Chapter 11
Covalent Protein Modification as a Mechanism for Dynamic Recruitment of Specific Interactors

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11.1 Introduction

The complement of proteins available to a human cell comprises ca. 20,000–25,000 members (Lander et al. 2004), a number not vastly greater than the number of proteins present in a unicellular organism such as *Saccharomyces cerevisiae* (the genome of which encodes ca. 6,000 different proteins (Goffeau et al. 1996)). An additional level of complexity is generated through the generation of multiple isoforms of many proteins via differential splicing and the use of alternate transcriptional start sites, which increases the repertoire of potential individual protein species. However, the mere presence of a large number of effector molecules does not create the dynamic interchange of information required to mediate the processes of cellular function, or mediate signal transduction to respond to environmental changes.

Additional complexity and flexibility is conferred upon the system by post-translational modifications. These either reversibly or irreversibly alter the configuration of proteins and cause changes in their function, including influences on enzymatic activity, interaction with other proteins, and other characteristics. Reversible modifications include phosphorylation, acetylation and methylation, as well as the covalent attachment of single or multiple moieties of ubiquitin or ubiquitin-related proteins, among others (see Fig.11.1). The addition of these covalent modifiers can be considered as equivalent to a "gain of function" for the target protein. The modified sites, or combinations thereof, can constitute recognition signals or "codes" for the recruitment of specific docking modules inherently present in single or multiple interaction partners, thus leading to the dynamic

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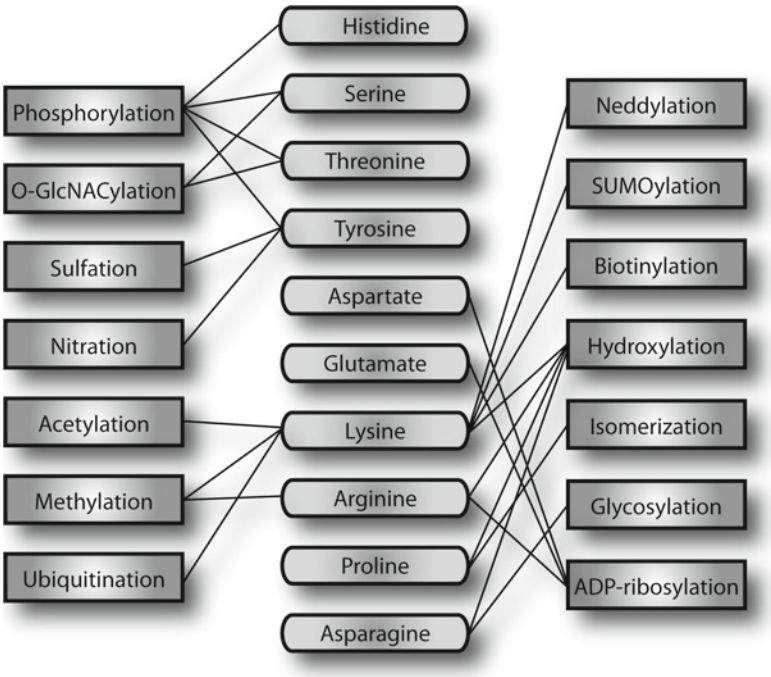

Fig. 11.1 Schematic representation of the most common post-translational modifications and the amino acids at which they can occur, demonstrating the potential for convergence of multiple modifications at specific residues, as well as the range of modifications and corresponding recognition domain-containing effectors that may functionally interact with each other

generation of a specific configuration of protein sets or complexes. Such partnership configurations can be finely controlled with respect to their spatiotemporal features, leading to specific signal transduction to activate or repress downstream functions. The reversibility of many of the covalent interactions involved allows for down-regulation of this activity via removal of the residues required for interactor recruitment when the task is complete, permitting dynamic control of the process.

Additionally, protein modifications can be utilized to not only generate a specific "fingerprint" for the recognition of an element by one or several interaction modules, but to determine which potential interactors are recruited via a particular amino acid. Several of the protein modifications discussed here are targeted to the same amino acid, such that the presence of one modification prevents the establishment of another. For example, acetylation of a lysine residue can act to prevent the addition of a ubiquitin moiety, and vice versa, via same-site competition.

In this chapter, we present an overview of several protein modification interaction domains currently considered to play an important role in the dynamic recruitment of effector or regulatory factors, as well indicate the complexity of the potential interaction space created by interactions between modifications. Where possible, we emphasize how different modifications are linked to cellular signaling networks.

