5/rbx2 module to promote rpb1 ubiquitination (Yasukawa et al. 2008). This seems counter to the established role of Elongin complex in reducing the pausing of RNA synthesis, thus enhancing transcription rates. However, further experiments show that Elongin deficient cells suppress UV-stimulated rpb1 ubiquitination (Yasukawa et al. 2008). Hence, Elongin may have dual roles: enabling efficient transcription under "normal" cellular circumstances, but assisting in transcription arrest following cell damage.

The von Hippel Lindau tumor suppressor (pVHL) is associated with regulation of HIF-1α (see Sect. 12.5.6 of this chapter). However, studies show that pVHL also ubiquitinates rpb1, dependent on Ser5 phosphorylation (Mikhaylova et al. 2008). Interestingly, this effect is also dependent on the prior hydroxylation of a proline residue by Prolin-hydroxylase (PHD1). PHD1 activity is enhanced under conditions of low oxidative stress. PHD1 hydroxylates Pro1465, enabling phosphorylation of the Ser5 residue and the subsequent binding of pVHL to rpb1 (Mikhaylova et al. 2008). pVHL-mediated rpb1 ubiquitination is not associated with degradation. This would suggest that pVHL-mediated poly-ubiquitination is via a Lys63-linked or another non-degradation associated poly-ubiquitin modification. Indeed, the pVHL–mediated ubiquitination of rpb1 enhances the recruitment of rpb1 to chromatin (Mikhaylova et al. 2008).

What is unclear from these studies on the role of RNA polymerase ubiquitination is whether the system has redundancy, or whether specific E3-ligases were identified because of the test system used to investigate the biology. However, these studies suggest that the regulation of RNA polymerase II by E3-ligases is biologically complex, depending on the context of the cell cycle timing and the biological stimuli.

12.4.2 Regulation of the Transcription Co-factors CBP/p300 by the Ubiquitin–Proteasome System

The CREB binding protein (CBP) and p300 are critical regulators of transcription by forming a bridging complex between chromatin-bound transcription factors, RNA polymerase and RNA helicase. CBP and p300 integrate signals from multiple transcription factors such as Smad, STAT, CREB, AP1 and c-myc transcription factors. Hence, their levels and functions are very tightly controlled.

CBP has both E3- and E4-ligase properties, but is also a target of E3-ligases, which regulate its function and degradation. In addition to their ubiquitin ligase roles, CBP and p300 acetylate histones, to relax chromatin and promote transcription, as well as non-histone proteins, to regulate their function (a general review of CBP has been provided by Barco and Kandel 2006). CBP functions are tightly regulated by phosphorylation by multiple protein kinases, some of which regulate E3-ligase targeting of CBP for ubiquitination.

CBP is regulated by the E3-ligase MDM2 (Sanchez-Molina et al. 2006). Following stimulation of PDGF receptors, ras activation leads to an intracellular signaling cascade resulting in ERK and Akt activation and the subsequent degradation of CBP. The decrease of CBP was hypothesized to alter transcription rates by increasing the local competition for CBP between several transcription factors (Sanchez-Molina et al. 2006). A number of phosphorylatable residues on CBP have been shown to regulate its function (Impey et al. 2002). However, it is unclear from this study whether the direct CBP phosphorylation regulated its ubiquitination. The knockdown of MDM2 with siRNA lowered MDM2 levels and caused a reciprocal enhancement of CBP levels and function. In addition, an MDM2 mutant (C246A) was unable to promote the degradation of CBP (Sanchez-Molina et al. 2006). The identification of MDM2 as an E3-ligase for CBP is interesting given that these two proteins interact to regulate p53 transcription (see Sect. 12.5.4).

p300 was identified as a substrate for the SCF Fbx3 E3-ligase in a proteomics analysis of K652 cells and confirmed by immunoprecipitation (Shima et al. 2008). Loss of Fbx3 by siRNA resulted in stabilization of p300 and HIPK2 (another transcription cofactor). Interestingly, p300 associates with PML protein in nuclear bodies, resulting in the stabilization of p300. The effect on HIPK2 was not via a blockade in ubiquitination, but rather preventing the proteasomal degradation of the ubiquitinated HIPK2 in the nuclear bodies. In contrast, the non-nuclear body-associated p300 and HIPK2 were degraded by the proteasome (Shima et al. 2008). The loss of PML blocks p53 activation, suggesting that PML blocks p300 degradation thereby enabling it to regulate p53-mediated transcription.

p300 has E4-ligase function resulting in the poly-ubiquitination of a previously mono-ubiquitinated protein, thereby promoting its proteasomal degradation (Grossman et al. 2003). In addition to having intrinsic E3/E4-ligase properties CBP and p300 have been shown to interact with other nuclear E3-ligases to help regulate their function. The anaphase promoting complex (APC) is a multi-subunit regulatory protein which has E3-ligase properties and requires CBP/p300 (Turnell et al. 2005).

The APC regulates the transition of G_1 phase in the cell cycle. The APC E3-ligase has a similar organization to the SCF multi-subunit E3-ligases, in that APC11 consists of the catalytic RING domain and APC2 forms the bridge between the substrate and catalytic domain. The substrate is brought to the APC via either cdh1 or cdc20, depending on the temporal context of the cell cycle. These substrate-binding proteins recognize overlapping targets (Turnell et al. 2005). Cdc20 functions in mitosis to promote ubiquitination of Securin, a scaffold protein, and cyclins. In contrast, cdh1 is utilized in late mitosis and early G_1 to regulate cyclin, cyclin kinase and other G_1 regulatory protein degradation. A loss of CBP in cells results in slower degradation of cyclin B and slow cell cycle progression (Turnell et al. 2005).

The APC however has other functions in regulating gene transcription, and this is brought about via its interaction with CBP/p300. Loss of CBP/p300 binding to the APC results in the loss of the p21 response to UV light-induced DNA damage, a p53-mediated response (Turnell et al. 2005). The effect of the APC is not via a direct p53-mediated mechanism but rather the regulation of transcription events (Turnell et al. 2005). In addition, association of the APC with p300 increases its histone 4 acetylase activity. Hence, CBP/p300 directly regulates protein ubiquitination and also regulates the ability of the APC to ubiquitinate targets.

The p300/CBP associated factor (PCAF) has an E3-ligase RING domain in its amino terminus (Linares et al. 2007). PCAF (HDM2) of mouse targets the human homologue of MDM2 for ubiquitination and degradation. In addition, PCAF acetylates HDM2. Acetylation of the HDM2 homologue MDM2 results in an enhancement of p53 function. However, MDM2 is not acetylated by PCAF, but rather by p300 and CBP (Wang et al. 2004b). The significance of this difference in substrates between the species is not yet clear. To further complicate the system, it has been shown that MDM2 ubiquitinates and promotes the degradation of PCAF (Jin et al. 2004). The unraveling of this system shows a highly complex whereby transcription co-factors, such as CBP/p300, are under very tight regulation. The regulation of CBP/p300 by MDM2 and the converse regulation of MDM2 by CBP/p300 suggest that these proteins function under tight regulation in response to various biological stimuli. This regulation involves integration and convergence of multiple post-translational modifications of CBP/p300 including acetylation, ubiquitination and phosphorylation.

12.5 Regulation of Transcription Factors by the Ubiquitin–Proteasome System

There are many examples of transcription factors falling under the regulation of the ubiquitin–proteasome system. In general, their function is reduced due to their rapid degradation. However some transcription factors use multiple E3-ligases to regulate their function, thereby integrating multiple signaling pathways. Recent studies are beginning to unravel the additional complexities of non-degradation

signaling by the ubiquitin–proteasome system, whereby ubiquitination may affect the function of the transcription factor by regulating the binding of co-factors, or via the regulation of the cellular location of the transcription factor. Here we will focus on some specific transcription factor families and highlight the mechanisms whereby the ubiquitin–proteasome system may regulate their function.

