

# Recognition mechanism of p63 by the E3 ligase Itch

## Novel strategy in the study and inhibition of this interaction

Alessia Bellomaria,<sup>1</sup> Gaetano Barbato,<sup>1</sup> Gerry Melino,<sup>2,3</sup> Maurizio Paci<sup>1</sup> and Sonia Melino<sup>1,\*</sup>

<sup>1</sup>Dipartimento di Scienze e Tecnologie Chimiche; University of Rome "Tor Vergata"; Rome, Italy; <sup>2</sup>Medical Research Council (MRC); Toxicology Unit; Leicester University; Leicester, UK; <sup>3</sup>Laboratorio di Biochimica IDI-IRCC c/o Dipartimento di Biochimica e Chirurgia; University of Rome "Tor Vergata"; Rome, Italy

**Keywords:** p63, p53 family, E3 ubiquitin ligases, HECT, ubiquitination, cyclization, metal-peptide

**Abbreviations:** CSI, <sup>13</sup>C-chemical Shift Index; DTNB, 5,5'-DiThiobis-(2-NitroBenzoic acid); HECT, homologous E6-AP carboxyl terminus; HSQC, heteronuclear single quantum coherence; IPTG, IsoPropyl-β-D-ThioGalactoside; LAPT5, lysosomal-associated protein transmembrane 5; LATS 1, large tumor suppressor homolog 1; LC/ESI-MS, liquid chromatography electrospray ionization mass spectrometry; NOESY, nuclear overhauser effect spectroscopy; Melan-A, melanoma antigen recognized by T-cells 1; RP-HPLC, reverse phase high performance liquid chromatography; PPII, possible polyproline II; pep63, p63(534-551) peptide; TNB, 2-Nitro-5-Thiobenzoic acid; TCEP, Tris (2-CarboxyEthyl)-Phosphine; TOCSY, total correlated spectroscopy

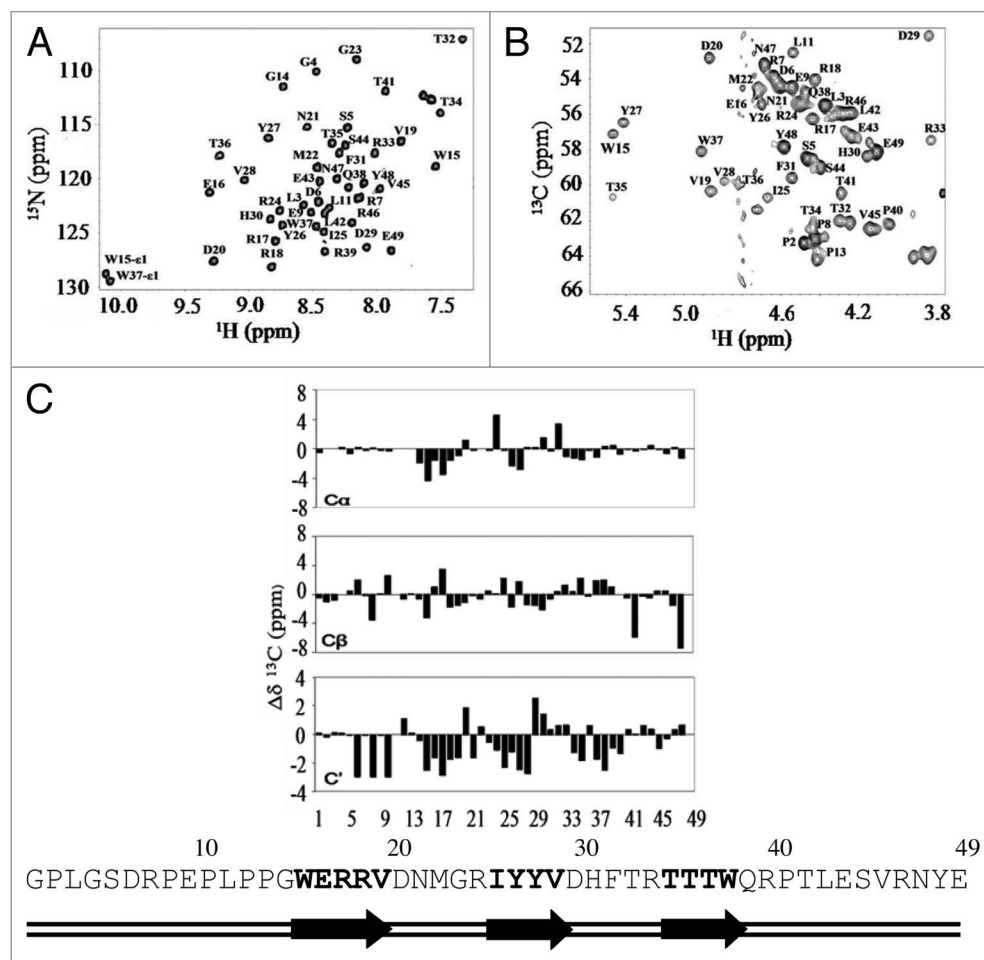
The HECT-containing E3 ubiquitin ligase Itch mediates the degradation of several proteins, including p63 and p73, involved in cell specification and fate. Itch contains four WW domains, which are essential for recognition on the target substrate, which contains a short proline-rich sequence. Several signaling complexes containing these domains have been associated with human diseases such as muscular dystrophy, Alzheimer's or Huntington's diseases. To gain further insight into the structural determinants of the Itch-WW2 domain, we investigated its interaction with p63. We assigned, by 3D heteronuclear NMR experiments, the backbone and side chains of the uniformly <sup>13</sup>C-<sup>15</sup>N-labeled Itch-WW2. In vitro interaction of Itch-WW2 domain with p63 was studied using its interactive p63 peptide, pep63. Pep63 is an 18-mer peptide corresponding to the region from 534–551 residue of p63, encompassing the PPxY motif that interacts with the Itch-WW domains, and we identified the residues involved in this molecular recognition. Moreover, here, a strategy of stabilization of the conformation of the PPxY peptide has been adopted, increasing the WW-ligand binding. We demonstrated that cyclization of pep63 leads to an increase of both the biological stability of the peptide and of the WW-ligand complex. Stable metal-binding complexes of the pep63 have been also obtained, and localized oxidative damage on Itch-WW2 domain has been induced, demonstrating the possibility of use of metal-pep63 complexes as models for the design of metal drugs to inhibit the Itch-WW-p63 recognition in vivo. Thus, our data suggest a novel strategy to study and inhibit the recognition mechanism of Itch E3-ligase.

### Introduction

Ubiquitin ligases play a pivotal role in modulating intracellular protein levels, by ubiquitylation of the protein substrates for their proteasomal or lysosomal degradation.<sup>1</sup> The differential status for ubiquitination (mono- or poly-) may further contribute to the mark for proteasomal or lysosomal degradation.<sup>2</sup> E6-AP carboxyl terminus (HECT)-type family of E3 ubiquitin ligases<sup>3</sup> (EC 6.3.2.19) transfer ubiquitin directly to substrate proteins by acting as catalytic intermediates through a conserved cysteine residue.<sup>4,5</sup> They are involved in a wide variety of cellular processes, including differentiation, endocytosis, signaling and transcription.<sup>6-12</sup> The oncogenic potential of the HECT-type

E3s is emphasized by the recognition of tumor-suppressor molecules among their protein targets.<sup>13</sup> The E3 ubiquitin ligase Itch or atrophin-1-interacting protein 4 (AIP4, here, after referred to as Itch; UniProtKB accession number Q96J02) is a member of the NEDD4 family of E3 ligases. Itch plays a key role in different cellular contexts and contributes to many cellular processes by targeting regulators of multiple signaling pathways.<sup>14</sup> Itch-null mice develop systemic and progressive immunological disorders.<sup>15</sup> Itch catalyzes the poly- and monoubiquitination of important globular and membrane proteins such as Melan-A, ErbB4, CXC-chemokine receptor 4 and LAPT5, regulating transport of intracellular vesicles to lysosome,<sup>2,16-18</sup> and transcription factors as c-Jun, JunB, p73, p63 and LATS 1, which

\*Correspondence to: Sonia Melino; Email: melinos@uniroma2.it  
Submitted: 08/03/12; Accepted: 08/21/12  
<http://dx.doi.org/10.4161/cc.21918>