11.2 Phosphorylation-Dependent Interactions

Historically, protein phosphorylation has been the most intensively studied of the covalent modifications, and its role as a key element in signal transduction has long been recognized. Although phosphorylated tyrosine residues were the first to be identified as interaction recruiters, the importance of serine and threonine residues as sites for transient generation of new binding sites is reflected in the number of modules recognized as binding to these motifs. The widespread nature of this modification is demonstrated through phosphoproteomic analyses. 6,600 phosphorylation sites could be identified from ca. 2,200 proteins investigated in HeLa cells; 14% of these changed in abundance within 15 min of exposure to the membrane receptor ligand EGF (*e*pidermal *g*rowth *f*actor), with many proteins containing multiple phosphorylation sites exhibiting different kinetics (Olsen et al. 2006).

11.2.1 Phosphotyrosine-Dependent Interactions

11.2.1.1 SH2 Domain

First identified in 1986 (Sadowski et al. 1986), this domain remains the archetype for recognition of a post-translationally modified amino acid within a sequence-specific context (reviewed in Pawson and Nash 2003). As well as recognizing phosphotyrosine, individual SH2 (*S*rc *h*omology *2*) domains interact with specific sequences of flanking residues, imparting additional specificity to the interactions. SH2 domain-containing proteins are classically considered to act as adapter molecules, coupling tyrosine phosphorylation of proteins such as membrane-localized receptor tyrosine kinases to positive control of downstream signaling events. Additionally, SH2 domains are present in protein tyrosine phosphatases, indicating the flexibility inherent in this interaction motif.

11.2.1.2 PTB Domain

Another class of phosphotyrosine recognition motifs is represented by the PTB (*p*hospho*t*yrosine *b*inding) domain family. These motifs share a low degree of sequence homology, yet adopt similar secondary and tertiary structure conformations (reviewed in Yan et al. 2002). They also exhibit a common binding affinity for phospholipids (reviewed in Uhlik et al. 2005). However, one group of PTB domains, the Dab-like PTBs, exhibits phosphotyrosine-independent binding. This group includes ca. 75% of PTB domains identified (Uhlik et al. 2005), illustrating the heterogeneity in substrate recognition present in this domain family.

11.2.1.3 C2 Domain

Previously recognized as a module responsible for binding phospholipids in a calcium-dependent manner (Davletov and Sudhof 1993; reviewed in Newton and Johnson 1998), this domain has recently been identified as a phosphotyrosine-binding motif (Benes et al. 2005; reviewed in Sondermann and Kuriyan 2005). The interaction is sequence-specific, and mediates the phosphorylation-dependent binding of the C2 domain-containing serine/threonine protein kinase PKCδ (*p*rotein *k*inase *C*δ) to CDCP1 (*C*UB *d*omain-*c*ontaining *p*rotein *1*). In this model, the kinase Src initially phosphorylates and subsequently binds to a tyrosine residue on CDCP1 via the Src SH2 domain, promoting further CDCP1 tyrosine phosphorylation events. This in turn leads to the recruitment of PKCδ via its C2 domain, creating a multimeric signaling complex (Benes et al. 2005).

As well as recruiting modification-dependent binding partners, the interaction of phosphotyrosines with specific recognition motifs can be exploited to create additional signaling pathways. Pyruvate kinase is an important enzyme in the glycolytic metabolic pathway. The *p*yruvate *k*inase *M2* splice isoform (PKM2) represents a variant that promotes the switch to anaerobic glycolysis and is preferentially expressed by cancer cells (Christofk et al., 2008a). PKM2 can interact with phosphotyrosine via a novel motif; this binding competes with that of the allosteric activator fructose-1,6-bisphophate, resulting in decreased PKM2 enzymatic activity (Christofk et al., 2008b). Thus, this motif can act as a sensor of cellular tyrosine phosphorylation levels, coupling this readout to the control of glycolytic metabolism (Christofk et al., 2008a, b).

11.2.2 Phosphoserine/Phosphothreonine-Dependent Interactions

11.2.2.1 The 14-3-3 Protein Family

The seven mammalian members of the 14-3-3 protein family exist in vivo as homo- or hetero-dimers. They play adapter and integrator roles in pathways promoting cell survival and inhibiting apoptosis (reviewed in Morrison 2009), as well as performing many other functional roles, including regulation of the subcellular localization of transcriptional corepressors, i.e., the histone deacetylase HDAC4, in a phosphorylation-dependent manner (Grozinger and Schreiber 2000; McKinsey et al. 2000; Wang et al., 2000a; Nishino et al. 2008; reviewed in Bertos et al. 2001). Their role in driving cell survival positions them as attractive targets for anti-cancer therapies.

11.2.2.2 BRCT Domain

Members of the *BR*CA1 *c*arboxyl-*t*erminal (BRCT) domain family are predominantly found in proteins involved in regulation of the DNA damage response (Koonin et al. 1996). These domains have been found to interact preferentially with

phosphoserine-containing peptides (Rodriguez et al. 2003; Yu et al. 2003), and somatic mutations in the BCRT domain of BRCA1 (*br*east *ca*ncer *1*) are linked to an increased risk of early-onset breast and ovarian cancer in affected individuals (Gayther et al. 1995).

