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Sonia Melino[a], Alessia Bellomaria[a], Ridvan Nepravishta[a], Maurizio Paci[a] & Gerry Melino[bc]
[a] Dipartimento di Scienze e Tecnologie Chimiche; University of Rome "Tor Vergata"; Rome, Italy
[b] Medical Research Council (MRC) Toxicology Unit; Leicester, UK
[c] Laboratorio di Biochimica IDI-IRCC c/o Dipartimento di Biochimica e Medicina Sperimentale; University of Rome "Tor Vergata"; Rome, Italy
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p63 threonine phosphorylation signals the interaction with the WW domain of the E3 ligase Itch

Sonia Melino[1,*], Alessia Bellomaria[1], Ridvan Nepravishta[1], Maurizio Paci[1], and Gerry Melino[2,3]

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

Keywords: E3 ubiquitin ligases, Itch, Pin1, proline isomerization, p63, p53 family, ubiquitynation

Abbreviations: CXCR4, chemokine receptor; HECT, Homologous E6-AP Carboxyl Terminus; RHS, Rapp-Hodgkin syndrome; TFE, 2, 2, 2-trifluoroethanol; RP-HPLC, reverse phase high performance chromatography; IPTG, isopropyl-β-D-thiogalactoside; pep63, p63(534–551) peptide; Ppep63, phosphorylated pep63; cPpep63, cyclic phosphorylated pep63; TRAF6, TNF receptor-associated factor 6; TNF, tumor necrosis factor

Both in epithelial development as well as in epithelial cancers, the p53 family member p63 plays a crucial role acting as a master transcriptional regulator. P63 steady state protein levels are regulated by the E3 ubiquitin ligase Itch, via a physical interaction between the PPxY consensus sequence (*PY* motif) of p63 and one of the 4 WW domains of Itch; this substrate recognition process leads to protein-ubiquitylation and p63 proteasomal degradation. The interaction of the WW domains, a highly compact protein-protein binding module, with the short proline-rich sequences is therefore a crucial regulatory event that may offer innovative potential therapeutic opportunity. Previous molecular studies on the Itch-p63 recognition have been performed *in vitro* using the Itch-WW2 domain and the peptide interacting fragment of p63 (pep63), which includes the *PY* motif. Itch-WW2-pep63 interaction is also stabilized *in vitro* by the conformational constriction of the S-S cyclization in the p63 peptide. The *PY* motif of p63, as also for other proteins, is characterized by the nearby presence of a *(T/S)P* motif, which is a potential recognition site of the WW domain of the IV group present in the prolyl-isomerase Pin1. In this study, we demonstrate, by *in silico* and spectroscopical studies using both the linear pep63 and its cyclic form, that the threonine phosphorylation of the *(T/S)PPPxY* motif may represent a crucial regulatory event of the Itch-mediated p63 ubiquitylation, increasing the Itch-WW domains-p63 recognition event and stabilizing *in vivo* the Itch-WW-p63 complex. Moreover, our studies confirm that the subsequently *trans/cis* proline isomerization of *(T/S)P* motif by the Pin1 prolyl-isomerase, could modulate the E3-ligase interaction, and that the $(T/S)_pP_{trans}PPxY$ motif represent the best conformer for the ItchWW-*(T/S)PPPxY* motif recognition.

Introduction

Protein ubiquitylation is a post-translational modification leading to either target protein degradation through the proteasome or activation of the target protein via conformational changes, which depends on the ubiquitin linkage.[1-3] Ubiquitin E3 ligases, for their ability to accept the charged ubiquitin from the E2 and directly transfers it to a protein substrate, play an essential role in the protein ubiquitylation and, recently, their relevance has been recognized in the pathogenesis of a significant number of human diseases.[4,5] Itch is a crucial member of HECT (Homologous to E6-AP carboxyl terminus) ubiquitin E3 ligases [6] that impairs TNF-induced NF-κB activation by facilitating A20-mediated ubiquitylation and degradation of the adaptor protein RIP, in the TNF receptor complex in T cells [7] and macrophages.[8] Accordingly, patients with Itch mutations have autoimmune inflammatory cell infiltration in various tissues.[9] Itch is also required for negative regulation of TNF- and lipopolysaccharide (LPS)-mediated TNF receptor-associated factor 6 (TRAF6) ubiquitylation induced RING finger protein 11[10] and represent a negative regulator of osteoclastogenesis by promoting de-ubiquitylation of TRAF6.[11] Moreover, like other HECTs, Itch is also deregulated in cancer development and represents a potential target for anticancer treatment.[3,12] Since Itch is able to regulate chemosensitivity,[13] we attempted to develop specific inhibitors. Indeed, a recent high throughput screening for Itch inhibitors has identified compounds that regulate chemo-sensitivity by regulating autophagy.[14] Relatively few studies have been performed to identify E3 inhibitors, due to the intrinsic difficulties of the reaction involved, that is, the complexity of the protein components involved in the reaction makes the identification of specific E3 ubiquitin ligase inhibitors challenging in comparison

*Correspondence to: Sonia Melino; Email: melinos@uniroma2.it
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to the identification of inhibitors of other classes of enzymes such as kinases or proteases.[15] Much of the research in the quest for E3 inhibitors has been finalized for the p53-HDM2 pathway since p53 is an extremely powerful transcription factor crucial in DNA damage response.[16-21] Here, the cellular defense to DNA damage is based on sensors and effectors that activates the cell death pathway,[22-24] via p53 [25-29] or its family members.[30-37] Like for our recent Itch inhibitor screening,[14] we believe that in a near future innovative E3 inhibitors will be developed. Here, however, we adopted a different approach to inhibit their function, based on a deeper understanding of the interaction of Itch with its substrate.