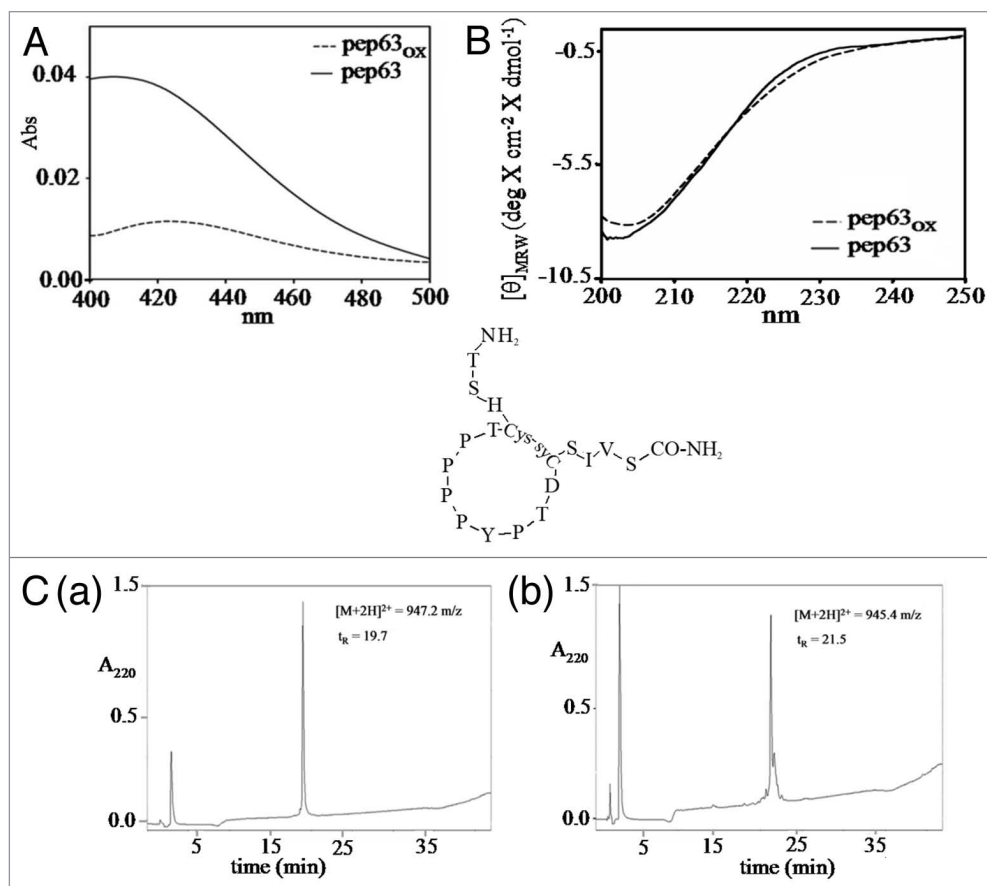


**Figure 1.** NMR assignment of Itch-WW2 domain. Region of the  $^{15}\text{N}$ - $^1\text{H}$ -HSQC spectrum (A) and  $^{13}\text{C}$ - $^1\text{H}$ -HSQC spectrum (B) of the 0.25 mM Itch-WW2 domain in 10 mM potassium phosphate buffer, pH 6.0, 50 mM KCl and 10%  $\text{D}_2\text{O}$  at 285K. (C) Secondary  $^{13}\text{C}$  chemical shift ( $\Delta\delta$   $^{13}\text{C}$ ) of Itch-WW2 domain for  $\text{C}\alpha$ ,  $\text{C}\beta$  and  $\text{C}'$  from CSI consensus values plotted and secondary structure prediction based on chemical shift of the backbone resonances using CSI.

are crucial to determine cellular fate.<sup>14,19-23</sup> Itch is a monomeric protein with a modular structural organization that consists of an N-terminal  $\text{Ca}^{2+}$ -dependent phospholipid-binding C2 domain and 2–4 WW domains, which confer substrate specificity.<sup>14,24</sup> The protein-protein recognition is a crucial event in the ubiquitination by the E3 ligases and it is determined by recognition of the WW domains with a specific proline-rich region of the targets. The WW domains are small stable proteins whose topology consists of a slightly curved, three-stranded  $\beta$ -sheet, comprising about 35 amino acids, including conserved tryptophan residues and proline-containing sequences that mediate the recognition mechanism.<sup>25,26</sup> According to their ligand binding preferences, the WW domains have been grouped into four classes, Itch has group I WW domains, specifically interacting with the PPxY motif, a proline-rich consensus sequence. Generally, the first tryptophan residue of this type of WW domain, in conjunction with an externally exposed tyrosine residue, forms a concave hydrophobic binding surface for the first two proline residues of the PPxY motif. The hydrophobic buckle formed by these two prolines stack against the W and Y residue, which likely contributes to maintain a more stable

folded structure of the entire domain.<sup>27</sup> The WW2 domain of Itch E3 has been shown to bind both p73 and p63<sup>28,29</sup> by interaction with the PPxY motif present in the C-terminal region of these transcription factors.<sup>30</sup> Indeed, the in vitro interaction between the p63 region of residues 534–551 (named pep63), encompassing the PPxY motif and WW2 domain of Itch, suggested a potential modulation effect of the downstream region to the PPxY motif on the Itch-p63 interaction. Accordingly, an extended PPxY consensus motif [P-P-P-Y-X(4)-(ST)-(ILV)] has been suggested to modulate the affinity and specificity of the interaction of p63 with Itch.<sup>30</sup>

In order to further investigate the influence of the individual residues in the recognition mechanism between Itch and p63, we have performed a structural analysis by NMR spectroscopy of the complex and identified the residues of Itch-WW2 domain involved in the interaction with the proline-rich domain of p63. Here, the NMR assignment of the Itch-WW2 backbone and the binding with a cyclic form of pep63 are indicated, demonstrating that more stable complexes can be obtained using the cyclic form of the pep63. The metal-binding ability of both forms of pep63 has also been investigated, demonstrating the ability of



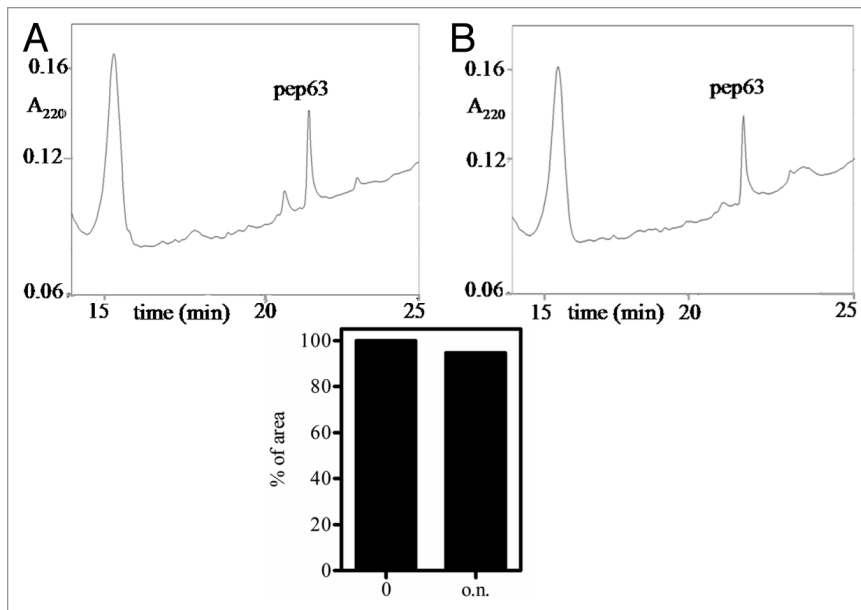
**Figure 2.** Oxidation reaction of pep63. (A) DTNB's assay of the pep63 after oxidation, UV-vis spectra of the for pep63 (—) and pep63<sub>ox</sub> (---), the maximum at 412 nm indicates the presence of TNB<sup>2+</sup> and the thiols; (B) RP-HPLC of the pep63 (a) and pep63<sub>ox</sub> (b) the molecular masses were obtained by ESI-MS of the corresponding peaks; (C) CD spectra of 50  $\mu$ M pep63(—) and pep63<sub>ox</sub> (---) in 10 mM sodium phosphate pH 6.2, buffer 25 mM KCl.

the metal-complexes to induce an oxidative damage to the WW2 domain. Since Itch regulates p63 functions, cyclic pep63 may represent a potential therapeutic pharmacological target. Taken together, our results open the way to obtain more stable complexes of Itch with its recognition target sites, in order to perform the structure of the complexes in solution, and to design potential selective competitive inhibitors of the E3 ligase activity of the recognition mechanism.

## Results

**Assignment of ITCH-WW2 domain by NMR spectroscopy.** From previous interaction studies,<sup>30</sup> we expressed the recombinant <sup>15</sup>N-<sup>13</sup>C-uniformly-labeled-Itch-WW2 domain and performed the backbone assignment of the Itch-WW2 domain, corresponding to region from D417 to E460 residues of the human Itch protein. Figure 1A shows the <sup>1</sup>H-<sup>15</sup>N HSQC spectrum with all the amide groups assigned; a total of 42 signals are labeled in the spectrum due to the presence of six prolines at the residue N-terminal of the protein, and 94% of the residues (46 out of 49) was assigned. Sequential backbone assignment was obtained using the "building blocks strategy,"<sup>31,32</sup> the <sup>1</sup>H $\alpha$  and <sup>1</sup>H $\beta$  frequencies were assigned from the corresponding HBHANH and

HBHA(CO)NH experiments and confirmed with the HCACO experiment. Side-chains were then assigned starting from the known <sup>1</sup>H $\alpha$  and <sup>1</sup>H $\beta$  frequencies, and the spin system was completed using the HCCH-TOCSY experiment and CC(CO)NNH and HCC(CO)NNH. Side-chain NH2 frequencies of asparagines and glutamine residues were assigned from the 3D <sup>15</sup>N-NOESY-HSQC and the CBCA(CO)NH spectra. Statistics for resonance assignments includes 42/43 HN, 42/43 N, 46/47 C $\alpha$ , 45/46 H $\alpha$ , 45/46 C $\beta$ , 71/72 H $\beta$ , 31/32 C $\gamma$ , 41/42 H $\gamma$ , 15/16 C $\delta$ , 23/24 H $\delta$ . Figure 1B shows the <sup>1</sup>H-<sup>13</sup>C  $\alpha$  region of the <sup>1</sup>H-<sup>13</sup>C HSQC with the relative assignments; only the N-terminal Gly, the Pro-12 and the Arg 39 are missing in this spectra. The secondary structure was predicted using the <sup>13</sup>C-chemical shift index (CSI).<sup>33</sup> Figure 1C shows the <sup>13</sup>C chemical shift deviations from the consensus CSI values for Itch-WW2; the lower panels report the predicted secondary structure. The data confirm a canonical structure for the Itch-WW2 domain, which encompasses an antiparallel three-stranded- $\beta$ -sheet. The residue Arg39, is positioned right after the end of the third strand of the  $\beta$ -sheet, in a position thus that could result in some conformational averaging, potentially explaining the broad signals detected experimentally as several resonances broadened beyond detection. The sequence-specific <sup>1</sup>H, <sup>13</sup>C and <sup>15</sup>N resonance assignments for



**Figure 3.** Biological stability of pep63<sub>ox</sub>. The chymotrypsin proteolysis of the pep63<sub>ox</sub> was monitored by RP-HPLC using the following solvent B gradient: 0–5 min, 0%; 5–25 min, 15%; 25–27 min, 15% and 27–30 min 90%. The eluted was monitored at 220 nm by UV detector; in (A) and (B) selected regions of the chromatograms of the digested products after 0 and overnight incubation with protease, respectively, are shown. Histogram of the percentages of the pep63<sub>ox</sub> peaks' area are also shown.