11.2.2.3 WD40 Domain

F-box-containing proteins, which play roles in targeting ubiquitinated protein for degradation, mostly also contain WD40 or *l*eucine-*r*ich *r*epeat (LRR) regions that have been linked to the recognition or binding of phosphoproteins, although this interaction has not yet been formally demonstrated (reviewed in Yaffe and Elia 2001).

11.2.2.4 FHA Domain

Proteins containing *f*ork*h*ead-*a*ssociated (FHA) domains include those with functions in DNA damage repair, as well as kinesins, RING-finger proteins, forkhead transcription factors and the proliferation marker Ki-67 (Durocher and Jackson 2002; Mahajan et al. 2008). FHA domains can be divided into 3 subcategories depending on their preferences for specific types of residues surrounding their target phosphoserine residue (Liang and Van Doren 2008). Interestingly, it has been reported that the FHA domain may also be able to bind to phosphotyrosine (Liao et al. 1999; Wang et al., 2000b), although the functional significance of this interaction is not currently clear.

11.2.2.5 Polo-Box Domain

Found in the *P*olo-*l*ike *k*inases (PLKs), the Polo-box domain is essential for proper localization of these important mitotic regulators. The specificity of the interaction of this domain with phosphoserine and phosphothreonine residues is related to the function of PLKs as molecular integrators. Appropriate PLK function requires that prior phosphorylation by other mitotic kinases must have occurred before the PLKs can be properly localized to their target sites, where they proceed to drive progression through the M phase of the cell cycle (reviewed in Lowery et al. 2004). A mass spectrometry-based screen for other Polo-box interactors identified proteins involved in other processes such as translational control, RNA processing and vesicular transport (Lowery et al. 2007), suggesting that this domain may function in additional processes beyond those previously identified.

11.2.2.6 WW Domain

Although generally considered to be modules responsible for interaction with proline-rich surfaces, a subset of WW domain-containing proteins utilize this sequence

for recognition of phosphorylated phosphoserine or phosphothreonine residues occurring immediately adjacent to proline (Rodriguez et al. 2003). These include the proline isomerase Pin1 (peptidyl-*p*rolyl cis/trans *i*somerase, *N*IMA-interacting 1) and the ubiquitin ligase Nedd4 (*n*eural precursor cell *e*xpressed, *d*evelopmentally *d*ownregulated *4*); in another illustration of the complexity of the control of such modification-dependent interactions, it has been reported that the WW domain of Pin1 must be phosphorylated to mediate binding to phosphoserine (Lu et al. 2002).

11.2.2.7 FF Domain

First identified as a novel motif often found near WW domains, FF domains contain two highly conserved phenylalanine residues (Bedford and Leder 1999). FF domains are found in the yeast protein Prp40 and the human protein TCERG1 (*t*ransc*r*iption *e*longation *re*g*u*lator *1*, also known as CA150), both of which bind to phosphoserines in the *c*arboxyl-*t*erminal *d*omain (CTD) of RNA polymerase II (Morris and Greenleaf 2000; Goldstrohm et al. 2001), as well as in other proteins known to interact with huntingtin (Faber et al. 1998; Passani et al. 2000). Interestingly, the FF domains of TCERG1 are also able to bind multiple transcription and splicing factors in a phosphorylation-independent manner through multiple weak interactions with motifs comprising negatively charged residues flanked by aromatic amino acids (Smith, et al. 2004).

11.3 Methylation-Dependent Interactions

Methylation of lysine or arginine residues can occur as monomethylation or dimethylation; in the case of lysine, trimethylation is also possible, while symmetric vs. asymmetric dimethylation expands the repertoire of possible configurations for methylated arginine (reviewed in Lee et al. 2005). Although there does not appear to be an obligate requirement for one methylation type vs. another for interaction with methylation-dependent recognition motifs, preferential binding to certain forms exists for specific methylation-specific domain-containing proteins. For example, the chromodomains of the HP1 (*h*eterochromatin *p*rotein *1*) and Polycomb proteins preferentially bind trimethylated lysine-9 of histone H3 (Fischle et al. 2003; Min et al. 2003), while the affinity of the tudor domain is highest for symmetric dimethylarginine (Sprangers et al. 2003).

11.3.1 Chromodomains

Classically found in chromatin-associated proteins, chromodomains exist across a wide variety of organisms, suggesting that they represent an ancient structural motif

(reviewed in Eissenberg 2001). In general, their most common binding partners appear to be nucleosomes, and thus it is not surprising that they exhibit an ability to recognize specific methylation patterns on this class of proteins. A screen for binding to specific partner elements conducted using a protein microarray approach, where differentially methylated peptides were used as probes (Kim et al. 2006), established that chromodomains possess specific affinities for methylated lysine residues present in the context of particular histone tail sequences.