12.5.1 Regulation of the Forkhead Transcription Factors

The Forkhead (winged helix) transcription factor family is defined by a large 100 amino acid binding domain. There are currently over 40 members encoded in the human genome (Katoh and Katoh 2004), which are the homolog of the DAF transcription factors described in *C. elegans*. Since their original identification, the nomenclature for the Forkhead proteins has changed, which can lead to some confusion in the literature: the common Forkhead proteins are FOXO-1 (FKHR), FOXO3a (FKHRL1), and FOXO4 (AFX). Gene deletion studies show that FOXO1 knockout mice are not viable past embryonic day 10, showing the importance of this transcription factor in development (Hosaka et al. 2004). In contrast, FOXO3a and FOXO4 knockouts are viable, and with no apparent defects (Arden 2008; Hosaka et al. 2004).

The Forkhead transcription factors are involved in the response of a cell to stress, nutrient deficiency and prevention of tumor progression. Activation of Forkhead transcription factor results in G1 arrest of cells due to the expression of cell cycle arrest proteins, such as p27kip1 (Stahl et al. 2002). In response to cell stressors, such as oxidative stress, seizures and ischemia, Forkhead regulates the expression of cell death mediators such as Fas ligand, TRAIL and the pro-apoptotic protein Bim (Gilley et al. 2003; Shinoda et al. 2004; Stahl et al. 2002). Members of the Forkhead family of transcription factors are regulated by multiple signaling pathways, resulting in the phosphorylation, ubiquitination, methylation and acetylation of Forkhead, each of which regulate Forkhead-mediated gene expression patterns. The reader is referred to some alternative reviews for further details of other post translational modifications of Forkhead (Vogt et al. 2005).

Forkhead transcription factors are activated following their phosphorylation by JNK in response to mild oxidative stress (Essers et al. 2004) (Fig. 12.2). The phosphorylation of Forkhead by JNK is on different residues to those phosphorylated by Akt, which turns off Forkhead-mediated gene expression. The stress activated GTPase ral activates JNK resulting in Thr447/451 phosphorylation of Forkhead (Essers et al. 2004). JNK-mediated phosphorylation of Forkhead induces gene expression and appears not to promote ubiquitination of Forkhead. When in the nucleus, the binding of active FOXO to CBP/p300 promotes acetylation of histone proteins, but may result in acetylation of FOXO. Acetylated FOXO recruits the deacetylase SIRT resulting in the preferential expression of GADD45, cell cycle and glucogenic genes, rather than pro-apoptotic genes such as Bim and Fas-ligand (Frescas et al. 2005).

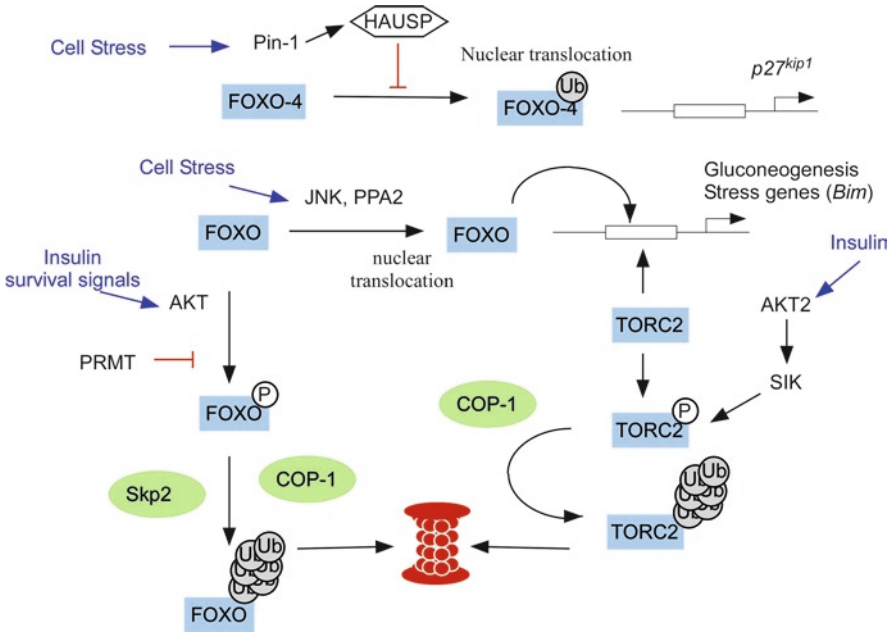

Fig. 12.2 Overview of forkhead transcription factor regulation by the ubiquitin–proteasome system. Forkhead transcription factors regulate gene expression in response to cell stress and also promote the expression of gluconeogenesis genes when glucose levels are low. Forkhead is normally repressed following its phosphorylation by Akt, which promotes its nuclear export and ubiquitination by Skp2. Forkhead is activated either by dephosphorylation by protein phosphatases or via phosphorylation of Forkhead by Jun N-terminal kinase (JNK) promoting its nuclear import. In response to conditions of high glucose availability Forkhead mediated-gluconeogenesis gene expression is rapidly turned off by the activation of Akt and the ubiquitination of Forkhead and its co-factor TORC2 by the E3-ligase Cop1. Forkhead proteins are also regulated by mono-ubiquitination, which affects their nuclear localization. The de-ubiquitinating enzyme HAUSP removes mono-ubiquitin modifications from Forkhead preventing the nuclear translocation of Forkhead (FOXO-4) and reducing gene expression

Forkhead FOXO-1 is inactivated by the phosphorylation of Ser253 by Akt (Brunet et al. 1999), where as IKKβ regulates FOXO3a phosphorylation (Hu et al. 2004). Phosphorylated Forkhead binds to 14-3-3 protein and chromosome region maintenance protein 1 and is exported from the nucleus (Kau et al. 2003). Phosphorylation blocks the nuclear location sequence in the Forkhead protein. In addition, Forkhead can be activated following its de-phosphorylation by protein phosphatase 2A (PP2A) (Yan et al. 2008). Forkhead is released from 14-3-3 revealing a nuclear localization sequence (NLS) motif resulting in translocation to the nucleus, where it can bind to its promoter region. Interestingly, Akt phosphorylation of Forkhead is prevented by methylation of two Arg residues (Arg 248/250) close to the Ser 253 site by PRMT revealing yet another layer of regulatory complexity of this transcription factor (Yamagata et al. 2008).

In addition to exporting Forkhead from the nucleus, Akt-induced phosphorylation of Forkhead promotes its degradation (Matsuzaki et al. 2003) (Fig. 12.2). Cytoplasmic FOXO-1 is ubiquitinated by Skp2, a member of the skp1/cullin/F-box E3-ligase family of proteins, which may play a role in tumorogenesis (Huang et al. 2005). Whether 14-3-3 facilitates ubiquitination or the assembly of the ubiquitination E3-ligase complex is not yet known. Alternatively 14-3-3 may protect Forkhead from the E3-ligase, preserving some of the cells Forkhead complement.

In response to cell survival signals, FOXO3a is ubiquitinated by MDM2 resulting in the proteasomal degradation of FOXO3a. Phosphorylation of FOXO3a on Ser294, Ser344 and Ser425 residues mediates the interaction between MDM2 and FOXO3a (Yang et al. 2008). Loss of these residues stabilizes the FOXO3a protein.

Forkhead proteins also play a role in the expression of genes associated with gluconeogenesis, and these studies have revealed an additional Forkhead E3-ligase, Cop1 (Fig. 12.2). In response to insulin, the E3-ligase Cop1 is expressed and this serves as an E3-ligase for FOXO-1, thereby reducing the expression of gluconeogenesis genes (Kato et al. 2008). For Forkhead to be ubiquitinated, it must be first phosphorylated on Ser256 by Akt. Akt2-mediated phosphorylation of FOXO, following insulin stimulation, results in FOXO ubiquitination by Cop1 (Kato et al. 2008). In addition to regulating Forkhead ubiquitination, Cop1 also regulates the ubiquitination of the Forkhead co-factor TORC2 (Dentin et al. 2007). Activation of Akt by insulin results in the phosphorylation and activation of the Ser/Thr protein kinase SIK (also known as Probable serine/threonine-protein kinase SNF1LK) on Ser378. Activated SIK phosphorylates TORC2 on Ser171 enhancing its Cop1-mediated ubiquitination and proteasomal degradation. This interesting example shows how a single E3-ligase may be responsible for the ubiquitination of multiple proteins to selectively turn down a specific gene expression pattern.