The substrate recognition and specificity of the HECTs is depending on specific protein-protein interaction domains, that account for their classifications in (i) HERC-RCC1-like domains (RLDs)-HECTs, (ii) SI(ngle)-HECTs or (iii) C2-WW-HECTs that have a tryptophan-tryptophan (WW) domain. Itch, indeed, recognizes its substrates via a specific WW domain. In the particular, Itch is able to regulate the ubiquitin-mediated proteasomal degradation of several proteins important in cancer, such as: c-Jun, JunB, Notch1, PLC-γ1, Notch1, Thioredoxin, tBid, Smad2, TIEG1, c-Flip-L, ErbB4, RASSF5, LATS1, LAPTM5, CXCR4, TRPV4, A20, itch, p73 and p63, reported in refs. 3,6,14,38. We have previously investigated the molecular mechanism of Itch recognition for the p63 transcription factor [39] in particular, because this powerful transcription factor is crucial for the development of epithelia,[31,40-45] it is involved in cancer,[46-59] and when it is mutated it causes severe genetic diseases.[60-62] While Itch is basically ubiquitous, the expression of the p53 family members, that is p63 and p73,[63-68] is strictly tissue- and cell-specific; moreover several disorders known as ectodermal dysplasias [12,69-75] as well as cancer show a deregulated expression levels of p63 and p73.[39,76] Physiologically, p63 and p73 protein levels are normally kept under strict control through Itch-mediated ubiquitylation, and indeed both p63 and p73 have a canonical PY motif (absent in p53 which is regulated by a different E3 ligase) located in their C-terminal which accounts for the binding to Itch through its WW domains. Both Itch and WWP1 target both isoforms of p63 for ubiquitin-mediated proteasomal degradation.[3]

The WW domains, comprising circa 35 amino acids, are not highly stable and slightly bended 3-stranded β-sheets with a high aromatic content, including several conserved tryptophan residues.[77-79] WW domains, widely present in all phyla, are present as single or multiple copies in several proteins with different functions, including regulation of transcription, RNA processing, ubiquitin ligation, protein trafficking and receptor signaling.[80-82] The WW domains physically interact with short proline-rich sequences and recently, it has been shown that this WW/PY interaction may also be regulated by tyrosine phosphorylation. According to their ligand binding preferences, the WW domains are in fact grouped into 4 classes: group I binds polypeptides with the core consensus PPxY (*PY* motif), where '*x*' can be any residue; group II recognizes the sequence PPLP (*PL* motif); group III selects for proline-rich sequences with arginine residues (*PR* motif); and group IV interacts with phospho(serine/threonine)-proline containing peptides (*p(S/T)P* motif), reviewed in refs 83–86. In the group I WW domains, the first conserved W residue, in concert with an exposed tyrosine residue, forms a concave hydrophobic binding surface for the first 2 proline residues in the *PPxY* motif of the ligand that packs against the W and Y residue forming a hydrophobic buckle that likely maintains the stable folded structure of the domain. Itch, a group I WW domain protein, and p63 interact with each other through the Itch-WW2 domain and the PPPY sequence (residues 540–543), which is the only *PPxY* motif in the p63 protein. Although the interaction among the WW2 domain of Itch and the *PPxY* motif of p63 is crucial for p63 degradation,[87,88] more detailed analysis of this interaction is lacking. This could be of relevance for its potential druggability. Previous molecular studies on the Itch-p63 recognition performed *in vitro* using the WW2 domain and the fragment pep63, which includes the PY motif, show that Itch-WW2-pep63 interaction is stabilized *in vitro* by the conformational constriction due to S-S cyclization of p63 peptide. The *PY* motif of p63, similarly to that in p73, is characterized by the close presence of *(T/S)P* motif, which is a potential recognition site of the WW domain of the IV group present in the prolyl-isomerase, Pin1,[89-92] a crucial regulator of p53 function.[93] In this study we have demonstrated that the phosphorylation of the threonine residue and proline *cis/trans* isomerization by prolyl-isomerase, Pin1, of the *(T/S)P motif* may represent molecular regulatory events of p63 ubiquitylation Itch-mediated, leading to an increase of the stabilization of the WW-p63 complex.

Results and Discussion

Phosphorylation of (T/S)P motif enhances the recognition of Itch-WW domains with the pep63

Early events of the physical recognition and interaction of p63 with the E3 ligase Itch, for its proteasomal degradation, seems to occur between the PPxY motif of p63 and WW2 domain of Itch.[94-97] In order to understand the biochemical properties of the interaction between these 2 proteins, the interaction between the Itch-WW2 and the synthetic peptide pep63, including the PPxY recognition motif, has been monitored using spectroscopic techniques; the apparent dissociation constant value of the Itch-WW2–pep63 complex has been reported previously.[96] The presence of the consensus TP motif, close to the PPxY recognition motif, and also the recent studies on the Pin1/p63 interaction [98,99] led us to evaluate the effect of the threonine phosphorylation of the (T/S)PPPxY motif on the binding of the Itch-WW domains to the pep63 peptide. The measured apparent dissociation constants of Itch-WW2 with both pep63 and the phosphorylated Thr form Ppep63 were obtained from the intrinsic fluorescence of the domain upon addition of increasing amounts of the peptides, resulting in a value of 42.09 ± 7.45 μM (for the WW2-pep63), which is in keeping with our previously reported value,[96] and 10.97 ± 1.45 μM for the Itch-WW2–Ppep63 (**Fig. 1A**). Molecular docking studies between a structural model of Itch-WW2 domain and p(T/S)PPPxY motif have been also performed in order to evaluate the effects of the threonine

