Role of the peptidyl-prolyl *cis/trans* isomerase Pin1 in the ubiquitin proteasome system (UPS)

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Pin1, a novel switch in the ubiquitin pathway. Jentsch and Siepe, *Cell Cycle*, **8** (23), 2009

Contents

1 Summary

Pin1, a unique and highly conserved eukaryotic peptidyl-prolyl *cis/trans* isomerase (PPIase), plays important roles in cellular regulation. Due to its activity to switch the conformation of peptidyl-proline bonds in polypeptide chains, Pin1 operates as a binary switch - often in fate-determining pathways. Unlike other post-translational modifications, Pin1-mediated *cis/trans* isomerization activity represents a non-covalent post-translational modification and is usually controlled by substrate phosphorylation. However, how Pin1 switches protein activities remains unclear.

This study reveals that yeast Pin1 (Ess1) Pin1 acts as a polyubiquitylation switch and controls the degree of substrate ubiquitylation and thereby protein fates. Pin1 is directly linked to the OLE pathway, which controls Spt23, an NFκB-related transcription factor that is crucial for providing cells with unsaturated fatty acids (Hoppe et al., 2000; Rape et al., 2001; Piwko and Jentsch, 2006). Interestingly, this study reveals that high Pin1 activity results in low ubiquitylation of Spt23, which triggers Spt23 precursor processing and hence transcription factor activation. By contrast, reduced Pin1 activity triggers robust Spt23 precursor polyubiquitylation, subsequently leading to its complete elimination by ERAD. Moreover, Spt23 phosphorylation of S/T-Pro sites is not only an essential prerequisite for Ess1 binding via its WW domain and PPIase activity, this work also shows that binding of Ess1 to Spt23 is stimulated by casein kinase 2 (CK2)-mediated phosphorylation of Spt23, coupling the isomerization activity to the tightly controlled activities of proline-directed kinases (and phosphatases).

Additionally, this study shows that inhibition of Pin1 *in vivo* in mammalian cells changes the ubiquitylation status of the tumor suppressor p53 from mono/oligoubiquitylation, which is known to trigger nuclear export, to polyubiquitylation, which causes nuclear p53 degradation. Furthermore, establishing a p53 *in vitro* ubiquitylation system to assay the direct influence of Pin1 on p53 ubiquitylation reveals that p53 polyubiquitylation is negatively correlated with the amount of Pin1 and that Mdm2 and Pin1 indeed exert opposing effects on p53 polyubiquitylation.

The discovery that Pin1 acts as a fate switch by controlling the degree of substrate ubiquitylation reveals a so far unrecognized role of this PPIase in the ubiquitin proteasome system (UPS). How Pin1 controls ubiquitylation is so far unclear, however, it most likely acts by altering the affinities of certain E3 ligases. This also suggests that the Pin1 toggle may affect the degree of substrate ubiquitylation and therefore fate in other pathways as well.

2 Introduction

2.1 The ubiquitin proteasome system (UPS)

The cellular proteome is in a dynamic state of synthesis and degradation. Intracellular protein half-lives vary from a few minutes to several hours for individual protein species in the same cell. Currently three major intracellular proteolytic systems are found in all eukaryotic cells: The endosomal/lysosomal system, the process of autophagy, which also involves the lysosome, and the ubiquitin proteasome system (UPS). The UPS is the principal non-lysosomal system for degrading cytosolic and nuclear proteins in the cell, serving both regulatory and quality control functions. It is instrumental to almost every aspect of cellular regulation including cell cycle control, immune response, transcriptional regulation and signal transduction. In order to act efficiently in these regulatory processes intracellular protein degradation must be highly specific.

Cellular proteases often recognize substrates *via* sequence-specific motifs located close to the actual cleavage site. Unlike conventional site-specific proteases, the 26S proteasome acts on numerous substrates and has no apparent preference for a specific amino acid sequence in the substrate. In fact to avoid promiscuous protein degradation and to achieve specificity and fidelity in the UPS a specialized system evolved in all eukaryotic systems in which substrates are selected and marked with the small protein ubiquitin. Once modified with several ubiquitin moieties the tagged substrate is destined for degradation and targeted to the 26S proteasome independently of its primary amino acid sequence. At the 26S proteasome, a 2.5 MDa multi-subunit protease with an inner cavity lined with a number of different proteolytic sites, the ubiquitin moieties are trimmed off and the protein is dragged into the barrel shaped cavity and degraded into small peptides (Figure 3). The specific molecular design of the proteasome provides efficient and processive peptide hydrolysis and in addition prevents unwanted degradation by sequestering the active proteolytic sites in the cavity. The covalent attachment of ubiquitin to a substrate constitutes a post-translational modification, which serves various purposes like protein degradation by the UPS or modulation of protein localization or protein function.

2.2 The ubiquitin-conjugating system

Ubiquitin is a small, evolutionary highly conserved polypeptide of 76 amino acids that is abundantly found in all eukaryotic cells. Ubiquitin is covalently conjugated to target proteins via an isopeptide bond between its carboxy-terminal glycine and the epsilon-amino group of lysine residues in substrate protein. In a first step ubiquitin is synthesized as a poly-ubiquitin fusion protein (Ubi4) or as a fusion protein to the N-termini of ribosomal proteins (Ubi1, Ubi2, Ubi3). Thus, ubiquitylation requires the proteolytic processing of the carboxy-terminus of the ubiquitin precursor proteins to its monomeric form that ends with a double glycine motif, a process also called ubiquitin maturation (Rose and Warms, 1983; Pickart and Rose, 1985).

The covalent attachment of ubiquitin to target substrates is orchestrated by an enzymatic cascade of at least three enzymes (Jentsch, 1992, Kerscher et al., 2006): E1 - ubiquitin activating enzyme, E2 - ubiquitin conjugating enzyme and E3 - ubiquitin protein ligase, where the E3 enzyme, which is largely responsible for the specificity of the reaction, associates with the substrate (Fang and Weissman, 2004; Hershko and Ciechanover, 1998; Pickart, 2001). After the initial ATP-dependent activation by an E1 ubiquitin-activating enzyme, the C-terminal carboxy group of ubiquitin is adenylated, this activated ubiquitin-AMP forms a high-energy thioester bond with an active cysteine (Cys) residue in E1, and is then transferred to a specific Cys residue of one of a family of E2 ubiquitin-conjugating enzymes (Ciechanover et al., 1982; Haas et al., 1982, Haas et al., 1983). The E3 ubiquitin-ligase plays then a pivotal role in the ubiquitin-conjugation machinery by recruiting the activated ubiquitin-E2-thioester-linked complex, and simultaneously recognizing specific target proteins. It functions either facilitating/handing over or directly catalyzing the ubiquitin transfer. This finally results in forming a isopeptide bond between the C-terminal carboxy-group in ubiquitin and in most cases the epsilon-amino group of an internal lysine or the N-terminus of their substrates (Hershko et al., 1986) (Figure 1). Once conjugated by this enzymatic cascade ubiquitin itself is often a target for further ubiquitylation reactions by using one of its 7 internal lysine residues and leads to the formation of polyubiquitin chains (Figure 2). Polyubiquitin-chain assembly is often a processive reaction that usually requires E1, E2 and E3. However, recent studies have shown that during chain elongation another class of proteins, the E4 enzymes, can catalyze an extension of the mono/oligoubiquitin chain and acts as an efficient chain elongation factor (Koegl et al., 1999; Hoppe et al., 2005). In some cases the initial

monoubiquitylation orchestered by the action of E1, E2 and E3 converts a substrate into its active state, which is e.g. involved in transcriptional regulation, DNA-Repair, endocytosis or intracellular transport. Inactivation can occur then by E4-dependent ubiquitin chain elongation that finally marks the substrate for subsequent proteasome dependent degradation.