Itch's second WW domain were deposited in BioMagResBank under the accession number BMRB 17715.

**Cyclization and characterization of pep63.** Peptide cyclization is often used to synthesize conformationally stable molecules, with limited flexibility when compared with their natural counterpart. Moreover, cyclic peptides are of considerable interest for several reasons, such as their resistant to proteolysis and degradation. In fact, cyclization affects metabolic stability, bioavailability and pharmacokinetics.<sup>34</sup> Oxidation in solution needs to be performed at high dilution, typically at 10–100  $\mu\text{M}$ , in order to avoid aggregation and intermolecular side reactions; accordingly, the oxidation of pep63 was performed at 100  $\mu\text{M}$  in 25 mM Tris-HCl, pH 8.0, buffer for 48 h at 25°C. The oxidation reaction was monitored according to Ellman's method (Fig. 2A).<sup>35,36</sup>

The major hydrophobicity in RP-HPLC of the pep63<sub>ox</sub> (RT 21.5 min), as compared with pep63 (RT 19.7 min), is in agreement with the formation of disulfide bonds (Fig. 2C). LC/ESI-MS analysis of pep63<sub>ox</sub> gives a  $m/z$  of 945.4  $[\text{M}+2\text{H}]^{2+}$ , further confirming the intramolecular formation of disulfide bond. The pep63 CD spectrum shows a significant negative band at 202/204 nm characteristic of a polyproline II (PPII) conformation. Comparing the CD spectra of two linear and cyclic forms of pep63, a small shift of the dichroic band vs.  $\beta$ -strand conformation of the oxidized form is detectable (Fig. 2B), probably due to the rearrangement of the poly-proline helix in the cyclic form. The biological stability of the peptide was also investigated by resistance to the proteolysis using chymotrypsin in E/S ratio 1:100 (w/w). The reaction products were analyzed by RP-HPLC (Fig. 3); the cyclic form pep63<sub>ox</sub> reveals a high resistance to the

proteolysis, probably due to a reduced accessibility of the recognition sites to the protease, in addition to the presence of proline residues. Indeed, only 5.4% of digestion was detected, comparing the area of the undigested peak after 15 min and overnight (Fig. 3).

**Interaction of Itch-WW2 with pep63<sub>ox</sub> by fluorescence and NMR spectroscopy.** The interaction between Itch-WW2 and both of pep63 forms was evaluated by the quenching of the intrinsic fluorescence of Itch-WW2 after addition of the peptides in the solution. The apparent  $K_d$  values, obtained by the curve fitting analyses, were  $47.7 \pm 0.2 \mu\text{M}$  and  $13.5 \pm 0.8 \mu\text{M}$  for pep63 and pep63<sub>ox</sub>, respectively (Figure 4). The cyclic peptide shows a higher affinity for the Itch-WW2 compared with the linear peptide.

The interaction was also investigated by NMR spectroscopy, and Figure 5A shows the HSQC titration of Itch-WW2 domain with pep63<sub>ox</sub>. The spectra show that the NH resonances of Itch-WW2 domain affected by the binding to the cyclic-form are the same of those detected in the binding to the linear-form of the peptide. The assignment of the free domain using the  $^{15}\text{N}$ - $^{13}\text{C}$ -labeled Itch-WW2

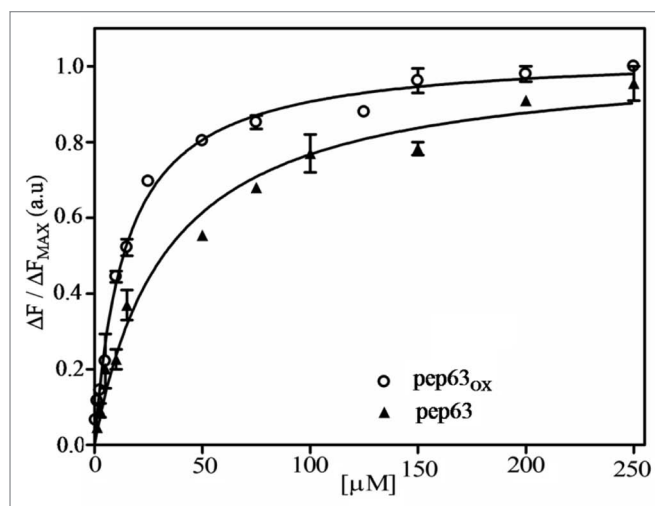
domain allowed the identification of the WW2 residues involved in the binding with the two forms of the peptide (Fig. 5A and B). The identical amino acid residues of Itch-WW2 domain are involved in the binding of both forms of the peptide, however, with relevant differences in the exchange regime (Fig. 5C and D). In fact, most of the residues affected by the binding for the cyclic form are in slow-exchange regime (Fig. 5C). In both interactions the residues of the C- and N-terminal regions of the domain are not affected by the binding. Table 1 summarizes the residues involved, with their respective exchange regimes. In particular, while R18 and the residues from T32 to W37 show a slow exchange regime in both complexes, the residues L11, E16, D20, R24, Y26, Y27, H30, Q38, T41 and V45 change their exchange regime from fast to slow in case the WW2-pep63<sub>ox</sub>. The slow-exchange regime indicates that the resident lifetime of the bound form is increased with respect of the open form, revealing a higher affinity between Itch-WW2 and pep63<sub>ox</sub> and the formation of more stable complexes.

**Metal binding of pep63 and oxidative damage to Itch-WW2 domain.** Pep63 is characterized by the presence of a histidine residue in third position forming a characteristic metal binding motif, the ATCUN motif, which binds metal ions such as  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$ .<sup>37,38</sup> In order to assess the formation of the ATCUN- $\text{Cu}^{2+}$  complex, we performed spectroscopic studies. Figure 6 shows the UV-Vis spectra of pep63 and pep63<sub>ox</sub> in the presence of equimolar concentrations of  $\text{Cu}^{2+}$  at different pHs. Both pep63 forms exhibit the characteristic maximum at 525 nm of the Cu binding in the pH range between 3.4 and 10.17 in presence of copper ions. The metal binding at low pHs indicated a characteristic of the formation of a

**Table 1.** Amino acid residues of Itch-WW2 involved in the interaction with pep63 and pep63<sub>ox</sub>

Residues	Exchange Regime	
	pep63	pep63 <sub>ox</sub>
L11	FAST	SLOW
W15	FAST	FAST
E16	FAST	SLOW
R17	-	SLOW
R18	SLOW	SLOW
V19	FAST	FAST
D20	FAST	SLOW
M22	-	SLOW
R24	FAST	SLOW
I25	FAST	FAST
Y26	FAST	SLOW
Y27	FAST	SLOW
V28	SLOW	SLOW
D29	SLOW	SLOW
H30	FAST	SLOW
F31	FAST	FAST
T32	SLOW	SLOW
R33	SLOW	SLOW
T34	SLOW	SLOW
T35	SLOW	SLOW
T36	SLOW	SLOW
W37	SLOW	SLOW
Q38	FAST	SLOW
R39	FAST	FAST
T41	FAST	SLOW
V45	FAST	FAST

stable ATCUN-Cu<sup>2+</sup>-peptide complex in agreement with previous studies.<sup>37,38</sup> Previously, specific Ni/Cu-ATCUN complexes have been shown to inhibit enzyme activity by inducing modification of the active site residues by reactive oxygen species cross-linking or cleavage.<sup>39,40</sup> In fact, several amino acid residues are susceptible to metal catalyzed oxidation.<sup>39,41</sup> We have investigated the effects of both metal-peptide complexes on the Itch-WW2 domain. **Figure 7** shows the SDS-PAGE of Itch-WW2 domain after incubation in the presence of either pep63 or pep63<sub>ox</sub> following the addition of ascorbic acid. A 25 kDa molecular weight band is detectable (**Fig. 7A**) after 30 min of incubation of Itch-WW2 in the presence of Cu<sup>2+</sup>-pep63 at 37°C, presumably for the formation of cross-links catalyzed by the formation of radical oxygen species during the reaction. Furthermore, the densitometry of the protein bands shows a 50% reduction of the WW2 band. By contrast, following 30 min incubation of Itch-WW2 in presence of CuCl<sub>2</sub> and ascorbic acid, the higher molecular weight band is not visible; only after 1 h do we observe an initial formation of a new band corresponding to a dimeric form of Itch-WW2 (**Fig. 7A**), indicating that the damage on the Itch-WW2 due to the metal-peptide