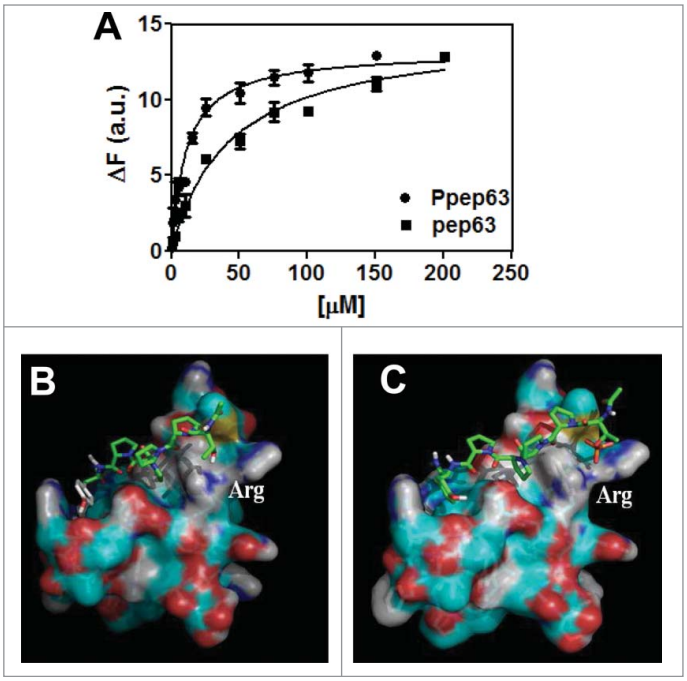

Figure 1. Itch-WW2 domain recognition of the phosphorylated form of pep63. (**A**) Interactions of Itch-WW2 with pep63 and Ppep63 were experimentally monitored by fluorescence spectroscopy. Intrinsic fluorescence changes of Itch-WW2 (5.0 μM) at the increase of pep63 (■) and Ppep63 (●) concentration. The quenching of the emission band of Itch-WW2 using an 280 nm λ_{ex} and 330 nm λ_{em} with a slit of 5 nm in 10 mM potassium phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, pH 6.0, at 37°C. Molecular Docking of the (**B**) TPPPPY or of the (**C**) TpPPPPY peptide with the WW2 domain model. The phosphate group is shown in orange. The 3D structures were obtained by molecular replacement as described in the materials and methods.

phosphorylation on the Itch-WW-PPxY motif binding. **Figure 1B, C** shows both the Itch-WW-TPPPPY complexes with and without phosphorylation at the Thr residue. These computerised model simulations are compatible with the above experimental data and suggest that the observed increase in the binding could be due to the electrostatic interaction between the phosphate group of the motif and the Arg residue close to the binding pocket of the WW domain.

The first 2 WW domains of Itch equally bind the Ppep63

Since the relevance of the second Itch-WW domain in the Itch recognition has been highlighted for both p73 and p63 proteins,[94,96,100] here we have analyzed the interaction of the Itch-WW1 domain with the phosphorylated form of pep63 by fluorescence spectroscopy, in order to compare the effects of the phosphorylation of *(T/S)PPPxY* motif on the recognition of the Itch-WW domains. Itch-WW1 domain, consisting of 47 residues, was expressed and purified as described in experimental materials (**Fig. 2A, B**) and the correct folding of the Itch-WW1 domain has been confirmed by CD spectra.[101] As shown in **Figure 2C**, its far-UV CD spectrum with a maximum negative signal at ~207 nm, was characteristic of a protein with a predominant β sheet secondary structure, in agreement with the conserved 3-stranded β-fold reported for the WW domains.[83,84,102] The dissociation constant of the complexes formed by the Itch-WW1 domain and pep63 was determined quantitatively by following the changes of the intrinsic fluorescence of the tryptophan residues in the domain upon addition of increasing amounts of the peptide. The intrinsic fluorescence of Itch-WW1 was quenched by the addition of the Ppep63 and in a concentration-dependent manner, see **Figure 3A**. The binding profiles of ΔF *vs* [Ppeptide] show a hyperbolic shape, allowing us to assume the formation of a 1:1 complex, considering the good quality of the fitting obtained using an equation for one-binding site and the Scatchard plot (data not shown). The apparent dissociation constant value, obtained by fitting of the titration binding curve, was 5.1 ± 0.5 μM.

The interaction and the effects on the secondary structures were also evaluated by CD spectroscopy; the data show conformational changes induced by the binding of Ppep63 to the protein. In **Figure 3B**, the CD spectra of the Itch-WW1 domain are shown in the presence of equimolar concentration of Ppep63 and, indeed, the arithmetic sum of Itch-WW1 domain and Ppep63 spectra does not account for the CD spectrum generated by the Itch-WW1-Ppep63 complex. Also in this case, as previously obtained for the interaction of the Itch-WW2-pep63,[96] these changes in the dichroic spectrum strongly indicates that relevant changes in the conformation of the molecules are induced upon binding. These changes, in fact, could be due to a different local conformation of Ppep63 induced by the Itch-WW binding. Notably, the partial (or unstable) fold of the WW domains in the absence of the ligands, similar to what has been described for WWP1 with the Nogo-A peptide,[103-106] undergoes a significant conformational transitions to become quite structured upon binding to the target peptides. Although, the Itch-WW1 domain shows a stable fold, as also observed in the case of Itch-WW2,[96] it is not possible to fully exclude that small conformational arrangements of the more flexible regions of the domain also occur after the binding to the peptide.

Pro cis/trans isomerization of TP motif modulates the Itch-WW-pep63 recognition

Pin1 isomerase catalyzes interconversion of proline isomers to restore *cis-trans* equilibrium at a biologically relevant timescale.[107] Regulation of the proline "conformational switch" due to peptidil prolyl-isomerases rules over the duration and amplitude of a variety of cellular processes.[107] As reported above, *p(T/S)P* motif is a characteristic recognition binding motif of the WW domain of Pin1 proline isomerase and recent data have shown a catalytic action of Pin1 on p63.[99] Therefore we have investigated the effect of the *cis/trans* isomerization of the first proline on the binding of the Itch-WW–*p(T/S)PPPxY* motif. Firstly, the binding between Itch-WW2 and Ppep63, following a previous incubation of the Ppep63 with Pin1 aimed at creating a proline *cis/trans* isomerization, has been analyzed by fluorescence spectroscopy (**Fig. 4A**). The Itch-WW-Ppep63 binding was analyzed by measuring the variation of the intrinsic fluorescence of the Itch-WW2 at different concentrations of Ppep63 in solution,