11.3.2 The "Royal Family"

Tudor, MBT (malignant brain tumor) and PWWP (proline-tryptophan-tryptophan-proline) Domains. These multiple subfamilies are also predominantly found in chromatin-associated proteins. Due to the structural similarity of some of their members to the chromodomain family, it has been suggested that they may function in a similar manner. In the same binding screen as discussed for chromodomains above (Kim et al. 2006), it was found that tudor domains appeared to be more sensitive to the degree of methylation than to the sequence context of the probe peptide, generally exhibiting a stronger affinity for di- or trimethylated lysine residues, while MBT domains preferentially interact with monomethylated lysines. The affinity of the PWWP domain for methylated lysine residues, on the other hand, has only recently been described (Wang et al. 2009). These differences in partner preference generate an expanded range of specific recognition modules for fine control of effector interaction with methylated proteins.

11.4 Acetylation-Dependent Interactions

11.4.1 Bromodomains

This motif is a ca. 110 amino acid module predominantly found in proteins involved with transcriptional control at the level of chromatin and the nucleosome (reviewed in Jeanmougin et al. 1997). Reversible acetylation of lysine residues is a common modification in the context of the components with which such proteins interact, especially for specific lysine residues in the N-terminal tails of histones H3 and H4. It has been shown that specific interaction of the bromodomain with acetyl-lysine residues occurs via a hydrophobic pocket located between the ZA and BC loops of the four-helix bundle (Dhalluin et al. 1999), thus tethering bromodomain-containing proteins to, e.g., lysine-acetylated histone tail regions.

Although the majority of bromodomain interactions identified to date have been with various acetyllysine residues located on the tail regions of histones H3 and H4, bromodomains have also been found to mediate binding to acetyllysines

present on other proteins, such as MyoD (*myo*genic *d*ifferentiation antigen 1) (Polesskaya et al. 2001), Myb (v-myb avian *my*elo*b*lastosis viral oncogene homolog) (Tomita et al. 2000), HIV Tat (*h*uman *i*mmunodeficiency *v*irus *t*rans-*a*ctivator of *t*ranscription) (Col et al. 2001) and p53 (Mujtaba et al. 2004). Furthermore, rather than binding to acetyllysines in general, bromodomains from different proteins also require additional sequence elements to mediate efficient interactions. This combination of specific context-dependent features, along with a "switchable" residue modification, imparts fine control of specificity and permits a wide range of individual interactions to be mediated by multiple bromodomain-containing proteins.

Acetylation and deacetylation of target proteins is mediated through two broad classes of enzymes, historically termed *h*istone *a*cetyl*t*ransferases (HATs) and *h*istone *deac*etylases (HDACs) from the initial substrates identified. Interestingly, several of these proteins, such as the HAT CBP (*C*REB-*b*inding *p*rotein)/p300, also contain bromodomains, which mediate interaction with their enzymatic targets, such as MyoD (Polesskaya et al. 2001). This potentially acts as a positive feedback mechanism potentiating the binding between the two proteins.

11.5 Hydroxylation-Dependent Interactions

Hydroxylation, a potential modification for proline, asparagine, arginine or lysine residues, occurs in the context of multiple proteins, including the hypoxia-inducible transcription factor HIF (*h*ypoxia-*i*nducible *f*actor) and the matrix component collagen. In the latter case, this modification has structural implications (Krane 2008), while in the former case, hydroxylation acts to create a specific recruitment platform for downstream signal modulators.

11.5.1 VHL Domain

The role of this motif in mediating the regulation of HIF (*h*ypoxia-*i*nducible *f*actor) activity, in combination with other post-translational protein modifications governing this central hub of the hypoxic response, illustrates the potential complexity of interactions between these elements, and will therefore be described in detail (see Fig. 11.2). The *v*on *H*ippel-*L*indau (VHL) factor is a crucial element in initiating the response to changes in intracellular oxygen concentrations. This function is mediated through its interaction with the HIF α subunit (reviewed in Ivan and Kaelin 2001). In normoxia, this interaction is promoted, leading to the degradation and subsequent loss of function of HIF via a polyubiquitin-dependent pathway (Huang et al. 1998; Iwai et al. 1999; Lisztwan et al. 1999; Maxwell et al. 1999). Under hypoxic conditions, the abrogation of this interaction leads to a suppression