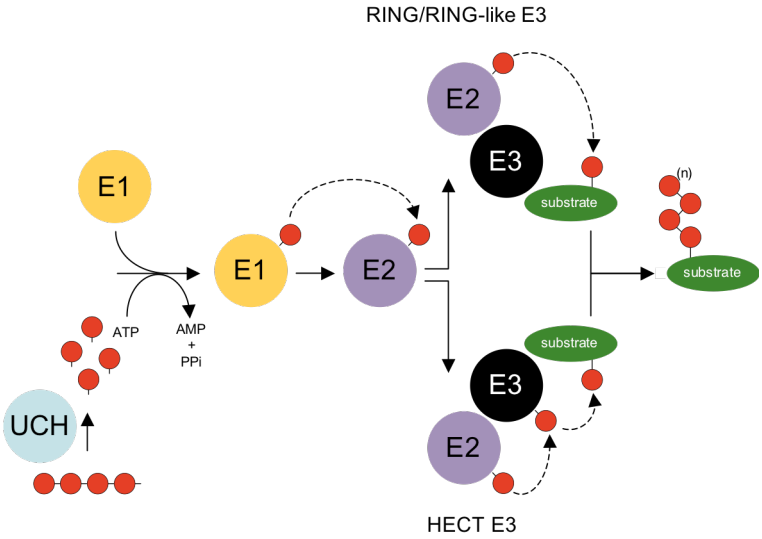

Figure 1. The ubiquitin-conjugation machinery. Schematic overview over the enzymatic cascade catalyzing the ubiquitylation reaction. A protein substrate is conjugated with a single ubiquitin moiety by the concerted action of E1, E2 and E3 enzymes (Initiation). Ub is first activated in an ATP-consuming reaction by an E1-Ub-activating enzyme and subsequently transferred to the active Cys of an E2-conjugating enzyme. With the aid of a third enzyme, called the E3-ubiquitin ligase, E2 catalyzes the transfer of (poly)ubiquitin onto the substrate that is destined for degradation. In some cases monoubiquitylation converts the substrate into its active state, which is involved in transcriptional regulation, DNA-Repair, endocytosis or intracellular transport. Inactivation occurs by E4-dependent ubiquitin chain elongation/assembly that marks the substrate for subsequent 26S dependent degradation.

2.3 E3 ligases determine substrate specificity

Specificity of substrate modification is largely conferred at the final step of the ubiquitylation cascade by the E3 ubiquitin ligase. The complexity of the E3 ligase is manifested by the fact that ubiquitin so far employs one to two E1 activating enzymes, dozens of E2 and numerous E3 enzymes to fulfill the conjugation requirements (Kaiser and Fon, 2007; Jin et al., 2007; Pelzer et al., 2007), unlike most other ubiquitin-like-proteins, which only utilize a single E1, E2, and a few E3 enzymes in the conjugation reaction.

E3 ubiquitin-ligases are generally classified based on the sequence homology of their E2-binding domain into the following two subclasses: the E3 ligases of the RING-type and the HECT (homologous to E6-AP carboxy-terminus) domain-containing E3s. Although unrelated in sequence or structure both enzymes are alike in their ability to establish selective substrate binding.

In the case of RING-E3 mediated catalysis, ubiquitin is directly transferred from the E2 to the substrate. The RING-E3, in most cases a multisubunit complex, functions as an adaptor between the activated E2 and the substrate, in contrast to the HECT domain the RING finger forms no thiol-ester with ubiquitin. A smaller set of E3 enzymes contain a U-box, a degenerate version of the RING-finger, which achieves the same general fold, but without coordinating metal ions (Koegl et al., 1999). HECT-E3s are quite unique among the E3 ligases, due to their intrinsic catalytic activity. In HECT-E3-mediated catalysis, ubiquitin is transferred from the E2 to a cysteine in the HECT-E3 as a thiol-ester conjugate and then transferred to the substrate, which is bound by the HECT-E3 enzyme (Pickart, C.M., 2001) (Figure 1). Substrate specificity of HECT-type E3s is mediated by protein-protein interaction domains. The C2-WW-HECT E3 ligases likely represent the by far best characterized subgroup of HECT ligases, they are highly conserved from yeast to mammals and include the family of Nedd4/Nedd-like ubiquitin ligases (neural-precursor-cell-expressed, developmentally down-regulated). There are nine members in humans, three in *S. pombe* and *D. melanogaster*, but only one Nedd4 homolog in *S. cerevisiae*: the Rsp5 enzyme. Rsp5, which is one of the best studied C2-WW-HECT E3 ligases is essential for viability and plays a crucial role in the regulation of many cellular processes including intracellular trafficking (Dupre et al., 2004; Horak, J., 2003; Staub and Rotin, 2006; Miranda and Sorkin, 2007), mRNA export (Rodriguez et al., 2003) and most intriguing the regulated ubiquitin/proteasome dependent processing (RUP, Hoppe et al., 2000) of the transcription factors Spt23 (and Mga2), both structurally related to NFκB, in the essential OLE pathway (Hoppe et al., 2000; Rape et al., 2001; Piwko and Jentsch, 2006).

The C2-WW-HECT E3s bear a common general modular structure composed of an N-terminal protein kinase C (PKC)-related C2 domain, known to function as a lipid-binding domain, two to four WW protein-interacting domains that recognize proline rich PPxY motifs (Rotin, 1998; Shcherbik et al., 2004), and the catalytic C-terminal HECT domain (Schwarz et al., 1998).

Regulated protein-protein interaction renders the basis for cellular signal transduction, a widely used molecular handle ensuring specificity in these signaling events are phosphate groups that are covalently attached to either tyrosine (pY), serine or threonine (pS/T) residues by site specific kinases. Although phosphorylation is a very common post-translational modification, there is actually only a handful of signaling domains known that recognize phosphorylated proteins with sequence specificity (Yaffe et al., 2002). The WW protein-protein interaction domain is in fact one of these rare examples.

WW domains are found in a variety of different proteins and are known to regulate cellular localization and substrate selectivity. The domain is highly conserved between species and consists of a small module of approximately 35 to 40 amino acids. WW domain-containing proteins have been shown to bind predominantly proline-rich motifs. Based on the recognition motif, they can be classified into four groups: PPxY (where x is any amino acid) (Chen and Sudol, 1995) including Nedd4 and Rsp5, PPLP (Bedford er al., 1997), PR (Bedford et al., 1998, 2000) and most interestingly the group IV WW domains, phosphoserine/threonine residues that precede a proline residue (pS/T-P) (Yaffe et al., 1997, Lu et al. 1999) including the peptidyl-prolyl *cis/trans* isomerase Pin1/Ess1. Interestingly there are certain substrates described that are targets for more than one WW domain binding protein; in fact it was demonstrated that the CTD of RNA Pol II is the target for various WW domain-binding proteins, including the peptidyl-prolyl isomerase Ess1 and the WW-HECT-E3 ligase Rsp5, both essential proteins in *S. cerevisiae* (Wu et al., 2001).

The UPS plays another fundamental role in protein quality control (PQC) by removing proteins that are misfolded and therefore unable to perform their designated functions. In the cytosol this process often seems to involve a specialized subset of molecular chaperones that directly cooperate with the UPS during protein quality control. In mammalian cells defective and aberrantly folded proteins of the cytosol are frequently recognized by Hsp70-Hsp90 chaperones that directly cooperates with CHIP, a chaperone associated E3 ligase to target proteins for ubiquitylation (Esser et al., 2004) that belongs to the U-box containing E3 ligases (Koegl et al., 1999). In contrast to misfolded proteins in the cytosol, proteins, which are translocated into the ER, and fail to fold or assemble properly, are subject to a proteolytic quality control pathway, termed "ER-associated degradation" or ERAD. The spatial separation between substrate selection and degradation in ERAD

requires substrate transport from the ER to the cytosol by a process termed dislocation where they are ultimately degraded by the cytosolic UPS (Sommer and Jentsch, 1993; Meusser et al., 2005). Interestingly, ERAD also requires the activity of the ubiquitin-selective segregase $Cdc48^{Ufd1/Npl4}$ (Braun et al., 2002), suggesting that the retrograde transport of short-lived substrates might be driven through the action of the AAA-ATPase $Cdc48^{Ufd1/Npl4}$ in conjunction with the proteasome.