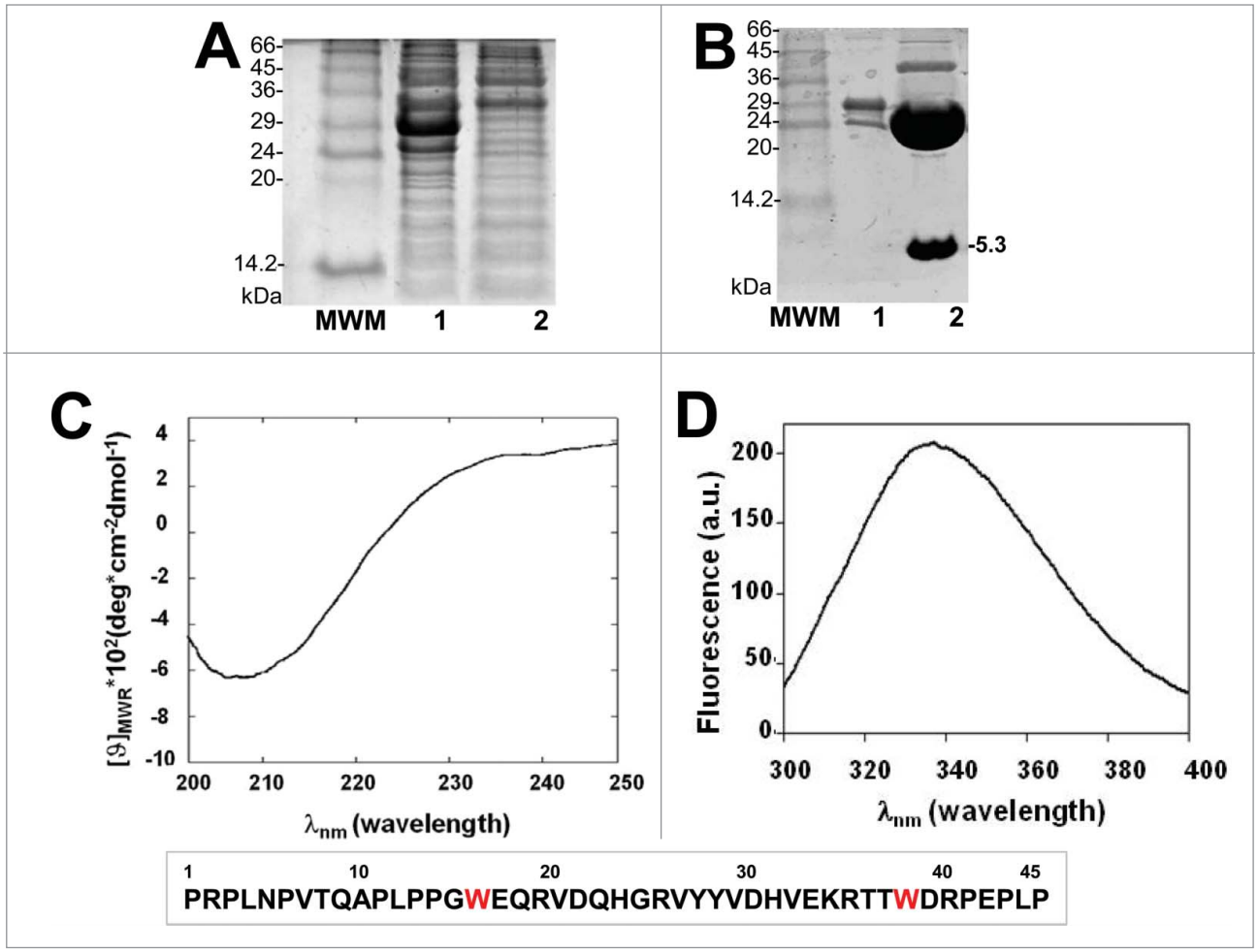

Figure 2. Production and characterization of the recombinant Itch-WW1 domain. (**A**) Expression of the recombinant GST-Itch-WW1 (29.7 kDa), SDS-PAGE 15% gel acrylamide before (lane 2) and after induction with 1 mM IPTG (lane 1); (**B**) purified Itch-WW1 before (lane 1) and after (lane 2) cleavage from GST (5.7 kDa); (**C**) Far-UV CD spectrum (50 μM) and (**D**) intrinsic fluorescence spectrum of the Itch-WW1 domain (5 μM), in 10 mM phosphate buffer, pH 7.0, with 0.15 M NaCl.

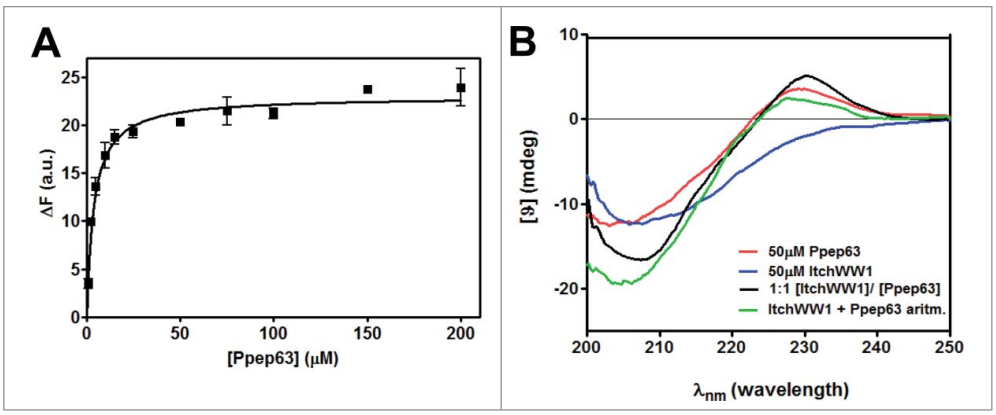

Figure 3. Interaction of Ppep63 peptide with Itch-WW1 domain. (**A**) Intrinsic fluorescence changes of Itch-WW1 (5 μM) at the increase of P-pep63 concentration. The quenching of the emission band of Itch-WW1 using an 280 nm λ_{ex} and 330 nm λ_{em} in 10 mM potassium phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, pH 6.0, at 37°C; (**B**) Far-UV spectra of 50 μM ItchWW1 (blue), of 50 μM Ppep63 (red), of the mixture Itch-WW1/Ppep63 in a molar ratio 1:1 (black) and of the arithmetic sum of the spectra of the single components (green). All CD spectra were acquired in 10 mM potassium phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, pH 6.0, at 37°C, using a 0.1 cm path length quartz cell.

without or with 2 h of pre-incubation of the peptide with Pin1 at 37°C. The results shown in **Figure 4A** indicate that the pre-incubation of the Ppep63 with Pin1 increases the interaction of the peptide with Itch-WW2, decreasing the apparent K_d from 10.09 ± 0.9 to 4.4 ± 0.5 μM.