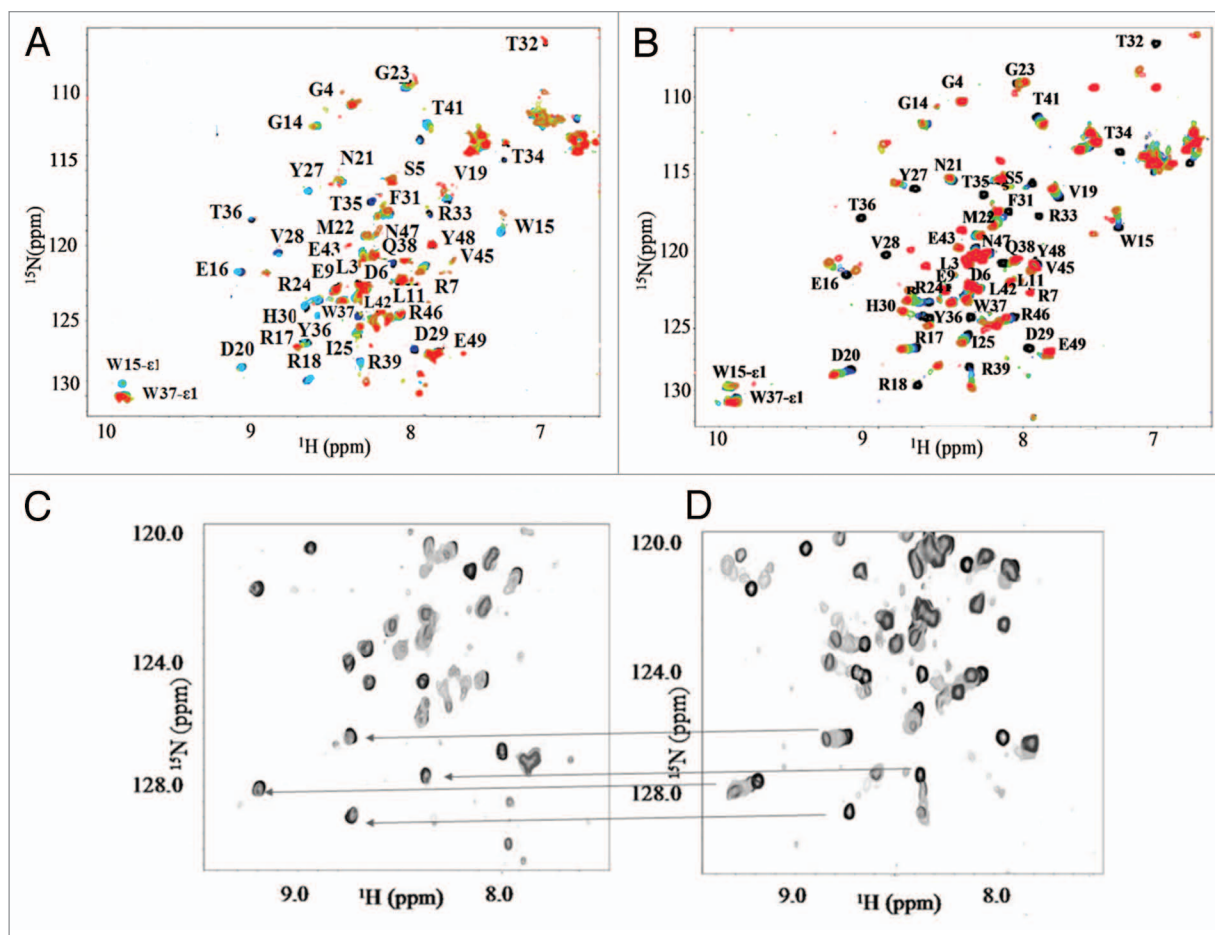
**Figure 4.** Interaction study between pep63<sub>ox</sub> and Itch-WW2. Intrinsic fluorescence changes ( $\lambda_{ox}$  280 nm and  $\lambda_{em}$  330 nm) of 5  $\mu$ M Itch-WW2 at increased concentrations of pep63 ( $\blacktriangle$ ) and pep63<sub>ox</sub> ( $\circ$ ) in 10 mM sodium phosphate, 6.0 pH, buffer, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT at 37°C.

complex is different from that with the metal ions alone. The same experiment performed using Cu<sup>2+</sup>-pep63<sub>ox</sub> complex showed only a reduction of the intensity of the band corresponding to the WW2-Itch domain (**Fig. 7B**) suggesting a possible fragmentation of the WW domain. In agreement to this hypothesis, the RP-HPLC analysis of the products after the reaction showed an increase of hydrophilic peaks concurrently to the reduction of the peak corresponding to the WW domain (**Fig. 8**).

## Discussion

The studies presented here shed light on the molecular determinants of the key WW-ligand interactions. Itch-WW2 shows a canonical structure of the WW domain in solution, with its antiparallel three-stranded- $\beta$ -sheet. The NMR assignment reported here represents a key step to assess the relevant residues and the conformational changes due to the molecular recognition mechanism of Itch with protein substrates. Titrating with increasing amounts of linear peptide, we detected distinct changes in the HSQC spectrum of WW domain. That is, we report resonance shifts, signal decrease or broadening and new signals, appearance, denoting a mixture of signal regimes respectively fast, intermediate and slow exchanging; all these characteristics well fit with a picture of a complex lifetime in the few ms timescale ( $< 4$  ms).<sup>30</sup> At atomic level description, the residues R18, T32, R33, T34, T35, T36, W37 are in slow exchange regime in the WW2-pep63 complexes, and the residues present in the third  $\beta$  strand seem to be predominantly involved in the binding of the peptide. Notable changes are induced by the interaction with the same peptide after cyclization, ten signals of Itch-WW2 domain change their exchange regime from fast to slow. The third strand signals exchange regime is invariant, while both the second and the first strands are involved in the change of regime from fast or intermediate to slow. These





**Figure 5.** NMR studies of the Itch-WW2-pep63 interaction. (A) Titration of 0.6 mM  $^{15}\text{N}$ -labeled-Itch-WW2 domain in 10 mM potassium phosphate buffer, 50 mM KCl, 5%  $\text{D}_2\text{O}$ , pH 6.0, with unlabeled pep63<sub>ox</sub> at molar ratios pep63<sub>ox</sub>/WW ranging from 0 to 35, monitored by HSQC, at 285K; (B)  $^{15}\text{N}$ - $^1\text{H}$ -HSQC spectra of 0.25 mM  $^{15}\text{N}$ -labeled-Itch-WW2; the titration was performed by sequential addition of pep63 (0–7.0 mM), seven different spectra were collected using pep63/Itch-WW2 molar ratios ranging from 0 to 35. Selected regions of the (C) Itch-WW2/pep63<sub>ox</sub>, (D) Itch-WW2/pep63  $^{15}\text{N}$ - $^1\text{H}$ -HSQC spectra, the lines underline some of the major differences observable.

data strongly suggest that the interaction of the domain with the cyclic form of the peptide results in a more stable complex with a longer lifetime than that resulting from the interaction with the linear form of pep63. This observation, from the signals point of view, is strongly backed up further by the 4-fold decrease in  $K_d$  of the interaction as measured in the fluorescence studies. Thus, the data presented here show unequivocally that pep63 is able to form a more stable complex when in cyclic form. It is notable that the residues Y26, V28, H30 and W37 seem to form a hydrophobic groove within Itch-WW2 domain. These residues are highly conserved across the family of WW domains that recognize PPxY ligands,<sup>42–46</sup> such as in the four WW domains of Itch, except for the substitution of the W with Y residue in the WW4 domain. In binding with cyclic form of pep63, the exchanging regime of two of these residues, Y26 and H30, change from fast to slow, indicating the formation of a less shaky complex induced by a more stable conformation of the peptide. Similarly, as described for Yap2-WBP2 complex,<sup>46</sup> in the WW2-pep63<sub>ox</sub> complex, the formation of a hydrogen bond between H $\eta$  phenolic hydrogen of Y within the PPxY motif and

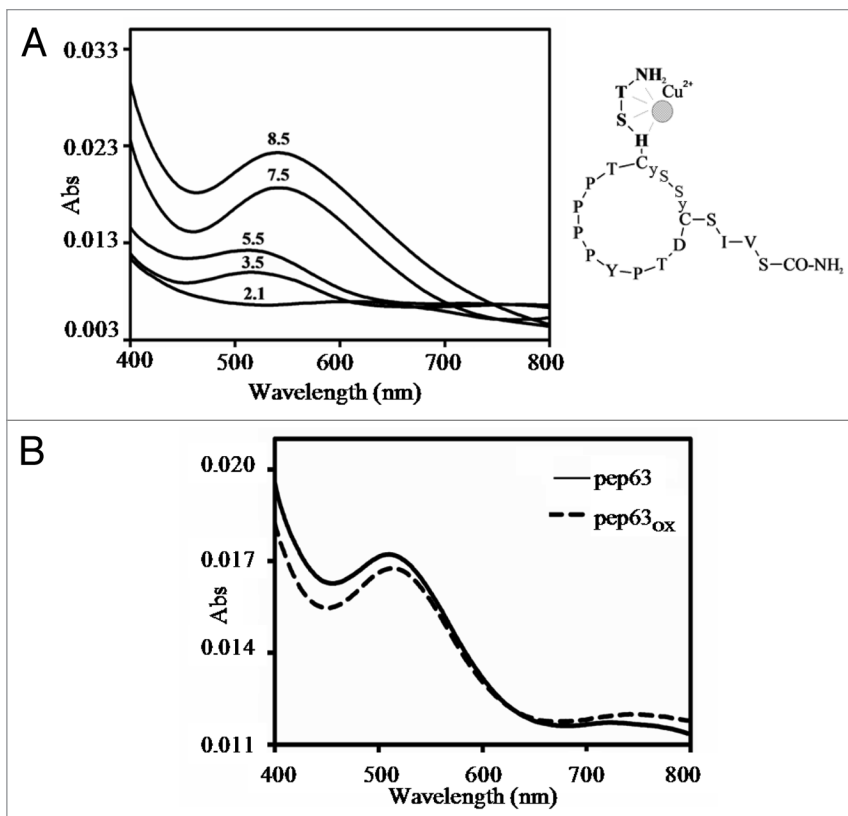
N $\delta$ 1 atom of H30 in the Itch-WW2 domain might be improved by the cyclization of the pep63 stabilizing the complex. Thus, the cyclization may also be advantageous for allowing the PPxY peptides to attain a close molecular fit within the hydrophobic grooves of the WW domains. A less flexible conformation of the PPxY peptide, achievable by cyclization, could also result in a lower entropic penalty upon binding to the WW domain. Hence, the overall free energy of WW-pep63 interaction could be favored by a reduction in the entropic penalty.