The two examples of E3-ligase identified for Forkhead highlight some of the challenges in identifying E3-ligases for a given protein substrate. Multiple E3-ligases can regulate protein ubiquitination when activated by different biological stimuli. For example Skp2 was identified in cancer cells and may regulate an anti-oncogenic phenotype and in contrast Cop1 was activated by insulin. However, care must be taken when interpreting such studies, as it is not yet clear what degree of redundancy E3-ligases may have, nor how truly selective an E3-ligase is for a given substrate protein.

Ubiquitination does not just promote degradation of Forkhead proteins. Mono-ubiquitination may also regulate Forkhead function. AFX/FOXO4 mono-ubiquitination results in its nuclear accumulation. Following cell stress, peptidylproline cis/trans isomerase 1 (Pin1) is activated and interacts with the deubiquitinating enzyme HAUSP/USP6 to attenuate FOXO-4 mono-ubiquitination and its nuclear accumulation (Brenkman et al. 2008). Blocking nuclear translocation of FOXO4 results in an attenuation of $p27^{Kip1}$ transcription. Interestingly, the FOXO-1 E3-ligase Skp2 also ubiquitinates the gene target of FOXO-4, $P27^{Kip1}$, as well as other cell cycle arrest associated proteins (CDK9) (Kiernan et al. 2001; Wang et al. 2003). Hence, a single E3-ligase can regulate a transcription factor family member and a family member gene product.

Taken together these examples show the rich crosstalk between intracellular signaling pathways which regulate the function of the Forkhead transcription factor family. Clearly much still remains to be investigated with respect to how different E3-ligases regulate differential gene expression depending on the biological stimuli and how Forkhead mitochondrial gene expression may also be regulated by the ubiquitin–proteasome system (Jacobs et al. 2008).

12.5.2 Regulation of bZIP Transcription Factors by the UPS

The AP1 transcription factors, also known as leucine zipper family, are regulated by ubiquitin-dependent proteasomal degradation. The AP1 family consists of the transcription factors Fos, Jun, CREB and ATFs. These transcription factors act as either homo- or heterodimers to regulate gene expression responses to multiple stimuli, including cytokines, growth factors, stress, and infections (for a more detailed review of AP1 transcription factors see Hai and Hartman 2001).

Fos is the protein encoded by the *c-fos* gene. Fos is known as an immediate-early gene due to its ability to be rapidly expressed and translated following stimuli. Fos protein is regulated by the E3-ligase Ubr1 in a signal transducer and activator of transcription protein (STAT)-dependent manner (Sasaki et al. 2006). The nuclear export and ubiquitination of Fos is regulated following the phosphorylation of Thr232 in the nuclear localization sequence NLS by Erk5 (Sasaki et al. 2006). Interestingly, in one of the earlier works studying the ubiquitin–proteasome system, a non-Ubr E3-ligase was also shown to ubiquitinate Fos protein (Stancovski et al. 1995). Ubr is an E3-ligase associated with N-end ubiquitination. This more exotic ubiquitination modification does not result in lysine residue modification, but is associated with the regulation of degron signals on proteins. Fos is an inherently unstable protein due to two degron motifs at the N- and C-termini, which promotes its degradation in a ubiquitin-independent manner (Basbous et al. 2007, 2008). Fos can also be ubiquitinated creating a second faster mechanism for its degradation by the proteasome and hence turning off Fos signaling. The ubiquitin-independent mechanism is inhibited by Erk1/2 phosphorylation of the C terminal domain on Ser363 and Ser374 (Basbous et al. 2007). One interesting hypothesis suggests that the 20S proteasome could partner with different 19S, 11S and an alternative 11S proteasome cap sub-units to form homogeneous or heterogeneous proteasome complexes, which may result in different proteasomal activities and requirements for substrates (Basbous et al. 2008; Rechsteiner and Hill 2005). It is generally believed that the 19S cap is required for Lys48-linked poly-ubiquitin-mediated degradation. Hence, alternative configurations of proteasome cap and core subunits may enable the degradation of non-K48 linked poly-ubiquitinated proteins and non-ubiquitinated proteins.

Fos is not the only AP1 transcription factor regulated by N-end rule ubiquitination. ATF5, which regulates suppression of gene expression, is also regulated by N-end rule ubiquitination (Wei et al. 2008). A number of E3-ligases have been associated with N-end ubiquitination, especially the Ubr class of E3-ligases. The

chemotoxic agent cisplatin induces cell death via a number of mechanisms, but has been shown to stabilize ATF5 protein levels via the reduced ubiquitination of ATF5 (Wei et al. 2008). Interestingly cisplatin treatment resulted in a relocation of cdc34 (aka UbcH3) from the nucleus to the cytoplasm, thereby depleting the E2-ligase necessary for ATF5 ubiquitination (Wei et al. 2008). This novel mechanism of action of cisplatin opens up the possibility that E2-ligases may also be molecular/drug targets for regulating ubiquitin–proteasome system-regulated cellular events.

Three E3-ligases have been identified for Jun: Fbw7, hCOP1/hDET and Itch (Bianchi et al. 2003; Gao et al. 2004; Nateri et al. 2004). Fbw7 and Itch are down-regulated by UV light, resulting in stabilization of c-jun levels (Anzi et al. 2008). In addition to ubiquitinating c-jun the Fbw7/hCdc4 SCF E3-ligase complex also ubiquitinates c-Myc, cyclin E and Notch transcription factors (Nateri et al. 2004; Welcker et al. 2004). Fbwx7-mediated c-jun degradation results in a loss in apoptotic signaling in neurons, and conversely the loss of Fbw7 results in stabilization of c-jun and neuronal apoptosis (Nateri et al. 2004). The effect of Fbxw7 knockdown is mitigated by overexpression of the inhibitory JNK interacting protein JIP (Nateri et al. 2004). To date it is not clear which residues are phosphorylated to regulate Jun degradation, both JNK and CSK3 (COOH-terminal Src kinase) phosphorylate Jun, thus promoting its ubiquitination (Nateri et al. 2004; Wei et al. 2005; Zhu et al. 2006).

Cyclic AMP mediated transcription events are mediated by CRE-response element binding protein (CREB). The transcriptional activity of CREB is enhanced following its phosphorylation on a critical Ser133 residue, which promotes its association with CBP (Barco and Kandel 2006; Johannessen et al. 2004; Mayr and Montminy 2001). Other residues on CREB are also phosphorylated resulting in enhanced or repressed transcriptional activity (Kornhauser et al. 2002). Activation of CREB results in the transcription of many genes, but interestingly it also drives the expression of its inhibitory proteins cAMP response element modulator (CREM) and inducible cAMP early repressor (ICER) (Barco and Kandel 2006). Both CREB and its repressor proteins are ubiquitinated by E3-ligases promoting their rapid degradation.

It is not yet clear which E3-ligase ubiquitinates CREB, but CREB ubiquitination and degradation is reported following hypoxia and PDGF-BB treatment of pulmonary smooth muscle cells (Garat et al. 2006). This effect is regulated by the Akt-mediated phosphorylation of Ser103 and Ser107 residues. Phosphorylation of this region results in nuclear export of CREB and its subsequent degradation by the proteasome. The export of CREB from the nucleus is blocked by Leptomycin B, which prevents the degradation of CREB (Garat et al. 2006). Akt is associated with the activation of CREB via the phosphorylation of the Ser133 residue (Perkinton et al. 2002; Walton and Dragunow 2000). In addition, brief ischemic conditions in brain cells activate both Akt and CREB, resulting in new gene expression and protection (Meller et al. 2005; Noshita et al. 2001; Tanaka et al. 1999; Walton et al. 1996). It is not clear how Akt signaling switches from promoting CREB activation to regulating the nuclear export and proteasomal degradation of CREB.