2.4 Regulation of ubiquitin conjugation

Understanding the crosstalk between different types of post-translational modifications is fundamental in delineating complex cellular signaling cascades. Regulation of a protein's function through a single modification is possible either through creating a new protein binding site, by abolishing protein protein interaction, or through allosteric effects. However, in many cases proteins are modified by multiple post-translational modifications. Hence a significant increase in informational content would be acquired if the different post-translational modifications would act in combination to serve as three-dimensional signal surfaces. Considering chromatin-based information, the interplay of covalent post-translational histone modifications are eminent examples (Bhaumik et al., 2007; Probst et al., 2009). Particularly prominent are the numerous connections between phosphorylation and ubiquitylation, which can either act positively or negatively in both directions to regulate cellular processes

The phosphorylation of serine/threonine (pS/T) or tyrosine (pY) residues is a dynamic and effective way to change protein fates and was also demonstrated to be a key in determining the interactions between E3 ligases and their substrates. It is therefore tempting to speculate that phosphorylation events not only play a role in substrate recognition but also in integrating multiple post-translational modifications into a signaling network. In fact the *S. cerevisiae* G2 cyclins Clb2, Clb3, or the Cdk inhibitor Sic1 are examples where phosphorylation is a prerequisite for subsequent substrate ubiquitylation, leading to a signal for the interaction with the corresponding SCF type E3 ligase (Willems et al., 2004). Activation of the "classical" NF-κB pathway for instance involves several phosphorylation and ubiquitylation events and converges on the IκB kinase (IKK) signalosome.

E3 ligase substrate recognition can as well be inhibited as a consequence of a post-phosphorylational event; e.g. binding of the E3 ligase Mdm2 to p53 is inhibited by phosphorylation of Ser15/Thr18 in direct response to genotoxic stress, which subsequently results in the stabilization of p53 (Clegg et al., 2008). In contrast to substrate phosphorylation, also E3 ligases themselves can be modified by phosphorylation and therefore change the affinity to bind a substrate, e.g. phosphorylation of the APC/C subunit cdc20 by protein kinase A (PKA; Searle et al., 2004). Furthermore, ubiquitylation can also be regulated by phosphorylation-mediated subcellular localization of substrates. In principle, phosphorylation can regulate access of an E3 ligase to its substrate through phosphorylation-dependent transport of either the target or the ligase between cellular compartments. p53 for instance, which is phosphorylated upon genotoxic stress, translocates to the nucleus to activate downstream targets (like p21) as an active transcription factor and can be polyubiquitylated by the E3 ligase Mdm2 and degraded by the proteasome in the nucleus. Furthermore monoubiquitylated p53 is a signal for nuclear export, a reaction carried out by the same E3 ligase Mdm2 (Li et al., 2003). Finally rather than phosphorylation regulates ubiquitylation, also protein kinase activity itself is regulated by ubiquitylation (Chen, Z.J., 2005).

In most cases it still remains unclear how phosphorylation triggers substrate E3 interaction and how this signal is translated into different cellular fates. Phosphorylation may change the substrate's conformation, or create specific binding surfaces to allow binding of auxiliary factors. Phosphorylation might also change the accessibility of certain lysines for ubiquitylation or change the composition of polyubiquitin chain linkage. Furthermore, post-translational modifications of the substrate might lead to a change of E3 affinities, which may be important for some E3 ligases that do not seem to act processively, like yeast Rsp5 or Mdm2. Non-processive E3s might need a higher affinity or a chain elongation factor (E4) for assembling a polyubiquitin chain and this could therefore be utilized as a mechanism for positively or negatively regulating the subsequent fate of the substrate by either complete degradation or proteasomal processing.

A key feature of the UPS is that ubiquitylation (analogous to phosphorylation) is a reversible modification. Ubiquitin can be specifically and efficiently removed from the substrate by a class of proteins termed deubiquitylating enzymes (DUBs; Amerik and Hochstrasser., 2004). This activity serves to switch off ubiquitin signals or to shift between different modifications on the same internal lysine of ubiquitin (Hershko and

Chiechanover, 1998; Newton et al., 2008). The regulation of substrate ubiquitylation *via* DUBs was interestingly shown for the active transcription factor Spt23 (p90). The NFκB-related transcription factor is a substrate for the deubiquitylating enzyme of the ovarian tumor family protein Otu1, which antagonized the HECT E3 ligase Rsp5 and the E4 enzyme Ufd2, respectively (Rumpf and Jentsch, 2006).

2.5 Ubiquitylation, linkage and substrate fate

The fate of ubiquitylated proteins is determined by the type of ubiquitin modification, the lysine used for polyubiquitin chain formation and furthermore the length of a polyubiquitin chain. Recently, mass spectrometry experiments indicated that basically all seven internal lysines in ubiquitin can be used for forming polyubiquitin chains. Based on semi-quantitative data K48 linked ubiquitin chains seem to be most abundant, followed by K11, K63, K6, K27, K29 and K33 (Xu and Peng, 2006). Additionally, ubiquitin can also be attached to its N-terminus in a head-to-tail fashion (Peng et al., 2003; Kirisako et al., 2006; Tokunaga et al., 2009). Newer studies based on absolute quantification of ubiquitin (Ub-AQUA) now show that the most prevalent linkage types in whole cell yeast extracts are K48-linked and K63-linked chains, approximately equally abundant, followed by K29 (Matiuhin et al., 2008). Whereas there is also data suggesting mixed linkages in one chain resulting in so called „forked“ or „barbed“ ubiquitin chain formation (Peng et al., 2003; Tagwerker et al., 2006). Remarkably, some auxiliary substrate processing factors are able to directly influence the degree of ubiquitylation; the E4 processivity factor Ufd2 for example seems to switch the type of ubiquitin linkage of a ubiquitin chain from K29-linked to K48-linked ubiquitin (Koegl et al. 1999; Saeki et al., 2004).

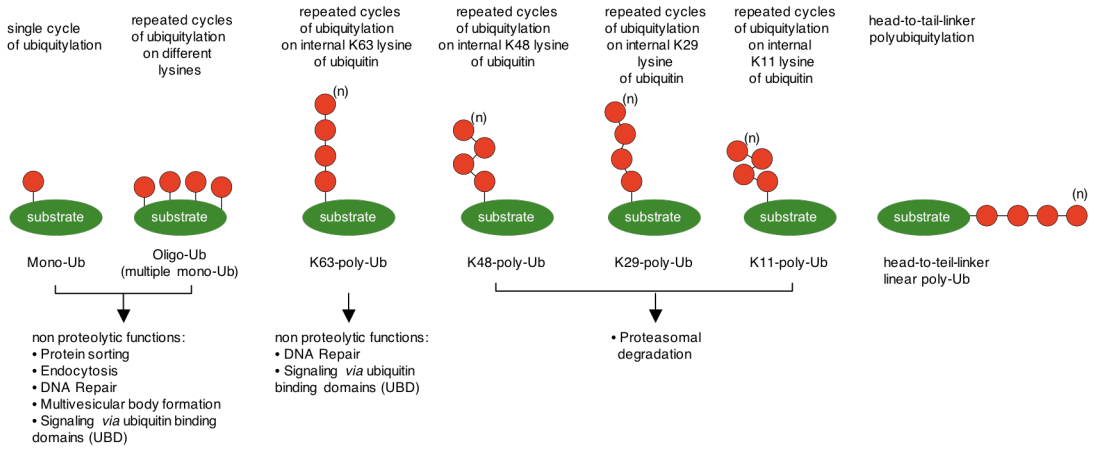

Figure 2. Linkage and fate of ubiquitylated substrates. Different ubiquitin modifications have distinct functions, substrates can be modified with a single ubiquitin (mono-Ub), multiple single ubiquitins or with ubiquitin chains (poly-Ub) or short. Ubiquitin chains differ in structure and function, depending on what lysine of ubiquitin is used for chain formation.