The above data are in agreement with recent thermodynamic studies that suggested strongly that non-consensus residues within and flanking the PPxY peptides are important for stabilizing the peptide conformation, and that peptides with distinct conformations are optimally suited for binding to WW domains.<sup>46</sup> The balance among rigidity and flexibility of both WW domains and protein targets might also be an *in vivo* critical factor in this molecular recognition mechanism. Accordingly, the WW-ligand interactions are usually studied *in vitro* using short, proline-rich peptides, and the relatively low affinities observed (in 10 to 100  $\mu\text{M}$ ) are generally characteristic of several similar

interactions.<sup>25,46-50</sup> The low affinity in many cases limited the possibility to obtain a high-resolution structure of the complex by NMR spectroscopy. Our results show the possibility that an increase of the stability of the complex in the solution may induce a major conformation stability of the fragment, probably improving the mimicking of the local structure of the protein by using a cyclic form of the PPxY peptide. Such optimized cyclic forms of PPxY peptides could represent a good model to study the determinants of the molecular recognition mechanism WW ligand. This cyclization strategy could also be used to optimize protein microarrays for analyzing the molecular determinants relevant in this key interaction. Furthermore, the S-S cyclization of pep63 leads also to the proteases' resistance. This feature can likely be attributed to a reduction in backbone flexibility of the peptide. Generally, in fact, potential cleavage sites within a protein are more susceptible to attack by proteases if they are located in flexible regions of the backbone.<sup>51</sup>

The cysteine residues in the pep63 peptide are present in native p63 protein. Although cyclization of the pep63 backbone provides an excellent protection from proteolytic attacks while increasing the interaction with the WW domain, this strategy cannot be directly applied to other WW-ligand interactions. Indeed, for each individual case, it is necessary to select a specific size of the linker in order to span the appropriate distance between the N and C termini of the ligand.

This optimization the WW-ligand interaction opens the way to the identification of novel potentially selective inhibitors of this molecular interaction. Protein-protein interactions play a key role in every biological process and represent a promising new class of biological drug targets.<sup>52,53</sup> The modulation of the E3 ligase-protein (namely Itch-p63) interactions could allow the development of innovative molecules able to modulate cellular functions, with potential therapeutic effects. In our study, the possibility to use the peptides to inhibit the p63-Itch recognition mechanism has been investigated. To this purpose, we evaluated the metal-binding ability of pep63, which presents a histidine residue in the third position; thus, at the N terminus of this peptide is present an N-terminal Cu<sup>2+</sup>- and Ni<sup>2+</sup>-binding motif.<sup>37,38</sup> The ATCUN motif has a well-defined structure of coordinated ligands around the metal center and is an optimal center for the design synthetic metal drugs. ATCUN metal peptides complexes have been studied for their ability to induce localized oxidative damage to nucleic acids or proteins, generating not diffusible radical species,<sup>39,41,54</sup> and, for this property, their potential application in biotechnological and pharmaceutical field has been proposed.<sup>55-57</sup> The biological effects that we describe here for both forms of the pep63 on the WW domain



**Figure 6.** Metal binding of the both forms of the pep63. UV-vis spectra of 0.5 mM pep63 (A) in the presence of equimolar concentration of CuCl<sub>2</sub> at different pHs; (B) UV-vis spectra of 0.5 mM pep63 (—) and pep63<sub>ox</sub> (---) in the presence of 0.5 mM CuCl<sub>2</sub> at pH 5.5, showing the characteristic maximum of the Cu<sup>2+</sup> bound at 525 nm.

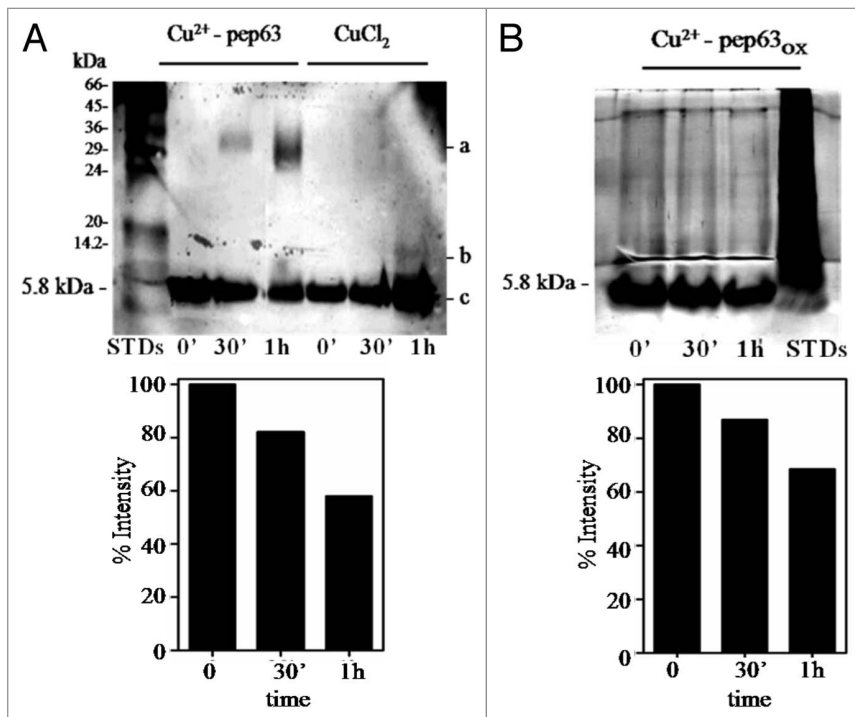
suggest the possibility of using pep63, and in the particular the cyclic form, as a model for the design of metal-drug inhibitor of Itch-recognition mechanism. Our future efforts will focus on unraveling the effects of the metal-pep63<sub>ox</sub> complex on the ubiquitilation of p63 and p73 in cell.

In conclusion, the above data provide a suggestion for a novel strategy for studying the WW-p63 interaction and, more generally, the key WW-ligand mechanism, with a potential use of the cyclic PPxY-containing peptide as a model for the inhibition of the recognition mechanism of HECT-E3-ligase.

## Materials and Methods

**Peptide synthesis.** Synthetic peptides were purchased from Spectra 2000. Analysis of the synthetic peptides by reverse phase high-performance chromatography (RP-HPLC) and mass spectrometry revealed a purity > 98%. The two sequences of the peptides had the following sequences: 18-mer pep63 NH<sub>2</sub>-TSH CTP PPP YPT DCS IVS-CONH<sub>2</sub> (1901.15 m/z).

**Circular dichroism.** CD measurements were performed using a Jasco 710 spectropolarimeter (Jasco) equipped with a thermal controller calibrated with camphor-sulfonic acid. Far-UV CD experiments were performed to explore the conformation of the peptides and of Itch-WW domain. CD spectra



**Figure 7.** Oxidative effects on Itch-WW2. SDS-PAGE at 20% gel acrylamide of 30  $\mu$ M WW2 in the presence of 0.3 mM CuCl<sub>2</sub> or Cu-pep63 complex (1:1 Cu<sup>2+</sup>/peptide c/c) in the linear (A) and cyclic form (B) after incubation of 0, 30 and 60 min at 37°C in 10 mM Tris-HCl, pH 7.0. The reactions were started by addition of 3 mM ascorbic acid and the densitometries of the Itch-WW2 bands (c bands) are shown.

were obtained between 200 and 250 nm using a path-length of 0.1 cm, 1.0 sec time constant, 2 nm bandwidth, 2 nm/min scan rate and at 20 or 50 mdeg of sensitivity. Each spectrum was averaged over four scans and subjected to smoothing following subtraction of the buffer background. The measured ellipticity data were converted to mean molar ellipticity per residue  $[(\theta), \text{deg} \times \text{cm}^2 \times \text{dmol}^{-1}]$ .

**Cyclization of the pep63.** The cyclic form of pep63 was obtained by disulfide bond formation incubating the peptide in 50 mM Tris-HCl, pH 8.0, buffer in presence of oxygen for 48 h at 25°C. The oxidation reaction was monitored by estimation of free thiol groups according to the method described by Ellman et al.<sup>35,36</sup> Briefly, the sample was added to the working solution: 100  $\mu$ M of Ellman's reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), which was previously solubilized in 50 mM sodium acetate solution, in 1M Tris-HCl, pH 8.0, buffer. The reaction between DTNB and thiol groups gave the mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB), which was quantified by monitoring of the absorbance of TNB anion at 412 nm. The oxidized form was then analyzed by LC/ESI-MS spectroscopy (C4T).