Functionally, the proteasome regulation of CREB may play a role in the development of long-term potentiation (LTP), a cellular model of learning. Following brief tetanic

stimulation, the response of a neuron to a given stimulation is increased. Many complex molecular pathways regulate LTP. Proteasome inhibitors have been shown to enhance the induction, but impair the maintenance of late phase LTP in the hippocampus (Dong et al. 2008). Proteasome inhibitors appear to block CREB function in LTP by preventing the degradation of the CREB repressor ATF4, reducing expression of BDNF (Dong et al. 2008). In the Aplysia model of LTP, serotonin induces the degradation of CREB repressors via the proteasome in a protein kinase C-dependent manner (Upadhya et al. 2004). These two studies suggest that under conditions of enhanced CREB function, its repressors are rapidly degraded.

The CREB ICER has been shown to be under tight regulation by the ubiquitin–proteasome system. Activation of MAPK has been shown to drive ICER phosphorylation (Ser41) and its ubiquitination in JEG-3 and mouse pituitary AtT20 cells (Yehia et al. 2001). MAPK also regulates CREB transcription via phosphorylation of CREB, CBP and affecting CBP recruitment to the CRE (Johannessen et al. 2004; Meller et al. 2005). ICER phosphorylation was blocked by MAPK inhibitors (PD98059) and cAMP (Yehia et al. 2001). Consistent with phosphorylation of ICER regulating the stability of this protein, loss of the Ser41 site on ICER prolongs the half life of the ICER protein (Yehia et al. 2001).

The SCF (skp1-fbox-cullin) complex has been identified as an ICER E3-ligase in yeast studies and utilizes the E2-ligases rad6 (UbcH2) and cdc34 (UbcH3) (Pati et al. 1999). Interestingly, loss of these two specific E2 ligases resulted in elevated levels of ICER, suggesting that both cdc34 and rad6 are necessary for ICER ubiquitination. This study shows how E2-ligases, in addition to E3-ligases, may regulate the target specificity of the ubiquitination reaction.

12.5.3 Regulation of Smad Transcription Factors by the UPS

The Smad family of transcription factors are activated by transforming growth factor (TGF) and bone morphogenic protein (BMP). The mammalian Smad proteins are homologues of the drosophila mother against decapentaplegic (MAD) and the *C. elegans* SMA transcription factors. These transcription factors are broken down into three different subgroups depending on their function. The receptor Smads (rSmads) interact with activated TGF receptors (Smad2 and 3) or BMP receptors (Smad1, 5 and 8). Following activation of their respective receptors, the receptor associated Smads (rSmads) are phosphorylated, bind to Smad4 and move to the nucleus. Once in the nucleus they bind to the Smad promoter and recruit additional transcription factors and cofactors such as CBP/p300 to initiate TGF and BMP-mediated gene expression (Shen et al. 1998; Yang et al. 2000; Zhang et al. 1998). Smad signaling is blocked by inhibitor Smads (iSmads) 6 and 7, which compete for the binding site at the receptor. In addition, the nuclear actions of active Smads are inhibited by SnoN and Ski (He et al. 2003). For a more detailed review, the reader is referred to the following review articles (Inoue and Imamura 2008; Ross and Hill 2008; Schmierer and Hill 2007).

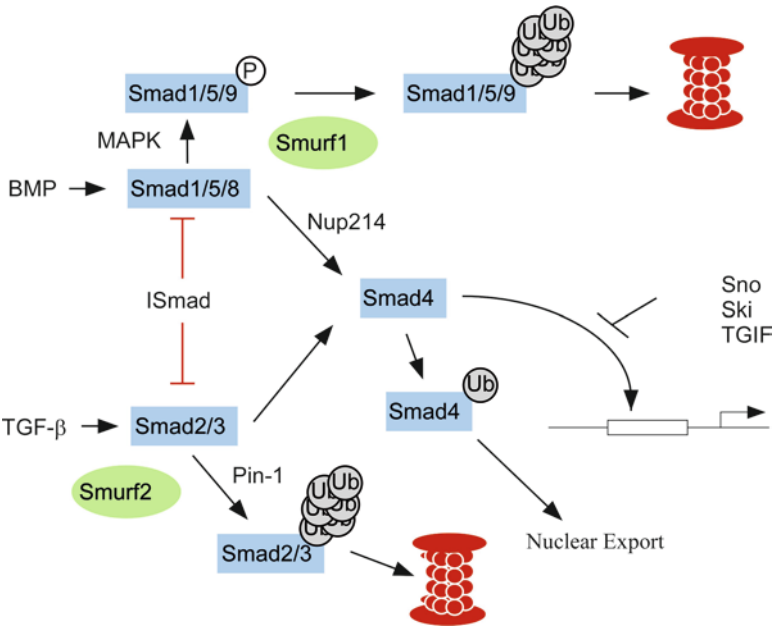

Fig. 12.3 Overview of Smad transcription factor regulation by the ubiquitin–proteasome system. Activation of receptor associated Smads by either TGF (Smad 2 and 3) or BMP receptors (Smads 1, 5 and 8) results in binding to a co-activator Smad4 and gene expression. Smad gene expression is inhibited by inhibitory co-factors Sno, Ski and TGIF. Smads are ubiquitinated by Smurf1 and Smurf2. Smurf1 ubiquitinates MAPK phosphorylated Smad1 associated with BMP signaling. Smurf2 ubiquitinates Smad2 and requires a co-factor Pin-1. In addition, mono-ubiquitination of SMAD4 results in its nuclear export

The Smad system is highly regulated by the ubiquitin–proteasome system (Inoue and Imamura 2008) (Fig. 12.3). Smad proteins are ubiquitinated by the HECT E3-ligase Smad ubiquitination regulatory factor1 (Smurf1), Smurf2 and a multi-subunit RING-type E3-ligase, ROC1-SCF (Fbw1a) (Fukuchi et al. 2001; Li et al. 2004). The E3-ligase CHIP has been shown to ubiquitinate Smad1 and Smad4 independent of TGF receptor activation (Li et al. 2005a). Additional E3-ligases associated with the poly-ubiquitination and degradation of Smad proteins are WWP1, Itch, Arkadia, SCFbwi1, SCF skp2, APC and ectodermin (Inoue and Imamura 2008). Here we will outline the role of some of these E3-ligases, but clearly the large number of E3-ligases with the potential to regulate Smad signaling emphasizes the complex regulation of this developmentally important pathway.

The E3-ligase Smurf1 ubiquitinates Smad1, 5 and 7, whereas Smurf2 ubiquitinates Smad1 and 2 as well as the inhibitor protein SnoN (Inoue and Imamura 2008). In addition, Smurf1 prevents nuclear import of active Smad1 by blocking its interaction with the nuclear import factor NUP214 (Sapkota et al. 2007). Ubiquitination of Smad proteins is associated with their degradation, thereby attenuating either TGF or BMP signaling.

Smurf binds to Smads promoting their ubiquitination and preventing signaling from upstream receptors (Fig. 12.3). Smurf-mediated Smad ubiquitination is regulated by prior phosphorylation of the substrate protein, similar to many protein ubiquitination pathways. The Smurf WW2 domain has been shown to bind to a PPXY motif in the linker region of Smads (Sangadala et al. 2007). In addition, Pin1, a peptidyl-prolyl cis–trans isomerase (PPIase) appears to be a necessary co-factor to regulate Smad ubiquitination by Smurf (Nakano et al. 2009). Pin-1 binds to Smad2 and 3, but not 4, due to their phosphorylation on a ST-P motif. Phosphorylation of this motif increases the association of Smads with Smurf (Nakano et al. 2009). Since Pin-1 enhances Smad ubiquitination, this suggests that Pin-1 may function as a regulator of protein ubiquitination, not only for Smads, but other proteins whose ubiquitination is regulated by phosphorylation.