The molecular docking between the model of the Itch-WW2 domain structure and *p(T/S) PPPxY* motif was also performed using both the proline (*cis/trans*) isomers of the motif (**Fig. 4B** and **C**). As shown in the **Figure 4B** and **C**, the proline *trans* isomerization of the TPPPPY peptide, in both phosphorylated and not phosphorylated peptide cases,

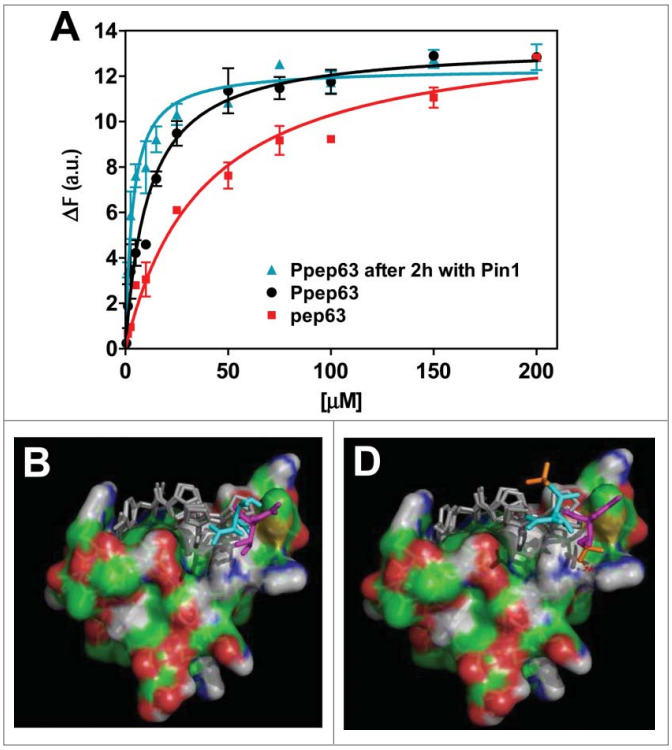

Figure 4. Proline *cis/trans* isomerization of *(T/S)PPPxY* recognition motif with the WW2-Itch domain. Intrinsic fluorescence changes (**A**) of Itch-WW2 (5 μM) at the increase of pep63 (—) and Ppep63 concentrations, after 0 (—) and 2 h (—) incubation of the Ppep63 in the presence of Pin1 enzyme. The quenching of the emission band of Itch-WW2 using an 280 nm λ_{ex} and 330 nm λ_{em} with a slit of 5 nm in 10 mM potassium phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, pH 6.0, at 37°C. Molecular Docking of the (**B**) $TP_{cis}PPPY$ (cyan) and $TP_{trans}PPPY$ (magenta) peptide and (**C**) $T_pP_{cis}PPPY$ (cyan) and $T_pP_{trans}PPPY$ (magenta) with the WW2-Itch domain. In orange is shown the phosphate group.

leads to an increase of the Itch-WW-TPPPPY binding with a decrease of the apparent K_d constants. The presence of the Arg residue close to the binding pocket of the WW domain can increase the molecular recognition and stabilize the complex. In the molecular docking, the proline *trans* isomerization draws up the phosphate group of the T_pPPPPY to 2.8 Å from the side chain of the Arg residue of the WW domain. This event can increase the binding leading to a decrease of the calculated apparent K_d from 177.04 to 39.54 μM, according to the experimental results. Altogether, the *in vitro* and *in silico* data suggest that the *(T/S)$_p$P$_{trans}$PPxY* phosphorylated conformation is the most probable form of the Itch recognition site in p63 protein.

Effect of the phosphorylation and the cis/trans isomerization in the Itch-WW-cyclic pep63 binding

The cyclic form of the Ppep63 (cPpep63) was obtained and characterized as described in previous work (**Fig. 5A**).[97] The intrinsic fluorescence changes of both WW1 and WW2 domains of the Itch protein were investigated by addition of the phosphorylated form of the cyclic pep63, in order to evaluate the effects of the phosphorylation on the molecular recognition and stabilization of the ItchWW-cPpep63 complex. **Figure 5B** shows the ΔFs of the maximal values of the intrinsic fluorescence of both Itch-WW1 and -WW2 at increased concentrations of the cPpep63. The observed apparent dissociation constants for Itch-WW1- and WW2-cPpep63 complexes were 2.1 ± 0.4 and 3.5 ± 0.6 μM respectively. In agreement with the results obtained for the linear form of pep63, the Thr phosphorylation increases the formation of the complex of the cyclic peptide with Itch-WW2, leading the K_d from 13.5 ± 0.8 [97] to 3.5 ± 0.6 μM. Moreover, the cyclization and the phosphorylation of the pep63 lead to an increase of about 12 folds in the binding of Itch-WW2 to pep63, with a decrease of the apparent K_d from 43.0 ± 1 to 3.5 ± 0.6 μM. The effect of the *cis/trans* proline isomerization on the Itch-WW2-cPpep63 binding was also investigated by pre-incubation of cPpep63 with Pin1 before interaction with the WW domain (**Fig. 5C**). After 2 h of pre-incubation of cPpep63 with Pin1 not significant changes were observed in the apparent dissociation constant. This result can be explained considering that the cyclic form of the pep63, as described for many other cyclic peptides containing proline residues, could preferentially assume, for the steric effects, the *trans* configuration in the proline residue of the *TP* motif. Moreover, our preliminary NMR studies indicated that the linear form of Ppep63 is characterized by the presence of about 20% of *cis* proline isomers. These results are in agreement, with other studies that showed the *trans* form is more favored than the *cis* form in peptides with Ser(P)-Pro motifs, with populations of *cis* isomer of 12–20% depending on the adjacent residues.[108,109] Thus, altogether these results may be explained with an increase of the *trans* proline isomers of Ppep63 catalyzed by the Pin1 isomerase during the pre-incubation. The Arg-373 residue of Itch E3-ligase is involved in the interaction with pep63 [97] and as previously observed it goes from fast to slow exchange regime when interacts with pep63 and pep63 cyclic form, respectively. This evidence seems to be in agreement with a *trans* proline conformer of the cyclic form and explain the absence of K_d variation between cPpep63 and both the WW domains after pre-incubation with Pin1.

Therefore, in keeping with very recent studies,[99] the Thr phosphorylation and the action of Pin1 on p63 directly regulates the physical recognition and interaction of Itch, similar to what has been described in the case of WWP1,[98,99,110] affecting the steady-state levels of the p63 protein. This could be also explained with a proline *trans* to *cis* isomerization of the *p(T/S)P* motif by Pin1.