**Fluorescence interaction studies.** The interaction of Itch-WW2 with pep63 peptides was monitored by quenching of the emission band of Itch-WW2 excited at 280 nm using an  $\lambda_{\text{ex}}$  and  $\lambda_{\text{em}}$  slit of 5 nm in 10 mM phosphate buffer, pH 6.0, buffer, 100 mM NaCl at 37°C. Binding constants were determined by fitting the titration curve of fluorescence changes vs. equivalents

of peptide provides a titration curve. The  $\Delta F$  was plotted vs. molar equivalent of peptide, and the following equation was used for generating a Scatchard plot from which binding constants were determined  $\Delta f = nK[\text{peptide}] \Delta F_{\text{complex}} / (1+K[\text{peptide}])$ .  $\Delta F$  was the observed fluorescence changing after addition of peptide. Fluorescence was measured at the chemical equilibrium at the earliest 2 min after addition of the peptide. The results were plotted using GRAPHPAD PRISM v. 4.0 for Windows (GraphPad Software; www.graphpad.com).

**Expression of the uniformly isotope labeled Itch-WW2 domain.** The GST-Itch-WW2 was overexpressed using *E. coli* BL21 strain in LB medium containing 100  $\mu$ g/ml ampicillin. Cells were grown at 37°C, and the induction of the expression of the protein was performed by addition of 1 mM IPTG. Cells were grown at 37°C for a further 4 h, collected by centrifugation and after disrupted by sonication. The GST-Itch-WW2 was purified using a GST-Trap FF column (5 ml, GE-Amersham) equilibrated with 100 mM Tris-HCl, pH 7.5, 0.3 M NaCl buffer at 1.0 ml/min and was eluted using 50 mM Tris-HCl, pH 8.0, 10 mM GSH buffer. The Itch-WW2 domain was cleaved from the GST using the PreScission Protease

(GE-Amersham). The uniformly isotopically labeled <sup>15</sup>N-<sup>13</sup>C-GST-Itch-WW2 for NMR spectroscopy was generated in BL21 *E. coli* strain using the protocol for isotope labeling described by Marley et al.,<sup>58</sup> the culture was grown at 37°C in the presence of (<sup>15</sup>N)-NH<sub>4</sub>Cl and (<sup>13</sup>C)-glucose as the sole nitrogen and carbon sources, respectively. The induction of the expression of the protein was performed by addition of 1mM IPTG. The U-<sup>15</sup>N-<sup>13</sup>C-GST-Itch-WW2 was purified using GST-Trap FF column (5 ml, GE-Amersham) equilibrated with 100 mM Tris-HCl, pH 7.5, 0.3 M NaCl buffer at 1.0 ml/min and was eluted using 50 mM Tris-HCl, pH 8.0, 0.3 M NaCl and 10 mM GSH buffer. The Itch-WW2 domain was cleaved from the GST using the PreScission Protease (GE-Amersham). The expression-purification procedure resulted in a 16 mg/L yield. The purified protein was concentrated and dialyzed with 10 mM phosphate buffer, pH 6.0, 50 mM KCl. The incorporation rate for <sup>15</sup>N and <sup>13</sup>C were estimated by NMR to be ~90%.

**NMR spectroscopy.** <sup>15</sup>N-<sup>13</sup>C-Itch-WW2 was prepared for NMR experiments at 600  $\mu$ M in 10 mM pH 6.0, 50 mM KCl and 95/5% H<sub>2</sub>O/D<sub>2</sub>O using 250  $\mu$ l of sample in Shigemi microtubes (Shigemi Inc.). All the NMR experiments were performed using a three channel 700 MHz Bruker AVANCE instrument equipped with a triple resonance probe. For the assignment of backbone <sup>1</sup>H, <sup>13</sup>C and <sup>15</sup>N resonance, HNCA, HN(CO)CA, CACBNH, CBCA(CO)NH, HBHANH, HBHA(CO)NH, HNCO, HN(CA)CO,<sup>59</sup> HCACO<sup>60</sup> and 3D <sup>15</sup>N-edited TOCSY-HSQC and NOESY-HSQC spectra



were recorded. For side chain resonance assignment, 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC, 3D  $^{13}\text{C}$ -edited-NOESY-HSQC, HCCH-TOCSY, CC(CO)NNH and HCC(CO)NNH spectra were recorded. All NMR spectra were recorded at 298 K, processed using NMRPipe<sup>61</sup> and analyzed using the program NMRViewJ (OneMoon Scientific Inc.).<sup>62</sup>

**Spectroscopic characterization of the Itch-WW2-pep63 binding.** The NMR experiments were performed using 250  $\mu\text{l}$  of sample in Shigemi microtubes. Itch-WW2 domain as well as peptide pep63 were dissolved in 10 mM phosphate buffer, 50 mM KCl, 1 mM tris(2-carboxyethyl)-phosphine (TCEP), pH 6.0. Titrations of the Itch-WW2 domain were conducted twice at 12°C and followed using two different techniques. In the first, a sample of Itch-WW2 at 250  $\mu\text{M}$  was titrated by addition of small aliquots of a 5 mM pep63 or pep63<sub>ox</sub> solution dissolved in the same buffer. Itch-WW2 uniformly  $^{15}\text{N}$ -labeled was used. The titrations were repeated following all the WW2 domain amide region with 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC experiments using a pulse sequence with water flip back selective pulses to avoid water saturation. For these experiments small aliquots of the peptide were added stepwise using a 50 mM stock solution of the pep63 or pep63<sub>ox</sub> dissolved in the same buffer. All the NMR experiments were performed using a 600 MHz Bruker AVANCE instrument equipped with a z-gradient triple resonance proton cry-probe. The NMR spectra were processed and analyzed with an Octane Silicon Graphics workstation operating under Irix 6.5, using NMRPipe<sup>61</sup> and NMRView J 6.2.2 (One Moon Scientific Inc., 2009).

Proteolysis resistance Chymotrypsin digestion was performed using a 1:100 E:S (w/w) in 50 mM Tris-HCl, pH 8.04, the reaction mixture was incubated at 37°C for the indicated times and then stopped by addition of 0.1% TFA solution, vortex and freezing. Before the RP-HPLC analysis, 10 mM DTT was added at the samples in order to have a reduction of the disulfide bond of the pep63<sub>ox</sub> and, potentially, the separation of the fragments. The samples were analyzed using RP-HPLC system chromatography (mod. LC-10AVP Shimadzu equipment) with an solvent B gradient (0–5 min, 0%; 5–25 min, 15%; 25–27 min, 15% and 27–30 min 90%), using 0.1% TFA as solvent A and 80% CH<sub>3</sub>CN, 0.1% TFA as solvent B and a Brounlee C-18 column (OD-300, 250x4.6mm, 7  $\mu\text{m}$ ). The eluted was monitored at 220 nm by UV detector (Shimadzu). The same conditions of analysis was also used to analyze the WW domain after oxidative damage using the metal-pep63<sub>ox</sub> complex.

**Spectrophotometric metal binding characterization.** The peptides were dissolved in water to a concentration of 0.5 mM CuCl<sub>2</sub> was added in a 1:1 molar ratio with the peptides. The pH

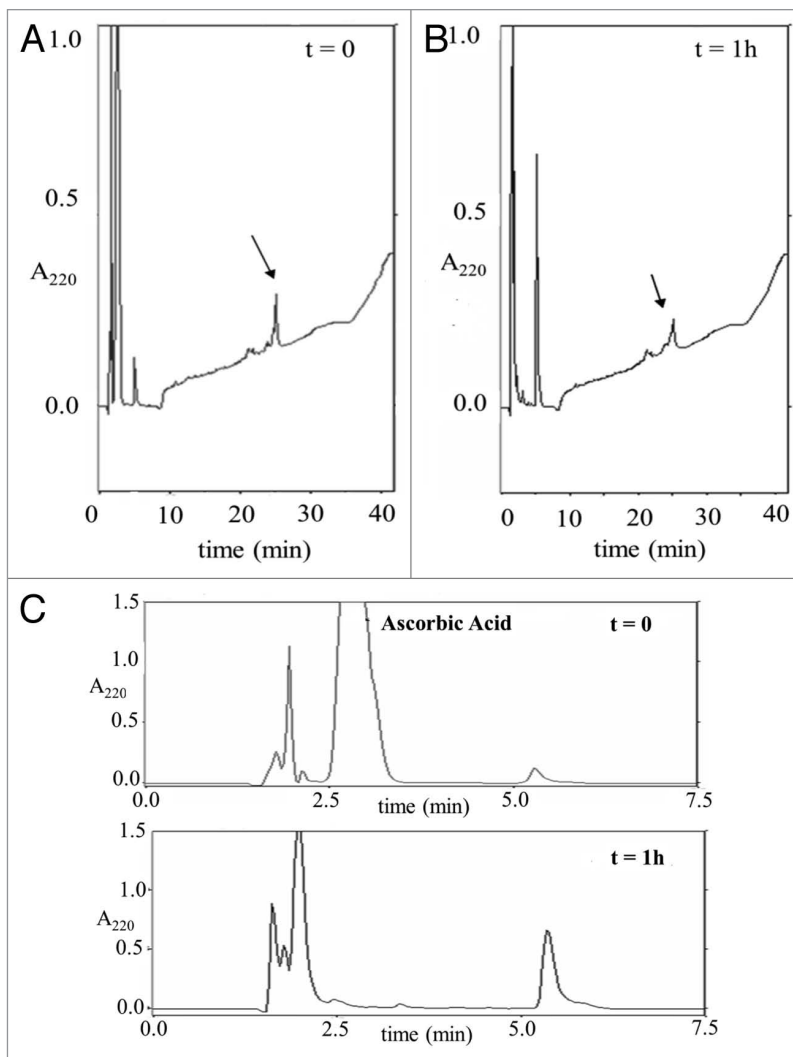
of the solution was decreased or raised using 0.1 M HCl or 0.2 M NaOH respectively, and the solutions were equilibrated at each pH before the spectrophotometric measurements. The visible spectra of the peptide-metal complexes were acquired on Perkin-Elmer Lambda-Bio 20 double-beam spectrophotometer using a 1 cm path length quartz cells at 25°C.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Acknowledgments

The authors are grateful to F. Bertocchi for technical support for the NMR experiments and to Dr. V. Fiordalice for her support in the preparation of some experiments.