Smad3 is ubiquitinated by ROC1-SCF, promoting Smad3 degradation in HaCaT human keratinocyte cells (Fukuchi et al. 2001). Smad3 ubiquitination was enhanced following TGF receptor stimulation, and resulted in a direct interaction between ROC1and the C-terminal MH2 domain of Smad3. p300 has biphasic effects on TGF signaling and Smad function. Binding of CBP to the Smad3 complex promotes Smad3-mediated gene expression. However, binding of Smad3 to CBP also promotes the ubiquitination of Smad3 by Roc1-SCF (Fukuchi et al. 2001). This may be part of a mechanism to "turn off" the TGF signal.

Smurfs ubiquitinate the trans-membrane receptors that activate Smad signaling. Both Smurf1 and 2 associate with Smad7 to promote the ubiquitination of both Smad 7 and the bound TGF receptor (Ebisawa et al. 2001; Kavsak et al. 2000). Smurfs are localized in the nucleus, but binding of the E3-ligase to Smad7 promotes their export to the membrane surface. Smad7 binding to Smurf may also enhance its ability to bind and utilize the E2-ligase UbcH7 (Ogunjimi et al. 2005). Smurfs can also facilitate Smad signaling via the degradation of the inhibitory factor SnoN. Smad2 activation promotes Smurf to ubiquitinate the inhibitory co-repressor SnoN (Bonni et al. 2001). Activated Smad3 also targets SnoN for degradation, but via the APC E3-ligase (Stroschein et al. 2001).

Additional E3-ligases shown to regulate Smad2 function are Nedd4-2 and Itch. Nedd4-2 ubiquitinates Smad2, reducing Smad2 mediated gene expression. Nedd 4-2 also ubiquitinates the TGF receptor 1 when bound to Smad2, and Smad4 when bound to Smad7 (Kuratomi et al. 2005). The protein kinase Itch, which is associated with NF-κB signaling, also regulates Smad2 function. Itch induces phosphorylation of Smad2, which increases its interaction with TGF receptor 1 and Smad7 to decrease TGF signaling (Bai et al. 2004).

Smad7 may play an important role in integrating Wnt/β-catenin signaling into the Smad signaling pathway. The Wnt signaling protein Axin binds to the E3-ligase Arkadia and Smad7 to promote Smad7 ubiquitination, thereby enhancing Smad signaling (Liu et al. 2006). Reducing Axin or Arkadia expression increases the stability of Smad7. Axin is inhibited by Wnt1 and Axin overexpression attenuates Smad7 degradation (Liu et al. 2006). In addition, it has been shown that Arkadia can also ubiquitinate Sno and Ski (Nagano et al. 2007). Interestingly, the binding of Axin to Smad7 competes with β-catenin (Tang et al. 2008). Smad7 binding to

Axin also displaces GSK3β and Smurf2 from the Axin complex, which reduces β-catenin phosphorylation and degradation (Tang et al. 2008). However the Smad7 stabilized β-catenin is preferentially transferred to the cell membrane, rather than the nucleus, where it interacts with cadherin proteins increasing cell-cell adhesion signals (Tang et al. 2008). As such, these examples show the complex regulation between transcription factor systems which are regulated by the ubiquitin–proteasome system.

In addition to regulating Smad function by poly-ubiquitination, mono-ubiquitination may also regulate Smad4 biological activity. Smad can be mono-ubiquitinated in the C-terminus domain (Lys507) promoting its binding to rSmads and enhancing transcriptional activity (Moren et al. 2003). In contrast to Lys507, Lys519 mono-ubiquitination has been shown to inhibit Smad4 function by blocking its interaction with phospho-Smad2. FAM/USP9x was identified by siRNA screen of Smad4 function, and ectodermin/Tif1γ is a potential mono-ubiquitin E3-ligase (Dupont et al. 2009). What is unclear is whether one form of mono-ubiquitination is dominant over the other form. In addition the E3-ligase which regulates the Lys507 modification has not yet been identified. These studies suggest that mono-ubiquitination of residues may have similarity to phosphorylation of residues on a protein, whereby seemingly adjacent phosphorylation sites have opposing effects on the function of the protein.

12.5.4 Regulation of the p53 Pathway by the UPS

The p53 pathway is a critical regulator of the response to cellular stressors (i.e. DNA damage, chromosomal aberrations, telomere erosion, hypoxia, or oncogenic responses) (Michael and Oren 2003). In unstressed cells, the p53 is shut off. In response to cellular stressors, the p53 pathway is activated thereby shutting down the multiplication of cells through cell cycle arrest, apoptosis, and as more recently shown, necrosis (Tu et al. 2009). An essential component of this response is the regulation of the levels and the activity of the transcription factor, p53. Upon activation of the pathway, p53 organizes into a tetramer, translocates into the nucleus where it upregulates and downregulates genes that are essential mediators of cell cycle arrest and apoptosis (for current reviews of p53 responsive genes, see references Riley et al. 2008; Bunz et al. 1998).

The levels of p53 are kept low in unstressed cells, but upon the introduction of stressors the levels of p53 dramatically increase (Kastan et al. 1991; Maltzman and Czyzyk 1984). The p53 pathway uses the ubiquitin–proteasome system to regulate p53 and a number of E3-ligases have been implicated in this process, including Pirh2, COP1, TOPORS, ARF-BP1, and MDM2 (Chen et al. 2005; Dornan et al. 2004; Honda et al. 1997; Leng et al. 2003; Rajendra et al. 2004). The most studied of these is the MDM2 which provides many examples of how ubiquitin–proteasome system is used to regulate transcription factors (Fig. 12.4). MDM2 regulates p53 levels through a negative feedback loop where p53 induces the expression of

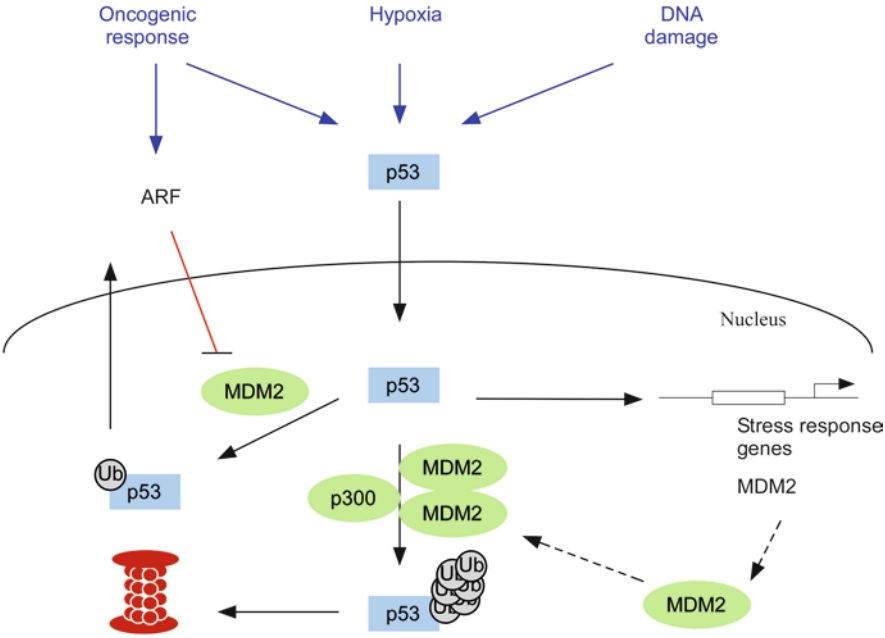

Fig. 12.4 Overview of p53 transcription factor regulation by the ubiquitin–proteasome system. p53-mediated gene expression is activated in response to oncogenic stressors, hypoxia and DNA damage. p53 regulates the expression of its own E3-ligase MDM2. When MDM2 levels are high in conjunction with p300, p53 is poly-ubiquitinated and degraded by the proteasome. When levels of MDM2 are low, p53 is mono-ubiquitinated which drives its export from the nucleus. The p53-regulated gene ARF inhibits MDM2 function

MDM2 (Barak et al. 1993; Wu et al. 1993). MDM2 then binds to p53 and promotes inactivation and proteasome-mediated degradation of p53. The level of p53 reduces the overall transcriptional activity of p53 and hence the levels of MDM2 transcribed. Continual p53 promoted expression is likely required as MDM2 is a very short-lived protein due to its self-ubiquitination and degradation (Chang et al. 1998). Interestingly, an in vitro assay using recombinant proteins showed that MDM2 modifies p53 through covalent attachment of mono-ubiquitin to multiple lysines of p53, although modification through a chain of Lys48-linked ubiquitin is associated with proteasomal-mediated degradation (Fang et al. 2000; Honda and Yasuda 2000). However, it is possible that in vivo MDM2 could be modified by p53 through poly-ubiquitination by an E4-enzyme.