The type of ubiquitin modification, mono-, oligo-, or polyubiquitylation with different kinds of linkage formation, determines different fates of the substrate in a cellular context (Figure 2). Hence, one feature of the UPS is the need and the capability to recruit or recognize polyubiquitylated substrates. Once modified with a K48-linked polyubiquitin chain of at least four ubiquitins (Thrower et al., 2000), the substrate can either bind to intrinsic ubiquitin receptors in the 19S regulatory subunit of the proteasome in a direct way through Rpn10 and Rpn13 or to a ubiquitin adaptor factor that mediates the binding between the polyubiquitylated substrate and the proteasome, acting as a ubiquitin adaptor receptor (Rad23, Dsk2, and Ddi1) (Welchman et al., 2005; Matiuhin et al., 2008). In fact a recent report suggests that for most substrates the transfer to the proteasome depends on the sequential action of ubiquitin binding factors (Rad23, Dsk2) that work in a hand-over mechanism to escort the ubiquitylated substrate on its way to the 26S (Richly et al., 2005).

The most extensively studied function of the proteasome in cells is the complete and irreversible elimination of specific cellular proteins tagged with a K48-linked polyubiquitin chain. In fact, as proteasomal degradation seems to be processive, it usually leads to the complete degradation of substrates once they have encountered the active sites of the inner cavity. However, emerging studies identified a growing number of cellular proteins with versatile functions that use ubiquitylation in

a non-proteolytic fashion. Addition of mono-ubiquitin, multiple mono-ubiquitin or modification with polyubiquitin chains through internal K63 linked ubiquitin are considered as non-canonical modifications that normally do not lead to subsequent proteasomal degradation, but provide a signal for processes like endocytosis, protein sorting, membrane trafficking, protein relocalization, ribosomal biogenesis and additionally have a large impact within the scope of DNA repair and transcription (Galan & Haguenauer-Tsapis, 1997; Hoppe et al., 2000; Hoege et al., 2002; Pickart & Fushman, 2004).

2.6 Protein activation through regulated partial proteolysis

Regulated nuclear translocation of transcription factors from cytosolic pools is a common conduit and a crucial regulatory mechanism by which the cell controls transcription. Transcription factors that are kept in a dormant inactive state in the cytosol or are anchored to membranes are excellent examples. The signal-induced activation of dormant, cytosolic pools of transcription factors seems to be a delicate process but guarantees an immediate transcriptional response and must be therefore highly specific and tightly regulated. Two fundamentally different mechanisms have been identified to activate proteins that are initially synthesized as inactive membrane-bound precursors. Both mechanisms include a release from membranes by proteolytic cleavage, which then enables the transcription factor to translocate into the nucleus and activate transcription of target genes. On the one hand cleavage of the precursor can be mediated by site specific proteases by a mechanism termed regulated intramembrane proteolysis (RIP) e.g. in Notch or SREBP signaling. On the other hand recent studies identified an increasing number of proteasomal substrates that are not degraded to completion; only parts of the polypeptide chains are eliminated by proteasomal degradation, whereas other parts are spared from degradation and released from the proteasome as stable protein fragments. Activation of the precursor can occur by a phenomenon coined regulated ubiquitin/proteasome-dependent processing (RUP) with the involvement of the 26S proteasome (Hoppe et al., 2000; Rape et al., 2001). Examples are maturation of the dormant transcription factors Spt23 and Mga2 which are structurally related to NFκB and which are essential for the regulation of unsaturated fatty acids in the OLE

pathway in *S. cerevisiae* (Hoppe et al., 2000; Rape et al., 2001; Piwko and Jentsch, 2006).

Proteasome mediated processing was described for several substrates before: e.g. maturation of the mammalian transcription factor NFκB1 from a dormant precursor in the cytosol (Palombella et al., 1994) or proteasome transformation of an active full-length transcriptional activator into a fragment that acts as a competitive repressor of the active precursor in response to changes in Hedgehog signaling (Ci and Gli proteins; Aza-Blanc et al., 1997; Sasaki et al., 1999). The actual mechanism for this unconventional activity of the proteasome was largely unknown at this time until RUP elegantly explained this non-canonical proteasomal activity (Hoppe et al., 2000; Rape et al., 2001; Piwko and Jentsch, 2006). Partial proteolysis mediated by RUP allows the direct switch of a signaling pathway from one state to another, positively or negatively for a cell's fate, and thus increases the dynamic range that can be achieved through one signaling molecule.

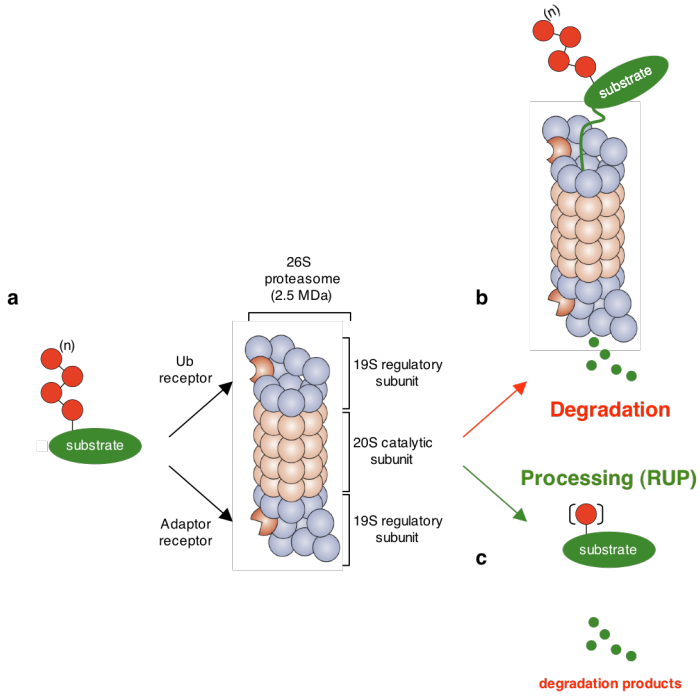

Figure 3. Regulated ubiquitin/proteasome-dependent processing (RUP). (a) Once modified by a polyubiquitin chain of at least four ubiquitins (Ub), the substrate can either bind directly to intrinsic Ub receptors in the 19S regulatory subunit or to adaptor proteins that contain both polyubiquitin- and proteasome-binding domains. **(b)** Binding of the the substrate to the 26S proteasome is followed by protein unfolding by ATPases that encircle the pore of the catalytic core, removal and recycling of the Ubiquitins by DUBs (not shown), and translocation into the central proteolytic chamber, where it is degraded to completion. **(c)** Emerging studies show a growing number of substrates that are mono/oligoubiquitylated in its inactive state and are subsequently processed *via* the 26S proteasome to an active state with different biological function instead of degraded to completion, this phenomenon was coined RUP (regulated ubiquitin/proteasome-dependent processing).

2.7 The OLE pathway

The regulation of membrane fluidity is of essential importance for the function and integrity of the membrane system of the cell. Unsaturated fatty acid levels in yeast are largely controlled by an intriguing pathway, coined the OLE pathway (Figure 5b). The ER-bound enzyme Δ-9 fatty acid desaturase Ole1 is essential for viability and the key enzyme for providing the cell with unsaturated fatty acids. Ole1 catalyzes the conversion of saturated to unsaturated fatty acids (UFAs) by introducing double bonds into their carbon chains. The balance between saturated and unsaturated fatty acids is a crucial parameter that determines membrane fluidity in the cell. Disproportionate activation of Ole1, resulting in low levels of unsaturated fatty acids, leads to a severe impairment of cellular membrane systems, most notably of the nuclear envelope and of mitochondria. In contrast high levels of UFAs are as detrimental as low levels for the cell with dramatic consequences for membrane composition and pleiotropic defects in the secretory pathways. The Ole1 enzyme itself is an integral membrane-bound protein of the ER. Ole1 is a short-lived protein and degraded *via* the ERAD machinery, with its half-life negatively regulated by the amount of UFAs resulting in a negative feedback loop (Braun et al., 2002).