**Figure 8.** Oxidative cleavage of Itch-WW2. RP-HPLC of Itch-WW2 (30  $\mu\text{M}$ ) after incubation in presence of Cu<sup>2+</sup>-pep63<sub>ox</sub> complex (0.3 mM) and of 3 mM ascorbic acid (A) after 0 and (B) 60 min of incubation at 37°C. (C) Selected region of the chromatograms (from 0 to 7.5 min.) showing the presence of fragments. The arrows indicate the peak corresponding to Itch-WW2.

## References

- Hershko A, Ciechanover A, Varshavsky A. Basic Medical Research Award. The ubiquitin system. *Nat Med* 2000; 6:1073-81; PMID:11017125; <http://dx.doi.org/10.1038/80384>.
- Ishihara T, Inoue J, Kozaki K, Imoto I, Inazawa J. HECT-type ubiquitin ligase ITCH targets lysosomal-associated protein multispansing transmembrane 5 (LAPTM5) and prevents LAPTM5-mediated cell death. *J Biol Chem* 2011; 286:44086-94; PMID:22009753; <http://dx.doi.org/10.1074/jbc.M111.251694>.
- Schwarz SE, Rosa JL, Scheffner M. Characterization of human hect domain family members and their interaction with UbcH5 and UbcH7. *J Biol Chem* 1998; 273:12148-54; PMID:9575161; <http://dx.doi.org/10.1074/jbc.273.20.12148>.
- Ingham RJ, Colwill K, Howard C, Dettwiler S, Lim CS, Yu J, et al. WW domains provide a platform for the assembly of multiprotein networks. *Mol Cell Biol* 2005; 25:7092-106; PMID:16055720; <http://dx.doi.org/10.1128/MCB.25.16.7092-7106.2005>.
- Kerscher O, Felberbaum R, Hochstrasser M. Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* 2006; 22:159-80; PMID:16753028; <http://dx.doi.org/10.1146/annurev.cellbio.22.010605.093503>.
- Abriel H, Loffing J, Rebhun JF, Pratt JH, Schild L, Horisberger JD, et al. Defective regulation of the epithelial Na<sup>+</sup> channel by Nedd4 in Liddle's syndrome. *J Clin Invest* 1999; 103:667-73; PMID:10074483; <http://dx.doi.org/10.1172/JCI5713>.
- Zhu H, Kavsak P, Abdollah S, Wrana JL, Thomsen GH. A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* 1999; 400:687-93; PMID:10458166; <http://dx.doi.org/10.1038/23293>.
- Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* 2000; 6:1365-75; PMID:11163210; [http://dx.doi.org/10.1016/S1097-2765\(00\)00134-9](http://dx.doi.org/10.1016/S1097-2765(00)00134-9).
- Flores SY, Debonneville C, Staub O. The role of Nedd4/Nedd4-like dependant ubiquitylation in epithelial transport processes. *Pflugers Arch* 2003; 446:334-8; PMID:12698368.
- Bai Y, Yang C, Hu K, Elly C, Liu YC. Itch E3 ligase-mediated regulation of TGF-beta signaling by modulating smad2 phosphorylation. *Mol Cell* 2004; 15:825-31; PMID:15350225; <http://dx.doi.org/10.1016/j.molcel.2004.07.021>.
- Anindya R, Aygün O, Svestrup JQ. Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1. *Mol Cell* 2007; 28:386-97; PMID:17996703; <http://dx.doi.org/10.1016/j.molcel.2007.10.008>.
- Liu YC. The E3 ubiquitin ligase Itch in T cell activation, differentiation, and tolerance. *Semin Immunol* 2007; 19:197-205; PMID:17433711; <http://dx.doi.org/10.1016/j.smim.2007.02.003>.
- Bernassola F, Karin M, Ciechanover A, Melino G. The HECT family of E3 ubiquitin ligases: multiple players in cancer development. *Cancer Cell* 2008; 14:10-21; PMID:18598940; <http://dx.doi.org/10.1016/j.ccr.2008.06.001>.
- Melino G, Gallagher E, Aqeilan RI, Knight R, Peschiaroli A, Rossi M, et al. Itch: a HECT-type E3 ligase regulating immunity, skin and cancer. *Cell Death Differ* 2008; 15:1103-12; PMID:18552861; <http://dx.doi.org/10.1038/cdd.2008.60>.
- Matesic LE, Copeland NG, Jenkins NA. Itchy mice: the identification of a new pathway for the development of autoimmunity. *Curr Top Microbiol Immunol* 2008; 321:185-200; PMID:18727493; [http://dx.doi.org/10.1007/978-3-540-75203-5\\_9](http://dx.doi.org/10.1007/978-3-540-75203-5_9).
- Lévy F, Muehlethaler K, Salvi S, Peitrequin AL, Lindholm CK, Cerottini JC, et al. Ubiquitylation of a melanosomal protein by HECT-E3 ligases serves as sorting signal for lysosomal degradation. *Mol Biol Cell* 2005; 16:1777-87; PMID:15703212; <http://dx.doi.org/10.1091/mbc.E04-09-0803>.
- Sundvall M, Korhonen A, Paatero I, Gaudio E, Melino G, Croce CM, et al. Isoform-specific monoubiquitination, endocytosis, and degradation of alternatively spliced ErbB4 isoforms. *Proc Natl Acad Sci USA* 2008; 105:4162-7; PMID:18334649; <http://dx.doi.org/10.1073/pnas.0708333105>.
- Marchese A, Raiborg C, Santini F, Keen JH, Stenmark H, Benovic JL. The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Dev Cell* 2003; 5:709-22; PMID:14602072; [http://dx.doi.org/10.1016/S1534-5807\(03\)00321-6](http://dx.doi.org/10.1016/S1534-5807(03)00321-6).
- Zhang P, Wang C, Gao K, Wang D, Mao J, An J, et al. The ubiquitin ligase itch regulates apoptosis by targeting thioredoxin-interacting protein for ubiquitin-dependent degradation. *J Biol Chem* 2010; 285:8869-79; PMID:20068034; <http://dx.doi.org/10.1074/jbc.M109.063321>.
- Ishihara T, Tsuda H, Hotta A, Kozaki K, Yoshida A, Noh JY, et al. ITCH is a putative target for a novel 20q11.22 amplification detected in anaplastic thyroid carcinoma cells by array-based comparative genomic hybridization. *Cancer Sci* 2008; 99:1940-9; PMID:19016753.
- Ho KC, Zhou Z, She YM, Chun A, Cyr TD, Yang X. Itch E3 ubiquitin ligase regulates large tumor suppressor 1 stability [corrected]. *Proc Natl Acad Sci U S A* 2011; 108:4870-5; PMID:21383157; <http://dx.doi.org/10.1073/pnas.1101273108>.
- Melino G. p63 is a suppressor of tumorigenesis and metastasis interacting with mutant p53. *Cell Death Differ* 2011; 18:1487-99; PMID:21760596; <http://dx.doi.org/10.1038/cdd.2011.81>.
- Levine AJ, Tomasini R, McKeon FD, Mak TW, Melino G. The p53 family: guardians of maternal reproduction. *Nat Rev Mol Cell Biol* 2011; 12:259-65; PMID:21427767; <http://dx.doi.org/10.1038/nrm3086>.
- Perry WL, Hustad CM, Swing DA, O'Sullivan TN, Jenkins NA, Copeland NG. The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice. *Nat Genet* 1998; 18:143-6; PMID:9462742; <http://dx.doi.org/10.1038/ng0298-143>.
- Macias MJ, Wiesner S, Sudol M. WW and SH3 domains, two different scaffolds to recognize proline-rich ligands. *FEBS Lett* 2002; 513:30-7; PMID:11911877; [http://dx.doi.org/10.1016/S0014-5793\(01\)03290-2](http://dx.doi.org/10.1016/S0014-5793(01)03290-2).
- Zarrinpar A, Lim WA. Converging on proline: the mechanism of WW domain peptide recognition. *Nat Struct Biol* 2000; 7:611-3; PMID:10932238; <http://dx.doi.org/10.1038/77891>.
- Sudol M, Hunter T. NeW wrinkles for an old domain. *Cell* 2000; 103:1001-4; PMID:11163176; [http://dx.doi.org/10.1016/S0092-8674\(00\)00203-8](http://dx.doi.org/10.1016/S0092-8674(00)00203-8).
- Rossi M, De Laurenzi V, Munarriz E, Green DR, Liu YC, Vousden KH, et al. The ubiquitin-protein ligase Itch regulates p73 stability. *EMBO J* 2005; 24:836-48; PMID:15678106; <http://dx.doi.org/10.1038/sj.emboj.7600444>.
- Rossi M, De Simone M, Pollice A, Santoro R, La Mantia G, Guerrini L, et al. Itch/AIP4 associates with and promotes p63 protein degradation. *Cell Cycle* 2006; 5:1816-22; PMID:16861923; <http://dx.doi.org/10.4161/cc.5.16.2861>.
- Bellomaria A, Barbato G, Melino G, Paci M, Melino S. Recognition of p63 by the E3 ligase ITCH: Effect of an ectodermal dysplasia mutant. *Cell Cycle* 2010; 9:3730-9; PMID:20855944; <http://dx.doi.org/10.4161/cc.9.18.12933>.
- Bax A, Ikura M. An efficient 3D NMR technique for correlating the proton and <sup>15</sup>N backbone amide resonances with the alpha-carbon of the preceding residue in uniformly <sup>15</sup>N/<sup>13</sup>C enriched proteins. *J Biomol NMR* 1991; 1:99-104; PMID:1668719; <http://dx.doi.org/10.1007/BF01874573>.
- Bax A, Ikura M, Kay LE, Barbato G, Spera S. Multidimensional triple resonance NMR spectroscopy of isotopically uniformly enriched proteins: a powerful new strategy for structure determination. *Ciba Found Symp* 1991; 161:108-19, discussion 119-35; PMID:1814691.
- Wishart DS, Sykes BD. The <sup>13</sup>C chemical-shift index: a simple method for the identification of protein secondary structure using <sup>13</sup>C chemical-shift data. *J Biomol NMR* 1994; 4:171-80; PMID:8019132; <http://dx.doi.org/10.1007/BF00175245>.
- Tyndall JD, Reid RC, Tyssen DP, Jardine DK, Todd B, Passmore M, et al. Synthesis, stability, antiviral activity, and protease-bound structures of substrate-mimicking constrained macrocyclic inhibitors of HIV-1 protease. *J Med Chem* 2000; 43:3495-504; PMID:11000004; <http://dx.doi.org/10.1021/jm000013n>.
- Ellman GL. Tissue sulphydryl groups. *Arch Biochem Biophys* 1959; 82:70-7; PMID:13650640; [http://dx.doi.org/10.1016/0003-9861\(59\)90090-6](http://dx.doi.org/10.1016/0003-9861(59)90090-6).
- Riddles PW, Blakeley RL, Zerner B. Reassessment of Ellman's reagent. *Methods Enzymol* 1983; 91:49-60; PMID:6855597; [http://dx.doi.org/10.1016/S0076-6879\(83\)91010-8](http://dx.doi.org/10.1016/S0076-6879(83)91010-8).
- Sigel H, Martin RB. Coordinating properties of the amide bond: Stability and structure of metal ion complexes of peptides and related ligands. *Chem Rev* 1982; 82:385-426; <http://dx.doi.org/10.1021/cr00050a003>.
- Laussac JP, Sarkar B. Characterization of the copper(II)- and nickel(II)-transport site of human serum albumin. Studies of copper(II) and nickel(II) binding to peptide 1-24 of human serum albumin by <sup>13</sup>C and <sup>1</sup>H NMR spectroscopy. *Biochemistry* 1984; 23:2832-8; PMID:6547847; <http://dx.doi.org/10.1021/bi00307a046>.
- Stadtman ER. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radic Biol Med* 1990; 9:315-25; PMID:2283087; [http://dx.doi.org/10.1016/0891-5849\(90\)90006-5](http://dx.doi.org/10.1016/0891-5849(90)90006-5).
- Nepravishita R, Bellomaria A, Polizio F, Paci M, Melino S. Reticulon RTN1-C<sub>(CT)</sub> peptide: a potential nuclease and inhibitor of histone deacetylase enzymes. *Biochemistry* 2010; 49:252-8; PMID:20000484; <http://dx.doi.org/10.1021/bi9012676>.
- Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu Rev Biochem* 1993; 62:797-821; PMID:8352601; <http://dx.doi.org/10.1146/annurev.bi.62.070193.004053>.
- Macias MJ, Hyvönen M, Baraldi E, Schultz J, Sudol M, Saraste M, et al. Structure of the WW domain of a kinase-associated protein complexed with a proline-rich peptide. *Nature* 1996; 382:646-9; PMID:8757138; <http://dx.doi.org/10.1038/382646a0>.
- Pires JR, Taha-Nejad F, Toepfer F, Ast T, Hoffmüller U, Schneider-Mergener J, et al. Solution structures of the YAP65 WW domain and the variant L30 K in complex with the peptides GTPPPPYTVG, N-(n-octyl)-GPPPY and PLPPY and the application of peptide libraries reveal a minimal binding epitope. *J Mol Biol* 2001; 314:1147-56; PMID:11743730; <http://dx.doi.org/10.1006/jmbi.2000.5199>.
- Huang X, Poy F, Zhang R, Joachimiak A, Sudol M, Eck MJ. Structure of a WW domain containing fragment of dystrophin in complex with beta-dystroglycan. *Nat Struct Biol* 2000; 7:634-8; PMID:10932245; <http://dx.doi.org/10.1038/77923>.
- Kanelis V, Rotin D, Forman-Kay JD. Solution structure of a Nedd4 WW domain-ENaC peptide complex. *Nat Struct Biol* 2001; 8:407-12; PMID:11323714; <http://dx.doi.org/10.1038/87562>.