The regulation of p53 export out of the nucleus may also be regulated by MDM2. Co-expression of MDM2 along with p53 promoted nuclear export of p53 (Boyd et al. 2000; Geyer et al. 2000). Furthermore, an expressed ubiquitin–p53 fusion protein promoted nuclear export of p53. It has been proposed this ubiquitination of p53 by MDM2 causes a conformation change in p53 and exposes or activates the nuclear export signal (NES) in p53, thereby promoting nuclear export of p53 (Gu et al. 2001; Lohrum et al. 2001). This nuclear shuttling of p53 by MDM2

is highly dependent on the type of ubiquitin modification. Expression of varying levels of MDM2 along with p53 in the p53-null human lung carcinoma cell line revealed that low levels of MDM2 promoted mono-ubiquitination and rapid translocation of p53 out of the nucleus, whereas high levels of MDM2 promoted poly-ubiquitination and nuclear degradation of p53 (Li et al. 2003). The necessity of having a two-step process for ubiquitination of p53 is unknown, but translocation of p53 to the cytoplasm could prevent any extraneous transcriptional activity by p53 in a rapid and reversible fashion (Brooks et al. 2004).

The interactions between p53 and MDM2 are modulated by a number of proteins. One known modulator of this interaction is the tumor suppressor, Arf (Arf is known as p19Arf in mouse and p14Arf in humans). Arf is induced in response to oncogenic signals, such as overexpression of the oncogenes E1A, Myc and Ras (de Stanchina et al. 1998; Palmero et al. 1998; Zindy et al. 1998). Inactivation of Arf is known to commonly occur during cancer development and its inactivation is associated with upregulation of oncogenes such as retinoblastoma (Chang et al. 2007; Matheu et al. 2008). Arf prevents MDM2-mediated degradation of p53 by binding to MDM2 thereby inhibiting MDM2 activity as well as preventing export of the p53-MDM2 complex out of the nucleus (Honda et al. 1997; Midgley et al. 2000; Zhang and Xiong 1999).

Another important modifier of MDM2 activity is the transcriptional co-activator p300/CBP which has E3-ligase activity (Fig. 12.4). In vitro, MDM2 alone can only promote the mono-ubiquitination of p53, but co-expression of MDM2 with p300 promotes poly-ubiquitination of p53 (Grossman et al. 2003; Lai et al. 2001). Although it was not examined whether the proteasome-targeted K48 linked polyubiquitin chains were formed, it has been speculated that p300 is necessary for promoting proteasome-mediated degradation of p53 (Grossman et al. 2003). However, work using the p53 null fibroblasts found that the expression of p300 was able to promote ubiquitination of p53, but failed to promote degradation of p53 (Zhu et al. 2001).

12.5.5 Regulation of the Wnt/β-Catenin Pathway by the UPS

Wnt-1 signaling pathway is a highly conserved pathway that plays a critical role in cell adhesion, cellular proliferation, cellular differentiation, and stem cell maintenance, thereby affecting many developmental processes such as neurogenesis, hematopoiesis, and body axis formation (Clevers 2006). Three major cascades of Wnt have been identified: one pathway commonly referred to as the canonical Wnt signaling and two later identified non-canonical Wnt signaling pathways. The transcriptional factor complex of β-catenin/Tcf has been identified as a major effector of the canonical Wnt signaling pathway and will be the focus of this review (Widelitz 2005).

Stimulation of transcriptional activity in Wnt signaling is essentially regulated by the levels of cytoplasmic β-catenin. In the absence of Wnt signaling, cytoplasmic

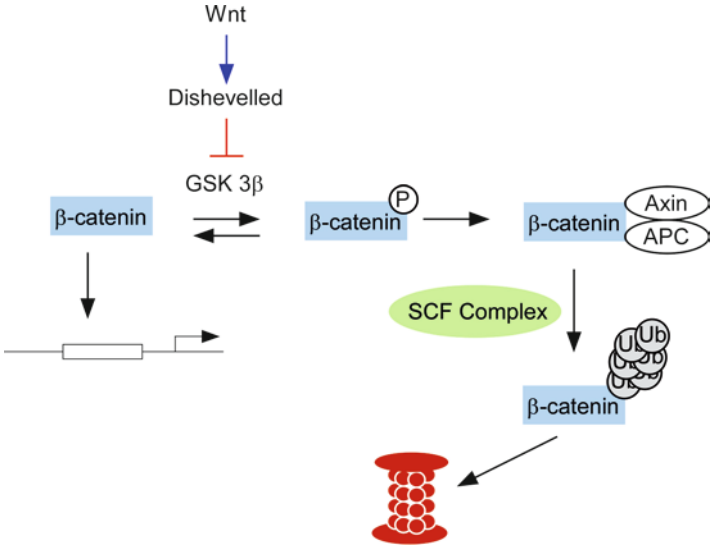

Fig. 12.5 *Overview of β-catenin transcription factor regulation by the ubiquitin–proteasome system*. β-catenin levels are normal low in the cell, due to the phosphorylation of β-catenin by GSK-3β, which increases the binding of β-catenin to axin and APC. Once bound to these co-factors β-catenin is ubiquitinated by a SCF E3-ligase and degraded. Following activation of Wnt signaling, dishevelled blocks GSK-3β function thus stabilizing β-catenin protein levels and enabling the nuclear translocation of β-catenin and promoting gene expression

levels of β-catenin are kept low through ubiquitin-mediated degradation (Aberle et al. 1997) (Fig. 12.5). Ubiquitin-mediated degradation of β-catenin is initiated through phosphorylation of β-catenin by glycogen synthase kinase-3β (GSK-3β) (Hedgepeth et al. 1997; Stambolic et al. 1996). This phosphorylation promotes the association of β-catenin with a degradation complex consisting of adenomatous polyposis coli (APC), Axin, Casein kinase-1α, and GSK-3β (Hart et al. 1998; Kishida et al. 2001; Sakanaka et al. 1998; Price 2006). The formation of this complex is necessary for subsequent ubiquitination of β-catenin by the SCF E3-ligase complex, which consists of Skp1, Cullin1, and Roc1/Rbx1 (Liu et al. 2004b; Winston et al. 1999). The recruitment of β-catenin to the SCF complex is mediated through F box motif ubiquitin ligase receptors, Slimb, Beta transduction repeat containing protein (β TrCP), and Homologue of Slimb (HOS) (Fuchs et al. 1999; Latres et al. 1999; Jiang and Struhl 1998). In the presence of Wnt, Dishevelled inhibits GSK-3β and β-catenin remains unphosphorylated (Wagner et al. 1997). β-Catenin can then form a transcriptional complex with β-catenin/Tcf/Lef and promote transcriptional transactivation of Wnt-responsive genes (Behrens et al. 1996).

Recent work has shed light on the purpose of the association of β-catenin to the APC/Axin/GSK-3β degradation complex. The association of phosphorylated β-catenin with the APC protein is critical for their recruitment to the SCF E3-ligase complex. Furthermore, the association β-catenin with APC is required to preserve

the phosphorylation of β-catenin (Su et al. 2008). This work would suggest that APC plays a critical protective role to ensure that β-catenin is ubiquitinated. The importance of the regulation of β-catenin levels in Wnt signaling is illustrated by the fact that β-catenin levels are not only regulated in the cytoplasm by the SCF complex, but also in the nucleus. The histone acetyltransferase (HAT) complex component, Transcriptional Histone Acetyltransferase Cofactor (TRRAP), recruits Skp1 to β-catenin residing on its target promoter in chromatin and promotes β-catenin ubiquitiation and degradation (Finkbeiner et al. 2008).