Importantly Ole1 is among the targets of the two transcription factors Spt23 and Mga2, which are kept initially synthesized as inactive precursors (p120) in the cytosol by anchoring to membranes of the ER/nuclear envelope via their C-terminal tails. Although deletion of either of the two transcription factors alone seems to have no obvious phenotype in yeast, the double deletion is lethal. Interestingly, growth can be completely restored by providing the cell with an external source of UFAs (oleic acid; Zhang et al., 1999), overexpression of *OLE1* itself or by overexpression of a truncated variant of the transcription factor Spt23 which resembles a “quasi soluble” processed and active form of the precursor (Hoppe et al., 2000; Rape et al., 2001).

2.8 Regulated ubiquitin/proteasome-dependent processing (RUP): The Spt23 life cycle

Transcription of *OLE1* gene is driven by the transcription factor Spt23 (and its homolog Mga2), structurally related to the mammalian transcription factor NFκB, and activation of the dormant transcription factor is regulated by RUP. The Spt23 life-cycle is determined by several ubiquitin-dependent events that take place in different compartments of the cell and can be subdivided into the maturation of p120 to p90 at the ER/cytosol and the inactivation of p90 in the nucleus via polyubiquitylation and subsequent 26S dependent degradation (Figure 4a, b; Hoppe et al, 2000; Rape et al., 2001).

Upon fatty acid restriction, homodimerization of the ER membrane-anchored Spt23 precursor (p120) takes place and one module of the dimer (p120/p120) is mono- or oligoubiquitylated (possibly by a stochastic mechanism) by the essential WW-HECT E3 ligase Rsp5. The ubiquitylated module is then subsequently processed by the 26S proteasome, both the 19S regulatory subunit and the 20S core are needed for processing (Piwko et al., 2006). Previous data elegantly show that the Spt23 and Mga2 precursors are endoproteolytically processed by the proteasome suggesting that the polypeptide chains of the precursors enter the openings of the proteasome as loops, which enables the contact to the active sites located in the inner cavity of the proteasome (Piwko et al., 2006). Processing of the dormant p120 precursor results in the complete degradation of the C-terminal, membrane-anchored domain of Spt23 p120, while the N-terminal transcription factor domain (p90) is left intact. Interestingly, processing of Spt23 by the proteasome has been shown to occur virtually exclusively at the ER membrane. Efficient processing requires dimerization *via* its N-terminus (the Ig-like/plexins/transcriptions, or IPT, domain) and is furthermore strictly dependent on the activity of Rsp5. In a second step, the processed transcription factor (p90) is liberated from its partner (p120) for nuclear targeting by the activity of the chaperone-like complex $Cdc48^{UFD1/NPL4}$ (Rape et al., 2001). This enzyme preferentially binds ubiquitylated substrates, and is thereby capable of segregating the ubiquitylated, processed molecule from its non-modified partner molecule (Rape et al., 2001). After mobilization from the ER membrane the liberated p90 migrates to the nucleus and activates *OLE1* transcription.

Interestingly, the UPS plays again a crucial role in the degradation of p90 in the nucleus, most likely after is has initiated *OLE1* transcription. Degradation of nuclear p90 again involves Cdc48 and additionally Ufd2 and Rad23. Nuclear Spt23 (p90) in fact still retained its initial monoubiquitin mark and this monoubiquitin can be further extended by the chain assembly factor Ufd2, which functions as an E4 ubiquitin elongation factor (Koegl et al., 1999; Hoppe et al., 2005). Ufd2 also binds to Rad23 and thereby enables targeting of polyubiquitylated p90 to the proteasome by the ubiquitin escort factor Rad23 (Richly et al., 2005). Interestingly Spt23 p90 is partially stabilized in a *ufd2* deletion and cells are hypersensitive to the addition of UFAs resulting apparently in an unbalanced regulation of *OLE1* transcription.

The mechanism of these specialized activities has been the subject of intense investigation (Hoppe et al., 2000; Rape et al., 2001; Piwko and Jentsch, 2006), however how these unconventional ubiquitylation events between mono/oligo -and polyubiquitylation are regulated is still largely enigmatic especially with regard to the differential control of Spt23's fate by the ubiquitin proteasome system.

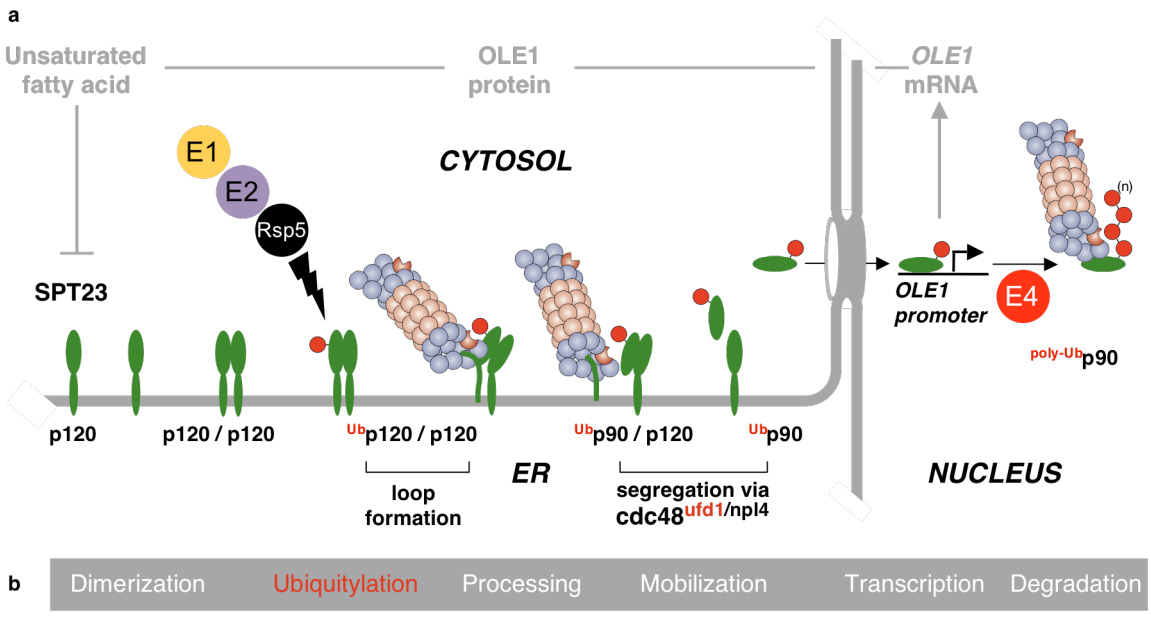

Figure 4. The OLE pathway. (a,b) The Spt23 life-cycle is determined by several ubiquitin-dependent-events that take place in different compartment of the cell to finally activate *Ole1* transcription in the nucleus: Spt23 p120 monoubiquitylation and processing at the ER, p90 mobilization from the ER to the cytosol, p90-mediated transcription in the nucleus, and finally, after E4-catalyzed polyubiquitylation of p90, degradation via the 26S proteasome in the nucleus.

3 Aim of this study

One of the most extensively studied functions of the 26S proteasome is unquestionably the complete degradation of ubiquitylated substrates, but previous studies show an increasing number of proteins escaping the canonical pathway of complete degradation in favor of proteasomal processing through specialized mechanisms. One such mechanism found in our lab was coined “OLE pathway” and previous work demonstrated that the synthesis of unsaturated fatty acids in the yeast *S. cerevisiae* is controlled by the essential, WW-domain containing ubiquitin ligase Rsp5. The E3 ligase Rsp5, being part of the OLE pathway, targets the NFκB-related transcription factor Spt23 (and its close homolog Mga2) for proteasomal processing (Hoppe et al., 2000; Rape et al., 2001). However, the regulatory mechanism and activation of this non-conventional activity of the proteasome favoring proteasomal processing (RUP) over complete substrate degradation remained still largely elusive.