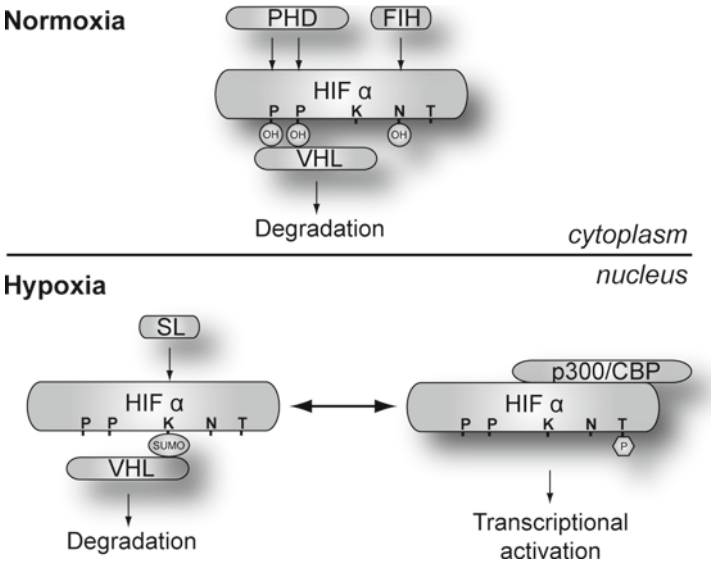

Fig. 11.2 Schematic representation of post-translational modifications involved in regulating HIF α activity under conditions of normal oxygen concentrations vs. hypoxia. PHD, prolyl hydroxylase domain-containing protein; FIH, factor inhibiting HIF; VHL, von Hippel-Lindau factor; p300/CBP, p300/Creb-binding protein; SL, SUMO ligase. Under hypoxic conditions, HIF1 α translocates to the nucleus where it partners with HIF β to exert its transcriptional regulatory function. Note that the role of SUMOylation in promoting HIF1 α degradation is not firmly established at this time

of degradation and thus an increase in the effective concentration of HIF, causing the transcriptional activation of HIF target genes.

Under conditions of normal oxygen concentrations, HIF α is hydroxylated on proline-564 and proline-402 through the action of PHDs (*p*rolyl *h*ydroxylase *d*omain-containing proteins), three of which have been identified so far in humans (Bruick and McKnight 2001; Epstein et al. 2001) (see Fig. 11.2). This process requires the presence of oxygen as a substrate for the hydroxylation reaction. Under hypoxic conditions, the rate of this reaction is reduced, leading to the loss of the hydroxyl groups at these residues. This suggests that the hydroxylation level of the HIF α subunit functions as a direct sensor of oxygen concentrations.

The mechanism through which this protein modification mediates control of HIF α subunit stability occurs via the hydroxylation-dependent recruitment of the von Hippel-Lindau factor, which serves as the recognition component of an ubiquitin ligase (Iwai et al. 1999; Lisztwan et al. 1999) that promotes proteosomal degradation of HIF α (Cockman et al. 2000; Kamura et al. 2000; Ohh et al. 2000; Tanimoto et al. 2000). This interaction is mediated by a specific domain within the von Hippel-Lindau factor, termed the VHL domain, which

binds to hydroxylated prolines of the HIF α subunit (Jaakkola et al. 2001). Mutation of this residue in the HIF α protein abrogates this interaction, as does the exogenous addition of a peptide corresponding to the surrounding HIF α sequence bearing a hydroxylated Pro-564 residue, which acts as a competitive inhibitor (Jaakkola et al. 2001).

Interestingly, the ability of prolyl hydroxylases to be regulated by intracellular oxygen concentrations appears to be utilized in a second mode of controlling HIF α activity. An asparagine residue (asparagine-803) located in the C-terminal transcriptional activation domain of HIF α is a target for the asparaginyl hydroxylase FIH (*f*actor *i*nhibiting *H*IF) (Hewitson et al. 2002; Lando et al. 2002a, b). Hydroxylation of this residue suppresses the transcriptional activation function of this domain by preventing its interaction with the transcriptional coactivator p300/CBP (Lando et al. 2002a). Illustrating the concept by which one covalent modification can affect another, the oxygen-independent phosphorylation of threonine-796 in HIF α by casein kinase 2 may be necessary for transcriptional activation (Gradin et al. 2002); phosphorylation at this residue abrogates hydroxylation of asparagine-803 by FIH (Lancaster et al. 2004), suggesting an additional level of control.

A second such instance is provided by the finding that hypoxia also induces the SUMOylation of HIF α, providing an alternative route for enhancement of binding to von Hippel-Lindau factor and subsequent proteosomal degradation (Cheng et al. 2007). This mode of control is depicted in Fig. 11.2; however, previous groups have reported that SUMOylation increases HIF α stability and increases transcriptional activity (Bae et al. 2004), or reduces the transcriptional activity of HIF α without affecting stability (Berta et al. 2007) These different observations may critically depend upon the balance between SUMOylating and deSUMOylating (e.g., SENP1) enzymes (Cheng et al. 2007), which act to modulate this signal. The question of whether different modifications vary with respect to time scale, such that their combination alters the nature of the induction of HIF-dependent transcriptional activity in response to transient vs. long-term decreases in oxygen concentrations, remains open.