Ubiquitination plays a role in the Wnt pathway by not only regulating β-catenin levels, but also ubiquitin ligase receptors as well. The Wnt pathway was demonstrated to regulate the mRNA levels of ubiquitin ligase receptor, βTrCP. The expression of a dominant negative Tcf decreased the levels of βTrCP1 mRNA whereas overexpression of stabilized β-catenin in HEK293T cells provided elevated levels of βTrCP mRNA. β-catenin/Tcf affect mRNA levels by inducing the expression of an RNA-binding protein, CRD-BP, which stabilizes βTrCP1 mRNA (Noubissi et al. 2006). Elevated levels of βTrCP resulted in enhanced β-catenin degradation (Latres et al. 1999). This would suggest that β-catenin/Tcf through induction of CRD-BP form a negative feedback loop to regulate the expression levels of β-catenin.

12.5.6 Regulation of the HIF-1α Pathway by the UPS

Hypoxia-inducible factor-1 (HIF-1) is a basic helix-loop helix-PAS domain transcription factor that regulates the cellular response to oxygen deprivation. Through induction of over 100 target genes, HIF-1 is able to promote adaptation of tissue to hypoxic conditions (i.e. increased oxygen delivery to tissues, increased angiogenesis and increased anaerobic glycolysis) (Yang et al. 1998) (Koh et al. 2008b). HIF-1 consists of two subunits: an oxygen-regulated subunit, HIF-α and a constitutively expressed subunit, HIF-1β (Wang et al. 1995). Currently, there are known three isoforms of HIF-α: HIF-1α, HIF-2α and HIF-3α. HIF-1α is expressed ubiquitiously, whereas HIF-2α shows tissue-specific expression. HIF-α/HIF-1β heterodimer translocates to the nucleus from the cytoplasm during hypoxic conditions where it binds to HIF binding sequence within the hypoxia-response element (HRE) (Wang and Semenza 1993; Wood et al. 1998).

Under normal oxygen levels (normoxia), HIF-α levels are kept low through continual ubiquitination and proteasome-mediated degradation (Fig. 12.6). The initiation of ubiquitination of HIF-α requires its hydroxylation on proline residues located within the oxygen-dependent domain (ODD) by specific proline hydroxylases (PHD1, PHD2 and PHD3s) (Ivan et al. 2001; Jaakkola et al. 2001). PHD activity depends on oxygen, so under hypoxic conditions PHDs are inhibited and incapable of hydroxylating HIF-α (Fong and Takeda 2008). Hydroxylation by PHDs under normoxic conditions allows HIF-α to bind to von Hippel-Lindau tumor suppressor gene (pVHL) (Jaakkola et al. 2001; Maxwell et al. 1999). HIF-α

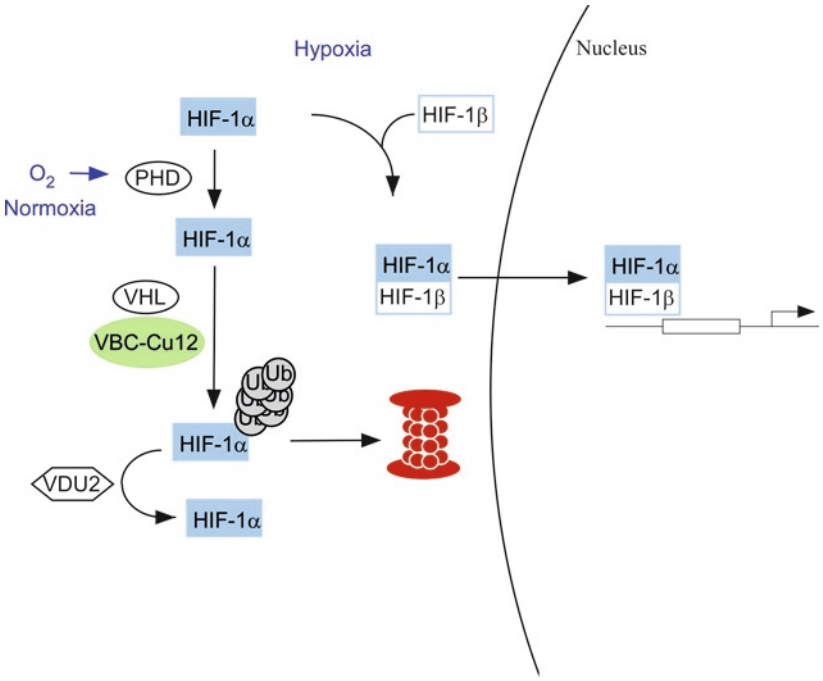

Fig. 12.6 Overview of HIF-1α transcription factor regulation by the ubiquitin–proteasome system. Under normal (normoxic conditions) HIF-1α levels are kept low. HIF-1α is hydroxylated on proline residues located within the oxygen-dependent domain (ODD) by specific prolyl hydroxylases (PHD) and then acetylated by arrest-defective 1 N-acetyltransferase. Acetylated HIF-1α binds to von Hippel–Lindau tumor suppressor gene (pVHL), is ubiquitinated by VBC-cu12 and then rapidly degraded by the proteasome. HIF-1α can be de-ubiquitinated by the DUB VDU2. Under hypoxic conditions, PHD is inhibited, which stabilizes HIF-1α and promotes its interaction with HIF-1β, leading to nuclear import and gene expression

and pVHL then form a complex with a member of the cullin-RING E3-ligase, the Elongin B/C-cullin2-Ring box 1 ubiquitin ligase (VBC-Cul2) (Kibel et al. 1995).

Other E3-ligases complexes can also ubiquitinate HIF-α. Thus, overexpression of SAG (sensitive to apoptosis gene), a member of the ROC1/RBX1 family, in the presence of pVHL can promote ubiquitination and degradation of HIF1-α. This effect is inhibited by a small interfering RNA against SAG. Like ROC1/RBX1, SAG forms a complex with the E3-ligase complex proteins, Cul-5 and pVHL (Tan et al. 2008). Receptor of activated protein kinase (RACK) is also involved in E3-ligase complex formation. RACK competes with Hsp90 of HIF-1α leading to instability. RACK then binds with Elongin B and C to promote formation of the E3-ligase complex leading to ubiquitination of HIF-1α (Liu et al. 2007). Hypoxia-associated factor (HAF), an E3-ligase expressed in proliferating cells, is able to promote ubiquitination irrespective of oxygen levels which results in a decrease in HIF-1α levels (Koh et al. 2008a).

Currently, p-VHL-interacting deubiquitinating enzyme 2 (VDU2), is the only DUB known for HIF-1α. VDU2 was found to interact with HIF-1α leading to the deubiquitination and stabilization of HIF-1α. Consequently, there is an increased level of expression of the HIF-1α responsive gene, vascular endothelial growth factor (VEGF) (Li et al. 2002, 2005b). The levels of pVHL are also known to be regulated by ubiquitination by its interaction with the E2-EPF ubiquitin carrier protein (UCP), a protein that is often overexpressed in liver and gastric cancers (Jung et al. 2006; Ohh 2006). pVHL and UCP were found to interact in HEK293T cells through co-immunoprecipitation experiments. Overexpression of UCP lead to decrease in pVHL levels in the presence of MG132, suggesting that UCP mediates proteasomal degradation of pVHL. As a result, HIF-1α levels were elevated (Jung et al. 2006).

These studies show the regulation of HIF1 and its regulatory protein by multiple ubiquitin ligases and demonstrate the necessity to control levels of these proteins during normoxic conditions. It would be interesting to determine the effect of ischemia and other cell stress conditions on the regulation of these E3-ligases.