46. McDonald CB, McIntosh SK, Mikles DC, Bhat V, Deegan BJ, Seldeen KL, et al. Biophysical analysis of binding of WW domains of the YAP2 transcriptional regulator to PPXY motifs within WBP1 and WBP2 adaptors. *Biochemistry* 2011; 50:9616-27; PMID:21981024; <http://dx.doi.org/10.1021/bi201286p>.
47. Kay BK, Williamson MP, Sudol M. The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. *FASEB J* 2000; 14:231-41; PMID:10657980.
48. Linn H, Ermekova KS, Rentschler S, Sparks AB, Kay BK, Sudol M. Using molecular repertoires to identify high-affinity peptide ligands of the WW domain of human and mouse YAP. *Biol Chem* 1997; 378:531-7; PMID:9224934; <http://dx.doi.org/10.1515/bchm.1997.378.6.531>.
49. Dalby PA, Hoess RH, DeGrado WF. Evolution of binding affinity in a WW domain probed by phage display. *Protein Sci* 2000; 9:2366-76; PMID:11206058.
50. Webb C, Upadhyay A, Giuntini F, Eggleston I, Furutani-Seiki M, Ishima R, et al. Structural features and ligand binding properties of tandem WW domains from YAP and TAZ, nuclear effectors of the Hippo pathway. *Biochemistry* 2011; 50:3300-9; PMID:21417403; <http://dx.doi.org/10.1021/bi2001888>.
51. Hubbard SJ. The structural aspects of limited proteolysis of native proteins. *Biochim Biophys Acta* 1998; 1382:191-206; PMID:9540791; [http://dx.doi.org/10.1016/S0167-4838\(97\)00175-1](http://dx.doi.org/10.1016/S0167-4838(97)00175-1).
52. Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. *Nature* 2007; 450:1001-9; PMID:18075579; <http://dx.doi.org/10.1038/nature06526>.
53. Dömling A. Small molecular weight protein-protein interaction antagonists: an insurmountable challenge? *Curr Opin Chem Biol* 2008; 12:281-91; PMID:18501203; <http://dx.doi.org/10.1016/j.cbpa.2008.04.603>.
54. Jin Y, Cowan JA. DNA cleavage by copper-ATCUN complexes. Factors influencing cleavage mechanism and linearization of dsDNA. *J Am Chem Soc* 2005; 127:8408-15; PMID:15941274; <http://dx.doi.org/10.1021/ja0503985>.
55. Brittain IJ, Huang X, Long EC. Selective recognition and cleavage of RNA loop structures by Ni(II).Xaa-Gly-His metallopeptides. *Biochemistry* 1998; 37:12113-20; PMID:9724523; <http://dx.doi.org/10.1021/bi9806605>.
56. Melino S, Gallo M, Trotta E, Mondello F, Paci M, Petruzzelli R. Metal-binding and nuclease activity of an antimicrobial peptide analogue of the salivary histatin 5. *Biochemistry* 2006; 45:15373-83; PMID:17176059; <http://dx.doi.org/10.1021/bi0615137>.
57. Hocharoen L, Cowan JA. Metallotherapeutics: novel strategies in drug design. *Chemistry* 2009; 15:8670-6; PMID:19685535; <http://dx.doi.org/10.1002/chem.200900821>.
58. Marley J, Lu M, Bracken C. A method for efficient isotopic labeling of recombinant proteins. *J Biomol NMR* 2001; 20:71-5; PMID:11430757; <http://dx.doi.org/10.1023/A:1011254402785>.
59. Bazzo R, Cicero DO, Barbato G. A new 3D HCACO pulse sequence with optimized resolution and sensitivity. Application to the 21 kDa protein human interleukin-6. *J Magn Reson B* 1995; 107:189-91; PMID:7599953; <http://dx.doi.org/10.1006/jmrb.1995.1077>.
60. Bazzo R, Cicero DO, Barbato G. A new 3D pulse sequence for correlating intraresidue NH, N and CO chemical shifts in C-13 N-15 labeled proteins. Application to the protein human interleukin 6. *J Magn Reson B* 1996; 110:65-8; <http://dx.doi.org/10.1006/jmrb.1996.0008>.
61. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 1995; 6:277-93; PMID:8520220; <http://dx.doi.org/10.1007/BF00197809>.
62. Johnson BA. Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Methods Mol Biol* 2004; 278:313-52; PMID:15318002.