11.6 Ubiquitination-Dependent Recognition Motifs

Ubiquitination, initially recognized as a modification involved in targeting proteins for intracellular degradation, has emerged in recent years as an important element in signal transduction. A key difference between the ultimate function of ubiquitination as a marker for degradation vs. as a modifier of function lies in the number of ubiquitin moieties added; while polyubiquitination is often associated with targeting of proteins for proteosomal degradation, monoubiquitination is more closely related to modulation of protein function by generation of a novel protein interaction site (representing a "gain of function"). Interestingly, many ubiquitin-binding domains can mediate autoubiquitination, thus potentially regulating the activity of the domain's "host" proteins.

11.6.1 UBA Domain

The first ubiquitin interaction domain to be described, the UBA domain (Hofmann and Bucher 1996; Bertolaet et al. 2001b) has traditionally been associated with polyubiquitin binding (Wilkinson et al. 2001; Funakoshi et al. 2002; Raasi and Pickart 2003), but is also capable of binding monoubiquitin moieties in vitro, as well as interacting with other UBA domains (Vadlamudi et al. 1996; Bertolaet et al. 2001a; Chen et al. 2001).

Surface plasmon resonance studies of the EDD (*E*3 identified by *d*ifferential *d*isplay) ubiquitin ligase UBA domain show that this module does not exhibit a strong preference for poly- vs. monoubiquitin as a binding partner (Kozlov et al. 2007). NMR (*n*uclear *m*agnetic *r*esonance) titration analysis suggest that the UBA domain of the p62 scaffold protein binds di-ubiquitin with slightly lower affinity than mono-ubiquitin, suggesting that this UBA domain may preferentially interact with extended polyubiquitin chains adopting more open structures (Long et al. 2008). Interestingly, in Paget's disease of bone, the primary defect appears to be mutation or truncation of the p62 UBA domain, which has deleterious effects on the NF-κB (*n*uclear *f*actor κ-*B*) signaling pathway in osteoclasts (reviewed in Layfield and Searle 2008).

The *r*eceptor *t*yrosine *k*inase (RTK) Met plays important roles in cell proliferation and survival, cell migration and epithelial morphogenesis (reviewed in Peschard and Park 2007). Therefore, tight control of its activity is required for normal cell function. One of the mechanisms for its ligand-induced downregulation occurs via phosphotyrosine-dependent recruitment of Cbl E3 ubiquitin ligases, which enhances Met degradation (Peschard et al. 2001, 2004; Abella et al. 2005; Mak et al. 2007).

The c-Cbl UBA domain, is required for both homodimerization and heterodimerization with the Cbl family member Cbl-b (Bartkiewicz et al. 1999; Liu et al. 2003), but does not appear to interact with ubiquitinated lysine residues (Davies et al. 2004; Raasi et al. 2005). Analysis of the crystal structure of this domain reveals that the same surface is used for both homo- and heterodimerization, while site-directed mutagenesis experiments demonstrate the requirement for UBA-mediated dimerization to enable ubiquitin ligase activity directed against the Met RTK (Kozlov et al. 2007). This finding further demonstrates that UBA domains, beyond their role in the recognition of ubiquitinated proteins, can be adapted to serve as protein-protein binding domains, further extending the repertoire of possible protein interactions, and illustrates a general trend where domain archetypes can be adapted to serve as recognition sites for multiple binding motifs.

11.6.2 CUE (*c*oupling of *u*biquitin conjugation to *e*ndoplasmic reticulum degradation) domain

This domain recognizes both mono- and polyubiquitinated residues, with different affinities depending on the "host" protein, as well as acting to promote intramolecular monoubiquitination (Donaldson et al. 2003; Shih et al. 2003).

11.6.3 PAZ/Znf/UBP/HUB Domain

The atypical class II histone deacetylases HDAC6, as well as possessing dual catalytic domains, also contains a zinc finger motif near its carboxyl terminal similar to that found in *u*biquitin-*s*pecific *p*rotease *3* (USP3) and *BR*CA1-*a*ssociated *p*rotein *2* (BRAP2) (reviewed in Bertos et al. 2001) which has been shown to act to interact specifically with ubiquitin (Seigneurin-Berny et al. 2001; Hook et al. 2002; Boyault et al. 2006). This interaction plays an important role in the recognition of misfolded proteins by HDAC6, which acts to transport them to the aggresome for eventual proteasome-independent degradation (Kawaguchi et al. 2003).

11.6.4 UBAN Domain

The NUB (*N*EMO *u*biquitin *b*inding) or UBAN (*u*biquitin *b*inding in *A*BIN and *N*EMO proteins) motif was previously identified as an ubiquitin-binding region present in NEMO (*N*F-κB *e*ssential *mo*dulator), the ABIN family (*A*20 *b*inding and *i*nhibitor of *N*F-κB) and optineurin (Ea et al. 2006; Wu et al. 2006; Zhu et al. 2007; Bloor et al. 2008; Wagner et al. 2008). Although the UBAN domain was previously thought to bind lysine-63-linked ubiquitin chains, recent studies have demonstrated that this motif preferentially interacts with head-to-tail linked linear ubiquitin dimers or multimers (Lo et al. 2009; Rahighi et al. 2009).