Conclusions

The presence of the conserved Arg (the Arg-373 in the Itch-WW2) (**Fig. 6**, http://www.ebi.ac.uk/Tools/msa/clustalw2/), close to the binding pocket of WW domains (**Fig. 1B** and **C**), seems to be a relevant determinant for the regulation of the WW interaction and phosphorylation. Clearly, the data shown in **Figures 1** and **4** indicate a different interactivity of the recognition domain which is depending on the phosphorylation status. Based

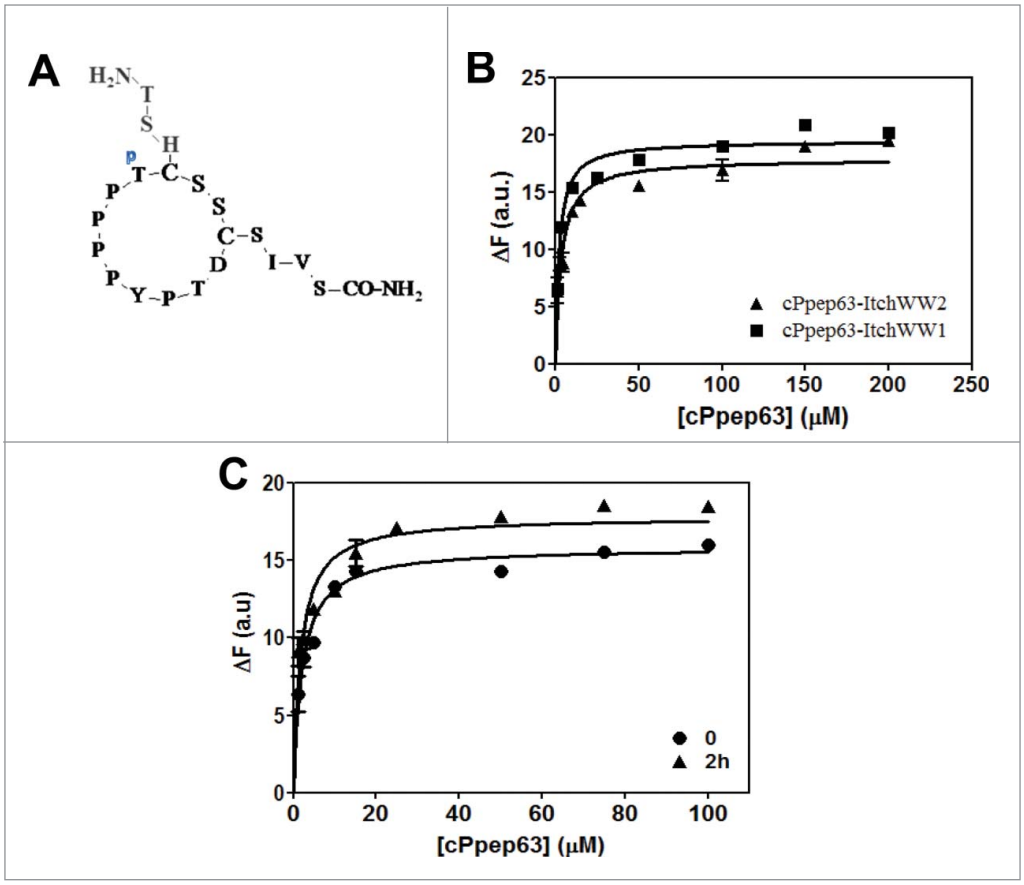

Figure 5. Interactions of Itch-WW domains with cyclic P-pep63 monitored by fluorescence. (**A**) Schematic representation of the cyclic form of Ppep63. (**B**) Intrinsic fluorescence changes of 5 μM Itch-WW1 (■) or Itch-WW2 (▲) at the increase of cyclic Ppep63 concentration and (**C**) of 5 μM Itch-WW1 at the increase of cyclic Ppep63 concentration after 0 (●) and 2 h (▲) of incubation of the cyclic peptide with Pin1 enzyme. The quenching of the emission band of Itch-WW domains using an 280 nm λ_{ex} and 330 nm λ_{em} with a slit of 5 nm in 10 mM potassium phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, pH 6.0, at 37°C.

on these result, we propose that the phosphorylation of p63 in the *(T/S)PPPxY* recognition motif dictates the interactivity with the WW domain of Itch, and therefore with the proteasomal degradation of p63 itself, while the prolyl isomerization by Pin1, affecting the interaction with the WW domain, diverts the pathway toward a stabilization of the p63 steady state levels. **Figure** 7 shows a schematic representation of the functionality of the *(T/S)PPPxY* recognition motif with the WW domain. Indeed, in this schematic potential diagram of the events occurring during the protein-protein interactions, (i) the Thr phosphorylation status of p63 seems to promote the interaction with both proteins containing WW domains, Itch and Pin1, and (ii) the catalytic action of Pin1 may result in a differential availability of p63 to the degradative interaction with Itch. The modulation of the p63-Itch binding by phosphorylation could be a mechanism more widespread and regulate also the interaction of Itch-E3 ligase with other proteins, such as observed in the interaction of Itch with the chemokine receptor CXCR4.[111] This last one occurs through WW-Itch domains and a phosphorylated serine residue of a recognition sequence that does not contain proline residues.[111]

The scheme in **Figure** 7 can be generalized for other PY interactions with WW domains, as the *PY* motif of p63, is also present in other proteins including for example RASSF5 where the PPxY motif interact with the WW domain of Itch[112] in a manner very similar to p63. All these proteins are characterized by the nearby presence of a *(T/S)P* motif, which is a potential recognition site of the WW domain of the IV group present in the prolyl-isomerase Pin1. **Table 1** shows that the *(T/S)PPPxY* recognition motif is present in different crucial cellular proteins, which stability and functional interaction with specific WW domains could affect the fate of the cell. Therefore, this motif could represent a consensus motif for the regulation of E3-ligase- dependent protein-degradation by *Ser/Thr* phosphorylation and Pin1 *trans/cis* proline isomerization.