Therefore the aim of this study was to investigate the underlying regulatory mechanism of this unconventional ubiquitylation event: mono/oligo- versus polyubiquitylation. The activation of the transcription factor Spt23 in the OLE pathway suggests the existence of a regulator for this molecular timing event that tightly regulates the ubiquitylation status of Spt23 between the different stages and compartments (ER/Cytoplasm, Nucleus). A change in membrane fluidity or composition might activate a signaling cascade to induce a conformational change into the Spt23 molecule or a binding partner, which thereby could subsequently trigger an association of Spt23 with the processing machinery.

Beyond the initial scope of the study we moreover became interested in what determines the specificity whether a substrate is mono/oligo- or polyubiquitylated and if there is a unifying regulatory mechanism that might act as a fate switch between mono/oligo- and polyubiquitylation.

4 Results

4.1 The peptidyl-prolyl *cis/trans* isomerase Ess1 is an essential new player of the OLE pathway

Previously, it was shown that the synthesis of unsaturated fatty acids in the yeast *S. cerevisiae* is tightly controlled by the essential WW domain-containing ubiquitin ligase Rsp5 (Figure 5a), which targets the transcription factor Spt23 (Figure 5a; and its close homolog Mga2) for proteasomal processing *via* a non canonical activity of the 26S proteasome (RUP; Hoppe et al., 2000; Rape et al., 2001; Piwko and Jentsch, 2006). To investigate additional regulatory components that might be linked to the essential OLE pathway, we reviewed earlier studies involving the WW-HECT E3 ligase Rsp5, which is the crucial E3 ligase in the OLE pathway.

We indeed observed a genetic link between Rsp5 and the peptidyl-prolyl *cis/trans* isomerase Ess1, the yeast orthologue of Pin1 (Wu et al., 2001). Both WW domain containing proteins act on the same substrate, namely the carboxy-terminal domain (CTD) of RNA Pol II (Morris et al., 1999; Chang et al., 2001). The CTD of RNA Pol II contains multiple repeats of a proline rich heptapeptide with the consensus sequence YSPTSPS (52 repeats in mammalians and 26-27 repeats in the yeast *S. cerevisiae*). Both WW domain containing proteins are essential for viability in *S. cerevisiae* and in both cases substrate specificity is mediated *via* the WW protein-interaction domain. RNA Pol II is regulated by CTD phosphorylation (Sudol and Hunter, 2000) and while Pin1 was shown to predominantly interact with the phosphorylated form of the CTD, Rsp5 was documented to interact preferentially with unphosphorylated CTD (Morris et al., 1999; Chang et al., 2000). Interestingly, Ess1 is thought to play a positive role in RNA Pol II transcription by generating conformational isomers of the CTD, while Rsp5 is thought to play a negative role in transcription by mediating RNA Pol II ubiquitylation and degradation *via* the 26S proteasome. The mechanism and the regulation of this mechanism are still largely unknown.

In fact, ubiquitylation of the transcription factor Spt23 (and Mga2) is mediated by the WW HECT E3 ligase Rsp5 and recognition and specificity of this interaction is mediated *via* one of the WW domains of Rsp5 (Hoppe et al., 2001), therefore we hypothesized that also Ess1 might be linked to the OLE pathway. Opposing effects of

Rsp5 and Ess1 on one substrate would be an interesting new regulatory mechanism, suggesting a model in which Ess1 and Rsp5 might also act in the OLE pathway.

Ess1, the yeast orthologue of Pin1, harbors a catalytic (PPIase) domain and and a single amino-terminal WW protein interaction domain through which the enzyme specifically targets its substrate. Ess1 belongs to the class of Pin1-type peptidyl-prolyl *cis/trans* isomerases, among which Pin1 is special, as its association with substrates involves a WW protein-protein-interaction domain that recognizes preferentially phosphorylated serine-proline or threonine-proline motifs (pS/TP motifs; "proline-directed phosphorylation"; Staub and Rotin, 1996; Sudol, 1996; Yaffe et al., 1997). Interestingly, the mammalian orthologue Pin functions as a regulatory toggle in fate-determining pathways (Lu et al., 2007; Yeh et al., 2007). Pin1 regulates diverse cellular processes, including differentiation, cell proliferation, cellular stress responses, neuronal function and immune responses. In line with the diverse physiological roles of Pin1, it has also been linked to several diseases that include tumorigenesis, Parkinson's and Alzheimer's disease. Among the known substrates are p53, β-catenin, c-Myc, cyclins, and tau. Furthermore, due to its unique activity to switch the conformation of peptidyl-proline bonds, Pin1 and the UPS are often linked, but how Pin1 controls protein stability remained largely enigmatic (Wulf et al., 2005; Lu et al., 2007).

4.1.1 Ess1 is a essential new player in the OLE pathway and functionally directly linked to Ole1

To test the hypothesis for a direct physiological role of the peptidyl-prolyl *cis/trans* isomerase Ess1 in the OLE pathway we assayed the otherwise lethal *ess1* deletion (*Δess1*) for suppression on oleic acid supplemented plates, which would mean a direct implication for Ess1 to act in the OLE pathway. For this purpose yeast shuffle-strains were created in which the only source of Ess1 was expressed from the endogenous *ESS1* promoter from an autonomously replicating centromeric vector containing the *URA3* marker (*Δess1; ESS1:URA3)*, as a control, the same strain was created for Rsp5 (*Δrsp5; RSP5:URA3)*. In such a setup viability of the strains can be tested by plating the transformants on medium containing 5-fluoro-oratic acid (FOA), which counterselects for the *URA3*-encoding plasmid. To test the hypothesis whether Ess1 is directly linked to the OLE pathway analogous to Rsp5, transformants were

either plated on medium containing FOA or on FOA plates supplemented with unsaturated fatty acids (0.2 % oleic acid). Interestingly, we found that the lethality of an *ess1* deletion strain (*Δess1;* Figure 5b, left panel) can be indeed suppressed by supplying the cells with unsaturated fatty acids (Figure 5b, right panel). Although growth was restored by the addition of oleic acid, *ess1* deletion (*Δess1*) leads to diminutive levels of *OLE1* transactivation (compared to *Δrsp5)*, measured by reporter assay (Figure 5d).