NEMO, the regulatory subunit of *I*κB *k*inase (IKK), a complex which plays a key role in regulation of NF-κB, is recruited to polyubiquitinated signaling mediators, leading to IKK recruitment and NF-κB activation. The crystal structure of the ubiquitin-binding region of NEMO reveals that conformational changes occur upon ubiquitin binding (Rahighi et al. 2009), suggesting a possible mechanism for permitting IKK transautophosphorylation and activation. Interestingly, NEMO is itself subject to linear polyubiquitination by the recently described LUBAC (*l*inear *ub*iquitin chain *a*ssembly *c*omplex) ligase complex (Tokunaga et al. 2009), suggesting a possible mechanism for NEMO cis- or trans-interactions (Rahighi et al. 2009).

11.7 Sumoylation-Dependent Interactions

The ubiquitin-related covalent adduct SUMO (*s*mall *u*biquitin-like *mo*difier) exists as four isoforms (SUMO-1, -2, -3 and –4, although the functional role of the last is currently unclear). While SUMO-1 is generally involved in monoSUMOylation, SUMO-2 and -3 are mostly added to their target proteins as polySUMO chains, thus recapitulating the mechanistic differences between mono- and polyubiquitination. The functional consequences of SUMOylation, however, are generally distinct

from those of ubiquitination. They are to a large part associated with alterations in protein-protein interactions (reviewed in Geiss-Friedlander and Melchior 2007) rather than targeting of proteins for degradation, although the latter has also been demonstrated to occur (Prudden et al. 2007). SUMOylation can promote protein interactions, e.g., SUMOylated RanGAP1 (*Ran G*TPase-*a*ctivating *p*rotein *1*) can interact with RanBP2 (*Ran-b*inding *p*rotein *2*) (Matunis et al. 1996; Mahajan et al. 1997) and SUMOylated p300 interacts with and acetylates HDAC6 (Girdwood et al. 2003); alternately, SUMOylation can lead to the abrogation of previously existing interactions, e.g., SUMOylated CtBP can no longer interact with the PDZ (*p*ostsynaptic density protein-95, *d*isk-large tumor suppressor protein, *z*onula occludens-1) domain of nNos (neuronal nitric oxide synthase) (Lin et al. 2003).

11.7.1 SIM Domain

The consensus sequence of SIMs (*S*UMO-*i*nteracting *m*otifs) is relatively elastic, although several overall features, such as hydrophobicity and charge clustering, are invariant (reviewed in Kerscher 2007). While multiple ubiquitin-binding domains have been identified, only one SIM has been characterized thus far; it is possible that the variable sequence permits the generation of SIMs with different affinities for the various SUMO isoforms, as well as for mono- vs. polySUMOylated sites.

Yet another level of complexity is generated by the fact that the interaction of some SIMs with their SUMOylated binding partners requires phosphorylation at serine residues adjacent to the hydrophobic core of the SIM domain, generating a phosphorylation-dependent SIM domain-SUMO interaction (Stehmeier and Muller 2009). Thus, the binding affinity of two proteins can be governed by post-translational modification both at the classical modification site, and within the recognition module of the partner protein, illustrating the linkage between SUMO binding and phosphorylation-dependent cellular signaling networks.

11.8 Examples of Multisite Modifications

11.8.1 "Histone Code" or "Chromatin Signature"

As a key integrator upon which multiple pathways converge, and being closely associated with direct control of transcription, it is not surprising that the histone components of chromatin are subject to a wide variety of post-translational modifications. This set of modifications has been proposed to constitute a "histone code", different configurations of which recruit specific combinations of interacting proteins to mediate downstream events (Strahl and Allis 2000). Covalent modifications of residues in the flexible histone amino-terminal tail regions include methylation (where mono-, di- or trimethylation is distinct), acetylation, phosphorylation,

ADP-ribosylation, SUMOylation and ubiquitination (Shiio and Eisenman 2003; reviewed in Bhaumik et al. 2007; Latham and Dent 2007; Munshi et al. 2009).

Multiple examples of the crosstalk between different modifications can be observed in this system. For example, acetylation of histone H4 at lysine-5 and lysine-12 promotes chromatin compaction and thereby gene silencing (Kelly et al. 2000); in the context of existing acetylation at lysine-8 and lysine-16 of the same protein, the same modification is linked to transcriptional activation (reviewed in Yang 2005). Histone H3 serine-10 phosphorylation acts to promote transcriptional activation (DeManno et al. 1999), unless serine-28 is also phosphorylated, in which case this combination constitutes a mark of condensed and therefore transcriptionally inactive chromatin (reviewed in Yang 2005).