Finally, the results in **Figure 5** indicate that a cyclic form of the peptide could specifically affect the PY interactions with the WW domains. According to the data shown, the Itch-WW2-pep63 interaction is stabilized *in vitro* by the conformational constriction of the S-S cyclization in the p63 peptide, offering a novel potential therapeutic target able to regulate the fate of the cell. Accordingly, cPpep63 peptide could represent a potential

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Itch-WW1 Q96J02|326-359 APLPPGWEQRVDQHGRVYYVDHVEKRTTWDRPEP
Itch-WW2 Q96J02|358-391 EPLPPGWERRVDNMGRIYYVDHFTRTTTWQRPTL
Itch-WW3 Q96J02|438-471 GPLPPGWEKRTDSNGRVYFVNHNTRITQWEDPRS
WWP1-WW1 Q9H0M0|349-382 ETLPSGWEQRKDPHGRTYYVDHNTRTTTWERPQP
WWP1-WW2 Q9H0M0|381-414 QPLPPGWERRVDDRRRVYYVDHNTRTTTWQRPTM
WWP1-WW3 Q9H0M0|456-489 GPLPPGWEKRVDSTDRVYFVNHNTKTTQWEDPRT
                         .**.***:* *   * *:*:*  : * *: *
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Figure 6. Multiple sequence alignment of the WW domains of Itch and WWP1 E3-ligases. Alignment of the WW1, WW2 and WW3 domains of Itch and WWP1 E3-Ligases involved in the ubiquitylation of p63. The Arg residue corresponding to the Arg-373 in ItchWW2 is shown in blue, and it is conserved in all sequences. The conserved Trp residues, which are characteristics of the WW domains, are in red.

model for the design of competitive inhibitors of Itch-protein recognition.

In conclusion, the data presented show, by *in silico* and spectroscopical studies using both the linear pep63 and its cyclic form, a significant structural difference during the interaction of the different isoforms of the p63 peptide with both the Itch domains, allowing as to predict a differential effect on the steady-state protein levels of p63, regulated by the interaction with the ubiquitin E3 ligase Itch. The threonine phosphorylation of the *(T/S)PPPxY* motif seems to represent a crucial regulatory event of the Itch-mediated p63 ubiquitylation, increasing the Itch-WW domains-p63 recognition event and stabilizing *in vivo* the Itch-WW-p63 complex. Furthermore, the identification of the tissue- specific kinases (and/or phosphatases) could clarify the regulation of p63 degradation, also explaining why different E3 ligases act on p63. Finally, our studies confirm that the subsequently *tran/cis* proline isomerization

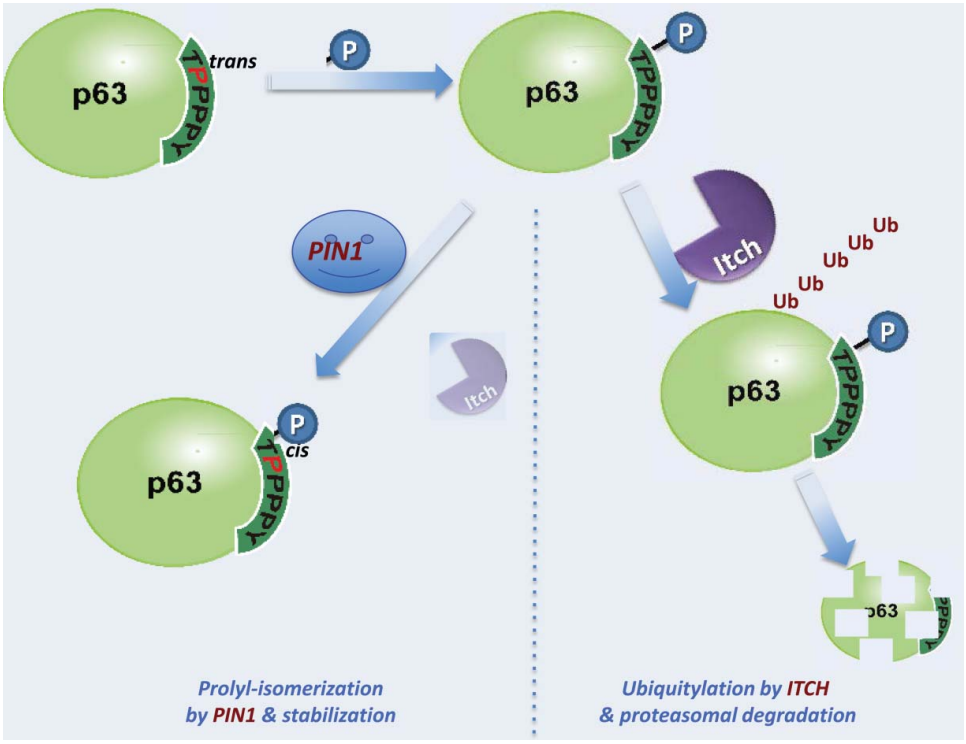

Figure 7. Schematic diagram of the physical interactions between p63 and Itch. Phosphorylation of the *TPPPxY* recognition motif of the p63 protein facilitates the Itch-p63 recognition promoting the ubiquitylation p63.[94,96,97,100,117] This recognition can be modulate by the Pin1 that interacting with the $(T/S)_pP$ motif catalyzes the proline isomerization from *trans* to *cis* configuration of the recognition motif in p63 and decreases the Itch-p63 interaction, resulting in a stabilization of the protein steady state level. Accordingly, the sequence of events is (i) phosphorylation by a kinase, (ii) recognition by Itch, poly-ubiquitylation and proteasomal degradation (right path), (iii) alternatively, Pin1 recognition and *trans-cis* prolyl isomerization of the proline, reducing interaction with Itch, hence stabilizing the steady-state protein levels of p63 (left path).