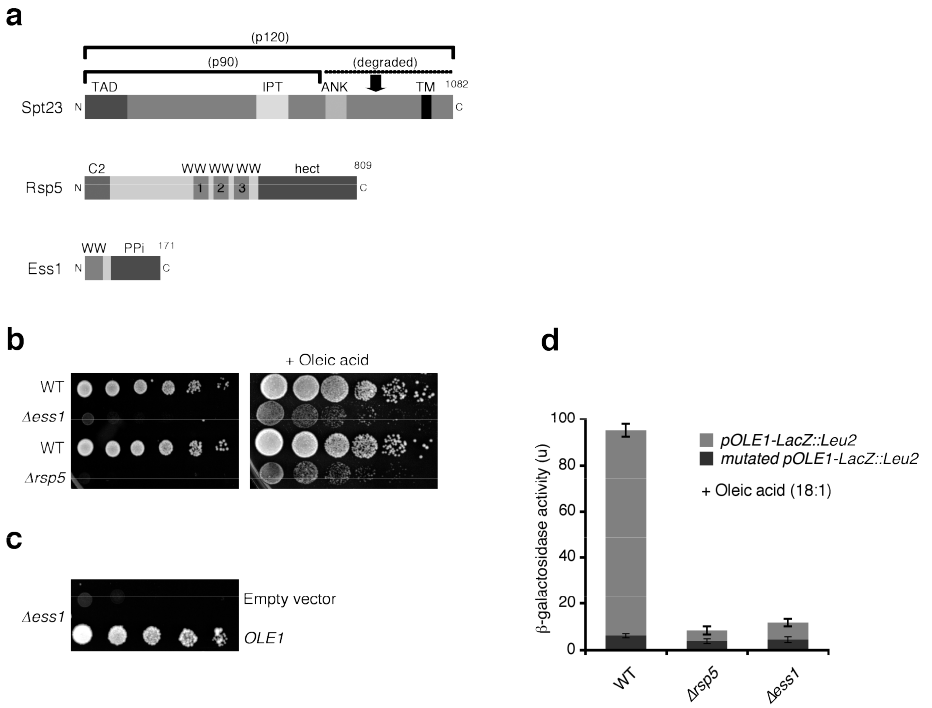

Figure 5. Ess1 is linked to the OLE pathway. (a) Schematic diagram of Spt23 showing the trans-activation domain (TAD), the NF-κB-like IPT domain, the positions of two ankyrin repeats (ANK), and the trans-membrane span (TM). Upon unsaturated fatty acid depletion, full-length Spt23 (p120) is ubiquitylated by Rsp5 and subsequently processed (arrow) at the ER membrane by the proteasome, yielding the active transcription factor (p90), whereas the carboxyl-terminal domain is degraded (dotted line). Schematic diagram of the E3 ligase Rsp5 and the peptidyl/prolyl isomerase Ess1 showing the double tryptophan (WW) substrate interaction motif(s) and the C-terminal catalytic domains, for Rsp5 the HECT E3 ligase domain (hect) and for Ess1 the PPiase (PPi) domain, respectively. **(b)** Growth of the otherwise inviable *ess1* deletion strain (*Δess1*) is restored by unsaturated fatty acids (0.2 % oleic acid) supplementation (upper panel), or by overexpression of *OLE* **(c)** An Ess1 shuffle strain (*Δess1; ESS1:URA3*) was either transformed with empty vector (*pADH1*-Empty) or with a construct constitutively overexpressing Ole1 (*pADH1-OLE1*), cells were then spotted either on FOA plates or FOA plates supplemented with 0.2 % oleic acid. An Rsp5 shuffle strain (*Δrsp5; RSP5:URA3)* strain was used as a control. **(d)** *OLE1* transactivation is dependent on Ess1. β-galactosidase expression (ONPG) assays comparing the *Ole1* transactivation activity of wild-type (WT), *rsp5* deletion *(Δrsp5)* or *ess1* deletion (*Δess1)* strains bearing an integrated *pOLE1-LacZ* fusion or a *mutated pOLE-LacZ* as a negative control as indicated. Strains were grown in media supplemented with 0.2 % oleic acid (18:1). Graphs represent the mean of three independent experiments done in triplicates; standard deviations are indicated.

To verify a direct involvement of Ole1 itself we additionally transformed the Ess1 shuffle strain (*Δess1; ESS1:URA3)* either with empty vector (*pADH1*-Empty) or with a construct constitutively overexpressing Ole1 itself (*pADH1-OLE1*). Cells were then spotted on plates with or without FOA. Notably, overexpression of *OLE1* fully restored growth of the otherwise lethal *ess1* deletion (Figure 5c) demonstrating that Ess1 is indeed directly functionally linked to the OLE pathway.

For further analysis of the role of Ess1 in the OLE pathway a yeast strain was created in which the only source of Ess1 was expressed from an autonomously replicating centromeric vector containing the *URA3* marker (*Δess1*; *ESS1:URA3*). This strain was then used to transform constructs encoding Ess1 mutants, viability of the Ess1 mutant variants was then tested by plating the transformants on FOA containing plates, which counterselects for the *URA3* encoding plasmid. Consequently, the plasmids encoding Ess1 mutants will remain as the only source of Ess1 in the cells plated on FOA containing plates. We tested two temperature-sensitive (ts) *ess1* mutants that were characterized in previous studies (Wu et al., 2000): One *ess1* ts-mutant (*ess1-W15R)* bears a mutation in the codon of the first signature tryptophan (W15R) that results in a ts phenotype at the non permissive temperature of 36°C. The other *ess1* ts-mutant variant (*ess1-H164R)* has a single amino acid substitution in the catalytic (PPi) domain. The equivalent histidine (H159) in Pin1 is characterized to stabilize the covalent intermediate formed with the substrate peptide (Wu et al., 2000) and in *S. cerevisiae* expression of the *ess1-H164R* mutant variant leads to a severe ts phenotype already at 34°C (Figure 6a, b).

The otherwise lethal *ess1* deletion strain (*Δess1::HIS)* was either transformed with *ESS1* (WT) or the two Ess1 mutant variants. The resulting strains were then either transformed with empty vector (*pADH1*-Empty; see Figure 6a) or with a constitutively overexpressing *OLE1* construct (*pADH1-OLE1*; see Figure 6b). Cells were either spotted on plates supplemented with or without 0.2 % oleic acid, and finally incubated either at permissive (25°C) or non-permissive temperature (34°C for *ess1-H164R;* 36°C for *ess1-W15R*). As expected both *ess1-ts* mutants were either rescued by supplementing the cells with unsaturated fatty acids (oleic acid) or by overexpression of *OLE1* itself when shifted to the otherwise lethal non-permissive temperature (Figure 6a, b).

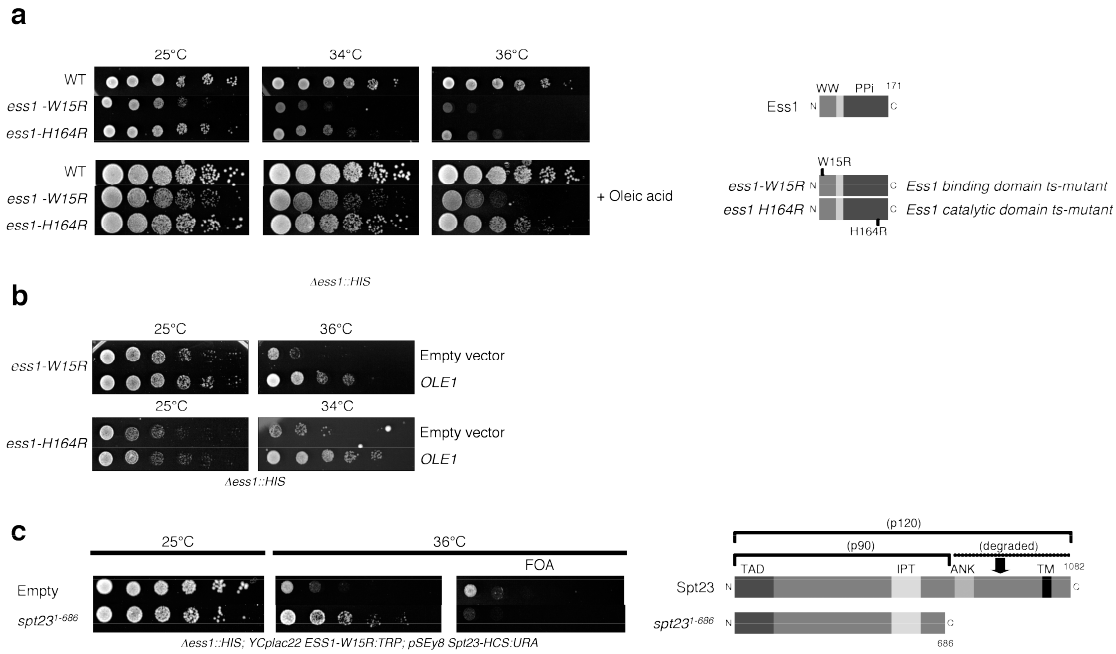

Figure 6. Ess1 is a new player in the OLE pathway. (a,b) For further analysis two *ess1-ts* mutants were used in this study (*ess1-W15R* and *ess1-H164R*). Growth of the otherwise inviable *ess1* deletion strain (*Δess1*) was either transformed with *ESS1* (WT), *ess1-W15R* or *ess1-H164R* and the strains were cultured at the permissive temperature (25°C). The strains were either transformed with empty vector (*pADH1*-Empty) or with a construct constitutively overexpressing Ole1 (*pADH1-OLE1*), cells were then spotted either on SC plates or SC plates supplemented with 0.2% oleic acid and finally shifted to the non-permissive temperature. Analogously to Figure 5b,c, growth was restored at the non-permissive temperature by either 0.2 % oleic acid supplementation **(a)** or by overexpressing *Ole1* itself **(b)**. **(c)** Suppression of *ess1-ts* mutant (*ess1-W15R*), colonies of serial diluted *ess1-W15R* mutant cells that were transformed with either empty vector (Empty) or a plasmid isolated as a high-copy suppressor of *ufd1-2* (*spt23$^{1-686}$:URA3*; Hoppe et al., 2001; see schematic diagram, right panel) that expresses a truncated SPT23 version. The plates were incubated at the temperatures indicated on either SC plates or FOA plates to counterselect against the *spt23$^{1-686}$:URA3* plasmid showing that the *ess1-W15R* strain is inviable withouth the *ufd1-1* high-copy suppressor after counterselection on FOA.