As the complexity of the spatiotemporal interactions between different modifications becomes more evident, it is apparent that, beyond the "histone code" that presents a specific temporally limited configuration for recruitment of effectors and interactors, chromatin modifications exist in a state of flux. The resulting regulatory paradigm can be thought of as being similar to a decision tree with multiple interacting branches. Thus, the current output at a specific location would be dependent upon the initial configuration of chromatin modifications as well as the availability and relative abundances of interactors recruited to those modifications, the state of these interactors as determined by their intrinsic modifications, the composition and membership of the interactor complexes themselves, and the dynamics of antagonistic, synergistic and regulatory interactions between these complexes. This output would then generate a novel combination of the factors listed above, while external pathways would also affect several of these parameters.

11.8.2 p53

As a central element in the regulation of the DNA damage response, p53 is subject to multiple levels of post-translational regulation, including a wide variety of post-translational modifications. These include phosphorylation (on serine and threonine residues), acetylation, methylation, ubiquitination, SUMOylation and neddylation (reviewed in Bode and Dong 2004; Yang and Seto 2008). Reminiscent of the regulation of HIF α, a primary mechanism for the control of p53 activity depends on the intrinsic instability of the protein; thus, post-translational modifications targeting this property play important roles in regulating p53 function.

In response to DNA damage, a phosphorylation-acetylation cascade leads to p53 activation; serine phosphorylation and lysine methylation promotes the association of p53 with HATs, which in turn acetylate lysine residues, altering DNA binding properties, creating docking sites for the recruitment of additional interactors, and competitively inhibiting ubiquitination at these sites, leading in turn to decreased degradation (reviewed in Yang and Seto 2008)). Additionally, acetylation of lysine-120 of p53, a site mutated in human cancer, appears to function as a switch determining differential assignments to apoptosis vs. cell cycle control.

Interestingly, transgenic mice in which multiple lysine acetylation sites were deleted by substitution with arginine do not demonstrate a severe phenotype (reviewed in Yang and Seto 2008), suggesting that multiple redundant levels of control may exist to regulate the activity of this key protein. Overall, we believe that multisite modifications constitute a set of still poorly understood regulatory programs for concerted actions in response to different cellular and environmental cues.

11.9 Concluding Remarks

The specificity of many of these interactions, which requires the presence of a covalently modified amino acid or a combination of modifications in the context of defined sequence elements, renders them particularly attractive as targets for the development of exogenous competitive inhibitors. These would have the advantage of blocking a specific interaction or set thereof, in contrast to the broad effects seen following pharmacological blockade of the enzymatic activity of a signaling cascade member. Although methods for delivery of such competitive inhibitors that can be used in the clinical context remain elusive, this approach holds out the promise of eventual specific targeting of therapeutics to abrogate protein-protein interactions.

As additional proteins are studied in detail, a picture is beginning to emerge in which the set of post-translational modifications present on each protein constitutes an additional level of information and control regarding its enzymatic activity, subcellular localization and interaction partners, where these factors may also be interdependent. It may be appropriate to characterize this set of modifications as comprising the "quinary structure" of a protein or protein assembly, which contains the necessary information to fully characterize and identify the role of the effector at a particular point in time.

Regulation via post-reversible post-translational modifications constitutes a dynamic spatiotemporal fine-tuning of cellular element function, thus permitting the cell, and by extension the organism, to continuously adapt and respond to its environment. Modifications at multiple sites can combine in an antagonistic, cooperative or synergistic manner. This further adds to the complexity of understanding the integration of protein modification recognition inputs and functional outputs.

The elucidation of significant portions of the "quinary structure" information set of certain proteins has occurred essentially serendipitously, as a consequence of multiple separate investigations into different aspects of protein function and regulation. The question of whether those proteins that have been identified as bearing multiple layers of functional regulation by a variety of post-translational modifications are indeed more subject to this type of control than others due to their key roles as information integrators, or whether these multiple levels have been revealed precisely because of the intensive investigations directed towards such key proteins, remains open.

Currently, we lack a comprehensive understanding regarding the full extent of post-translational protein modifications, and the degrees of functional interaction

between them. This situation likely underlies many of the instances in which conflicting reports arise regarding the effect of a specific modification, since the agonistic or antagonistic effects of other modifications may differ depending on cell type and environment.

A complete catalogue of protein modifications, their temporal relationship and their mutual interactions, as well as of the modules recruited by each moiety in the context of a specific sequence, is likely to be a long-term goal (Yang 2005). However, progress towards the understanding of this fundamental element in the regulation of protein activity is a key element in understanding normal biological processes, and in both elucidating the basic alterations underlying disease processes, and in the design of novel rational therapeutic modalities to target these perturbations.

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