Table 1. Sequences of the Proteins with the *(T/S)PPPxY* recognition motif

ID code	sequence alignment	protein
Q96AW1	160 ACPPPPAYCN**TPPPPY**EQVVKAK-	**VOPP1/ECOP_HUMAN EGFR**-coamplified and overexpressed protein Homo sapiens
Q9H3D4	538 PLSMPSTSHC**TPPPPY**PTDCSIVSFL	**p63_HUMAN** Tumor protein 63 Homo sapiens
O15350	482 AQSMVSGSHC**TPPPPY**HADPSLVSFL	**p73_HUMAN** Tumor protein 73 Homo sapiens
Q9NX94	117 PYEEVVNRPP**TPPPPY**SAFQLQQQQL	**OPA1L_HUMAN** Outcome predictor in acute leukemia Homo sapiens
Q6UUV7	443 FLPTEAQAQV**SPPPPY**PAPQELTQPLL	**CRTC3_HUMAN CREB**-regulated transcription coactivator 3 Homo sapiens
Q96J86	143 SPTPQGPAQR**SPPPPY**PGNARK	**CYYR1_HUMAN** Cysteine and tyrosine-rich protein 1 Homo sapiens
O43294	13 GYLPPKGYAP**SPPPPY**PVTPGYPEPAL	**PLS3_HUMAN** Phospholipid scramblase 3 Homo sapiens
Q9BZD6	202 AVALTRKHSV**SPPPPY**PGHTKGFRVFK	**TMG4_HUMAN** Transmembrane gamma-carboxyglutamic acid protein 4 Homo sapiens
O43294	33 ERPAEPL**TPPPSY**GHQPQTGSGE	**TGFI1_HUMAN** Transforming growth factor β-1-induced transcript 1 protein Homo sapiens
Q96G27	136 AYEDVVHRPG**TPPPPY**TVAPGRPLTA	**WBP1_HUMAN** WW domain-binding protein 1 Homo sapiens
O88902	857 LFPSQAPGIL**TPPPPY**PFTPQPGVLG	**PTN23_RAT** Tyrosine-protein phosphatase non-receptor type

The table shows the sequences of distinct proteins, with the consensus *(T/S)PPPxY* motif, that are or may be recognized by both group I and IV WW-domains.

of *(T/S)P* motif by the Pin1 prolyl isomerase, could modulate the E3-ligase interaction, and that the $(T/S)_pP_{trans}PPxY$ motif represent the best conformer for the Itch-WW-*(T/S)PPPxY* motif recognition.

Materials and Methods

Peptide synthesis

Synthetic peptides were purchased from Spectra 2000 (Rome, Italy). Analysis of the synthetic peptides by reverse phase high performance chromatography (RP-HPLC) and mass spectrometry revealed a purity >98%. The 2 sequences of the peptides had the following sequences: 18-mer pep63 NH_2- TSHCTPPPPYPTDCSIVS-$CONH_2$ (1901.15 *m/z*) and Ppep63 NH_2- $TSHCT_p$ PPPPYPTDCSIVS-$CONH_2$ (1981.15 *m/z*).

Expression and purification of the Itch-WW1 and -WW2 domains

GST-ItchWW1 and WW2 were overexpressed using *E.coli* BL21 strain in LB medium containing 100 μM /ml ampicillin. Cells were grown at 37°C and the induction of the expression of the proteins were performed by addition of 1 mM IPTG. Cells were grown at 37°C for a further 4 hours, collected by centrifugation and after disrupted by sonication. GST-Itch-WW1 and -WW2 were 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-WW1 and -WW2 domains were cleaved from the GST using the PreScission Protease (GE-Amersham) and purified.

Circular dichroism analysis

CD measurements were performed using a Jasco 710 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a thermal controller calibrated with camphor-sulfonic acid.[101,113-115] Far-UV CD experiments were carried out to explore the conformation of Itch-WW1 domain and the structural variation after addition of different concentrations of Ppep63. CD spectra were obtained between 200 and 250 nm using a path-length of 0.1 cm and between 600–300 nm using a path-length of 1 cm, a time constant of 1.0 s, a 2 nm bandwidth and a scan rate of 2 nm/min and at 20 or 50 mdeg sensitivity. Each spectrum was averaged over 4 scans and subjected to smoothing following subtraction of the buffer background. The measured ellipticity data were converted to mean molar ellipticity per residue ([θ], deg × cm^2 × $dmol^{-1}$).

Fluorescence interaction studies

The interactions of Itch-WW1 and -WW2 with the different forms of pep63 peptide were monitored by quenching of the emission band of Itch-WW domains excited at 280 nm using an λ_{ex} and λ_{em} slit of 5 nm in 20 mM phosphate buffer, pH 6.0, 25 mM KCl, 2 mM DTT at 37°C. Binding constants were determined by fitting the titration curve of fluorescence changes versus equivalents of peptide provides a titration curve. The ΔF was plotted *vs.* molar equivalent of peptide and the following equation was used for generating a Scatchard plot from which binding constants were determined $\Delta F = nK[peptide]\ \Delta Fcomplex/(1 + K[peptide])$

ΔF was the observed fluorescence changing after addition of the 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, San Diego, CA, USA; http://www.graphpad.com).

Before the fluorescence interaction analysis, the pre-incubation of pep63 isoforms with and without Pin1 was performed using 2 mM of peptide, 0.73 μM of Pin1 (specific activity >162 nmoles/min/μg) (PiN3001, ATGen Co Ltd) in 20 mM Tris-HCl buffer, pH 8.0, 5 mM DTT for 2 h at 37°C. The solutions were added at increased concentrations to the WW domain solution in 10 mM phosphate buffer, pH 6.0, buffer, 100 mM NaCl at 37°C. The spectra at time 0 of pre-incubation in the presence of Pin1 were obtained immediately putting the Pin1-peptide solution in ice. The fluorescence of the peptides-Pin1 solutions was subtracted at the respective WW fluorescence's spectra.

Molecular docking procedures

The structure of the Itch-WW2 domain was obtained by homology modeling using pdb file from the protein data bank-code 2 DMv (www.pdb.org). Subsequently, Autodockver 4.2 [115] and AutodockVina version 1.1 algorithms[117] were used for the docking of TPPPY, T_pPPPY peptides and their *trans/cis* proline to the Itch-WW domain. The binding of each peptide to the Itch-WW domain was performed by, using the docking procedure for rigid receptor and flexible or semiflexible ligand based on a knowledge guided Protein Docking as already reported by Lu et al.[118]

All the molecules were prepared for docking using Autodock Tools version1.4.[116] The default settings were used for all other parameters. Algorithms validation was conducted by re-docking native ligands to their receptors. The algorithms are considered valid if the re-docking results have a root square mean deviation (RSMD) less than 2 Å from original structure. After that, the binding energies of peptide to each receptor were calculated. Visualization of the binding site after docking analysis was performed by Autodock Tools version 1.4.[116,117]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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