12.6 Therapeutic Implications for Neurological Disease

Given the critical role of the ubiquitin–proteasome system in regulating cell function, it is perhaps not surprising that evidence for dysfunction of this system may be implicated in neuropathological disease. Experimental evidence supports a dysfunctional ubiquitin–proteasome system in Huntington's disorder, Alzheimer's and Parkinson's diseases. In addition, acute neurological disorders, such as stroke and seizure, also affect ubiquitin-proteasome function. While many studies may implicate the ubiquitin–proteasome system in the etiology of the disease, very few have identified an effect of ubiquitin–proteasome system dysfunction on gene expression regulation. Given the regulation of multiple transcription factors by the ubiquitin–proteasome system, this is clearly an area for further investigation.

12.6.1 Acute Neurological Conditions

Prolonged ischemic conditions result in neuronal cell death (stroke). Following harmful ischemia, ubiquitinated proteins accumulate in the brain due to proteasomal inhibition. The accumulation of ubiquinated proteins, or their subsequent proteasomal or autophagic processing may contribute to additional cellular stress (Hayashi et al. 1991; Keller et al. 2000; Liu et al. 2004a, 2005). Interestingly, the proteasome inhibitors MLN519 and bortezomib protect against ischemia induced brain damage (Henninger et al. 2006; Williams et al. 2003, 2006). However further studies suggest that the protective effect of proteasome inhibitor MNL519 may be due to the inhibition of neuro-inflammation associated NF-κB signaling, rather than preventing global proteasomal function (Williams et al. 2006).

Ischemic cell death is a delayed event and requires novel protein synthesis. The transcription factor HIF-1α is stabilized under hypoxic conditions where it mediates a program of gene expression (see Sect. 12.5.6). However, shorter periods of ischemia may help prevent cell death in a process called ischemic tolerance. Following brief ischemia (preconditioning) a different pattern of gene expression is observed compared to that occurring following harmful ischemia (Stenzel-Poore et al. 2003). Interestingly, the prior exposure to brief ischemia can re-program the genomic response to a harmful ischemic insult (Stenzel-Poore et al. 2003). Recent proteomic studies have started to identify proteins ubiquitinated following brief periods of ischemia. Of the proteins which were identified by proteomics some are associated with gene expression regulation (Meller et al. 2008). Hence, the ubiquitin–proteasome system may regulate gene expression following brain ischemia.

A role for the ubiquitin–proteasome system in seizures and epilepsy is less clear. Two studies have recently identified mutations in E3-ligase proteins in familial forms of epilepsy. For example a mutation in the FBX25 gene, which is a member of the SCF E3-ligase family was identified in a patient with familial epilepsy and mental retardation (Hagens et al. 2006). In a recent study of Angelman syndrome, a neurological disorder characterized by mental retardation and seizures, a mutation in the HECT family of E3-ligases UBE3A was observed in 10% of cases (Lalande and Calciano 2007). To date it is not clear whether these mutations are associated with specific types of epilepsy or with mental retardation. In addition, it is not yet clear what the consequence these mutations have on protein ubiquitination.

12.6.2 Progressive Neurological Disease

Alzheimers disease is characterized by the accumulation of β-amyloid proteins which form plaques and tangles in the brain. A shift in reading of the β-amyloid and ubiquitin genes results in the mutant expression of ubiquitin-B and β-amyloid (van Leeuwen et al. 1998). Brain plaques are enriched with microtubule proteins including tau. Interestingly the E3-ligase CHIP has been identified as a potential therapeutic target to degrade tau-enriched inclusions (Dickey et al. 2007). It is not yet fully clear whether the plaques that form in Alzheimer's disease are a self survival attempt by the cell or directly contribute to neurotoxicity.

The accumulation of ubiquitinated proteins results in the ER stress response. It has recently been shown that mutations in the SEL-1L gene which mediates ER stress gene expression may confer susceptibility to Alzheimer's disease (Saltini et al. 2006). To date, no studies have shown how ubiquitination with the mutant ubiquitin (+1) directly affects the function of transcription factors. Ubiquitin-mediated Wnt signaling may be aberrant in Alzheimers Disease. The phosphorylated form of β-catenin has been shown to accumulate in Alzheimer's patients. Furthermore, there appears to be enhanced levels of ubiquitinated form of β-catenin in these patients (Ghanevati and Miller 2005). Other groups report that tau hyperphosphorylation

results in a decrease in β-catenin phosphorylation and increased function (Li et al. 2007b). Hence, whether β-catenin is dysfunctional in Alzheimer's disease and its effect on disease progression is not yet clear.

Aberrant proteasome function has been noted in Parkinson's disease (Mandel et al. 2005). Multiple genes associated with the ubiquitin proteasome including E3-ligases (skp1A) and proteasome subunits show decreased expression in sporadic Parkinson's disease (Mandel et al. 2005; Zhang et al. 2005). In addition, there are 10 Parkinson's disease related genes, of which one is an E3-ligase (Parkin2) and one is a deubiquitinating enzyme (Parkin 5) (Le and Appel 2004). The Parkin2 gene, which encodes the E3-ligase, functions with CHIP and LIM-kinase 1 to ubiquitinate proteins (Imai et al. 2002; Lim et al. 2007). To date, no studies have shown a definitive dysfunction of a gene expression patterns mediated by the ubiquitin proteasome as a cause of Parkinson's Disease.

A better understanding of the impact of an aberration in the ubiquitin–proteasome system on gene expression regulation can be shown in Huntington's disease. Huntington's disease is due to a large poly-glutamine (polyQ) expansion in the Huntington gene (Htt). There is a number of poly-glutamine expansion diseases, such as spinocerebellar ataxia, and some non-coding expansions as observed in fragile X syndromes. Huntington's disease results in neuronal cell damage and inclusion bodies in the brain. The cells show abnormal phenotypes and one common observation is a decrease in proteasome activity in animal models of Huntington's. This has recently been confirmed by mass spectrometric experiments of brain samples from patients with Huntington's disease, which show an enhanced content of poly-ubiquitinated proteins in diseased brains, thus strongly suggesting a dysfunction in the proteasome (Bennett et al. 2007). In addition, proteasome activators reduce cell death in Huntington's disease models (Seo et al. 2007).

CRE-mediated gene expression is reduced in cell culture models of Huntington's, whereby Htt is overexpressed in PC12 cells. Microarray experiments show a reduction in CRE-mediated gene expression; whether this is due to reduced CREB levels or enhanced CREB inhibition is not clear (Wyttenbach et al. 2001). Overexpression of the mutant poly Q expanded Htt gene results in the formation of nuclear aggregates in hippocampal cells, which are enriched in CBP and then subject to ubiquitination and proteasomal degradation (Jiang et al. 2003). This observation is consistent with the observation of reduced CRE-mediated gene expression in Huntington's disease models (Wyttenbach et al. 2001). Given the importance of CREB-mediated gene expression for neuronal survival, this is likely to be detrimental to neurons (Walton and Dragunow 2000). In addition, the loss of CBP will increase competition for the remaining CBP thereby affecting the rates of transcription mediated by other transcription factors.

To further support this view, it has been shown that histone mono-ubiquitination is altered by mutant Htt (Kim et al. 2008). The decreased interaction between Htt and the E3-ligase Bmi results in a reduction in H2A mono-ubiquitination. Mono-ubiquitination of H2A is associated with reduced gene expression (Cao et al. 2005; Wang et al. 2004a; Zhou et al. 2008). Hence, this would suggest that Htt disrupts normal gene expression to change the pattern of genes regulated by histones.

This interesting example of so called "epigenetic re-modeling" of transcription suggests that the mutation of Htt would have a large global consequence on gene expression patterns and has implications for planned therapies for this disease.

12.7 Concluding Remarks

Many neurological diseases show dysfunction in their ubiquitin–proteasome systems either due to the selective loss of proteins, or a general downregulation of function. Since gene expression is under such tight regulation by the ubiquitin–proteasome system, a strong case for regulation of gene expression via transcription factor degradation may be made for multiple neurological diseases. However, the recent finding that the mono-ubiquitination of histones is altered in Huntington's disease strongly suggests that ubiquitin-mediated epigenetic reprogramming mechanisms may have profound effects in neurological diseases. Clearly, this identifies a novel area of biology, which requires further understanding in order to extrapolate from these observations into viable therapeutic options.

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