Previous studies have shown that Spt23 processing results in p90 bound to an unprocessed SPT23 p120 partner molecule at the membrane (Hoppe et al., 2000; Rape et al., 2001; Piwko et al., 2006). Interestingly, p90 has retained its ubiquitin modification after processing, and a chaperone-like enzyme, designated Cdc48$^{Ufd1/Npl4}$, finally separates the p120/p90 heterodimer *via* an ATP-dependent mechanism, thereby mobilizing p90 for nuclear targeting (Rape et al., 2001).

Notably, the lethality of the *ess1-W15R ts* mutant variant at the non-permissive temperature could also be suppressed by overexpression of a truncated clone of Spt23 (*Spt23$^{1-686}$:URA3*), which was initially genetically linked to the Cdc48 cofactor Ufd1 (Hoppe et al., 2000). This truncated version of Spt23 operates as a "quasi-solube", processed p90 (Figure 6c, schematic diagram) and is therefore able to translocate to the nucleus to drive *Ole1* transcription independent of the UPS

machinery. Although the rescuing Spt23 variant was overexpressed, these data supports a functional relationship between Ess1, Rsp5, Ufd1, Spt23 and Ole1. From these findings it can be concluded that the vital function of Ess1 at normal temperatures is its role in the essential OLE pathway.

4.1.2 Ess1 interacts physically with Spt23 *in vivo and in vitro* and binding is mediated via Ess1's WW domain

The implication of Pin1 as a new player in the OLE pathway and suppression of the otherwise lethal *ess1* deletion by overexpression of a "quasi-souble" Spt23 p90 strongly suggested that Spt23 is the target of Ess1 in the OLE pathway.

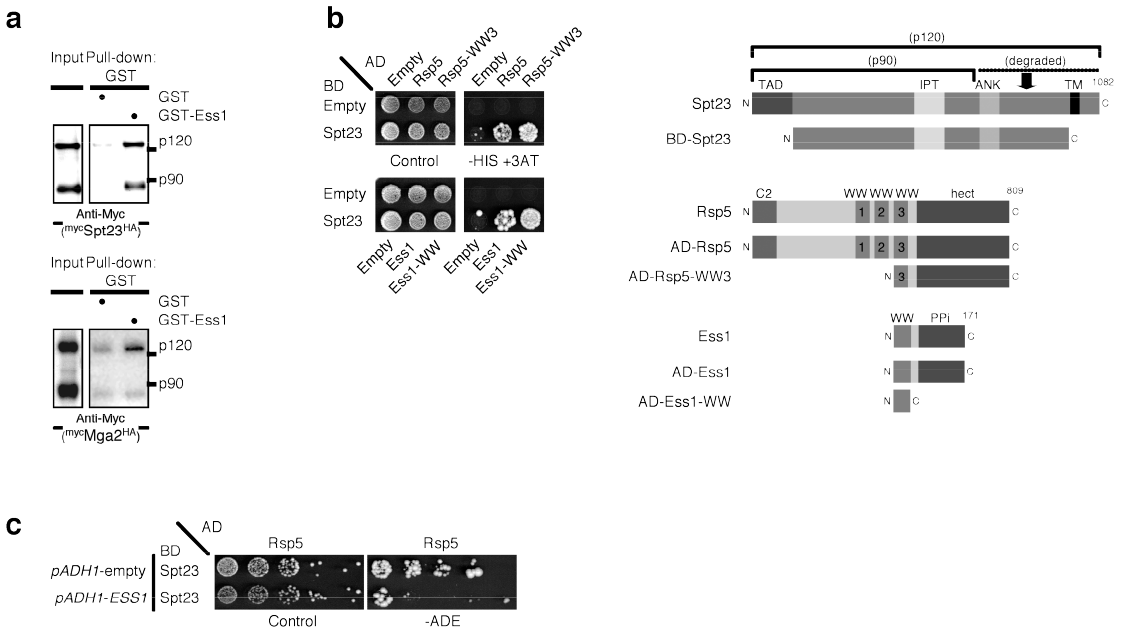

Figure 7. Ess1 binds Spt23 *in vivo* and binding is mediated via the WW substrate interaction domain. (**a**) Ess1 binds Spt23 p120 and p90 in GST pull-down experiments, lysates of cells that express myc-tagged Spt23 (*pGAL1-10*) were subjected to *in vitro* pull-down assays with either GST-Ess1 or GST as a control. **(b)** Two-hybrid interactions of full-length Rsp5 or Rsp5-WW3 (upper panel), or full-length Ess1 or Ess1-WW, with Spt23 (the amino-terminal trans-activation domain and the trans-membrane span were deleted, see schematic diagram). Interaction was identified on selective plates (-HIS, +3AT; without histidine, plus 3-aminotriazole). Fusions with the Gal4-activation domain (AD) or Gal4-DNA-binding domain (BD) are indicated (see schematic diagrams of the constructs used). **(c)** Competitive yeast two-hybrid analysis of Spt23, Rsp5 and Ess1. Rsp5 binding to Spt23 (see above) was monitored using cells that either contain only the vector (pADH1-Empty), or overexpress full-length Ess1 (pADH1-ESS1) from the pADH1 promoter. Interaction was identified on selective plates (-ADE; without adenine).

Like the WW-HECT E3 ligase Rsp5, Ess1 binds Spt23 (and Mga2) p120 and p90 directly as revealed by *in vitro* GST pull-down assays and two-hybrid analysis (Figure 7a, b). Additionally, binding is mediated via the WW interaction domain of Ess1 as indicated by two-hybrid analysis (Figure 7b). In fact, Rsp5 and Ess1 partially compete for Spt23 binding in two-hybrid assays (Figure 7c), suggesting that WW domains of both enzymes may target the same region of Spt23.

4.1.3 Ess1 activity is directly linked to *Ole1* transactivation *via* Spt23

When Ess1 was discovered and characterized (Hanes et al., 1989), it was reported that not only *ess1* deletion is detrimental for viability but also that overexpression of Ess1 leads to cytotoxicity (Figure 8a, left panels). Disproportionate levels of Ess1 might be connected to an imbalance in the transcriptional activation of *OLE1*. We therefore characterized this growth defect in the context of the OLE pathway by either overexpressing empty vector (Empty), full-length Ess1 (*ESS1)* or single domain mutants of Ess1, WW domain only (*ess1-WW)* or the catalytic domain of Ess1 (*ess1-PPi)* from the *pGAL1-10* promoter. Cells were then spotted in serial dilution on plates containing glucose (Figure 8a upper, panels) or galactose (to induce expression from the *pGAL1-10* promoter) either supplemented with or without 0.2 % oleic acid. Only overexpression of full-length Ess1 (*ESS1)* showed a severe growth defect, while the single domains of Ess1 alone (*ess1-WW, ess1-PPi*) showed no obvious phenotype upon overexpression (Figure 8a, left panels). Notably, this growth defect was even exacerbated when the cells were in addition spotted on galactose-plates containing unsaturated fatty acids (Figure 8a, right panels). Intriguingly, overexpression of *ESS1* directly results in disproportionate up-regulation (> 2.5 fold) of *OLE1* transcription as measured by reporter assays (Figure 8b). These results confirm that substrate recruitment via the WW domain is crucial, and that Ess1 activity is not only directly correlated to *OLE1* transactivation but *OLE* transcriptional activation is dependent on Ess1.

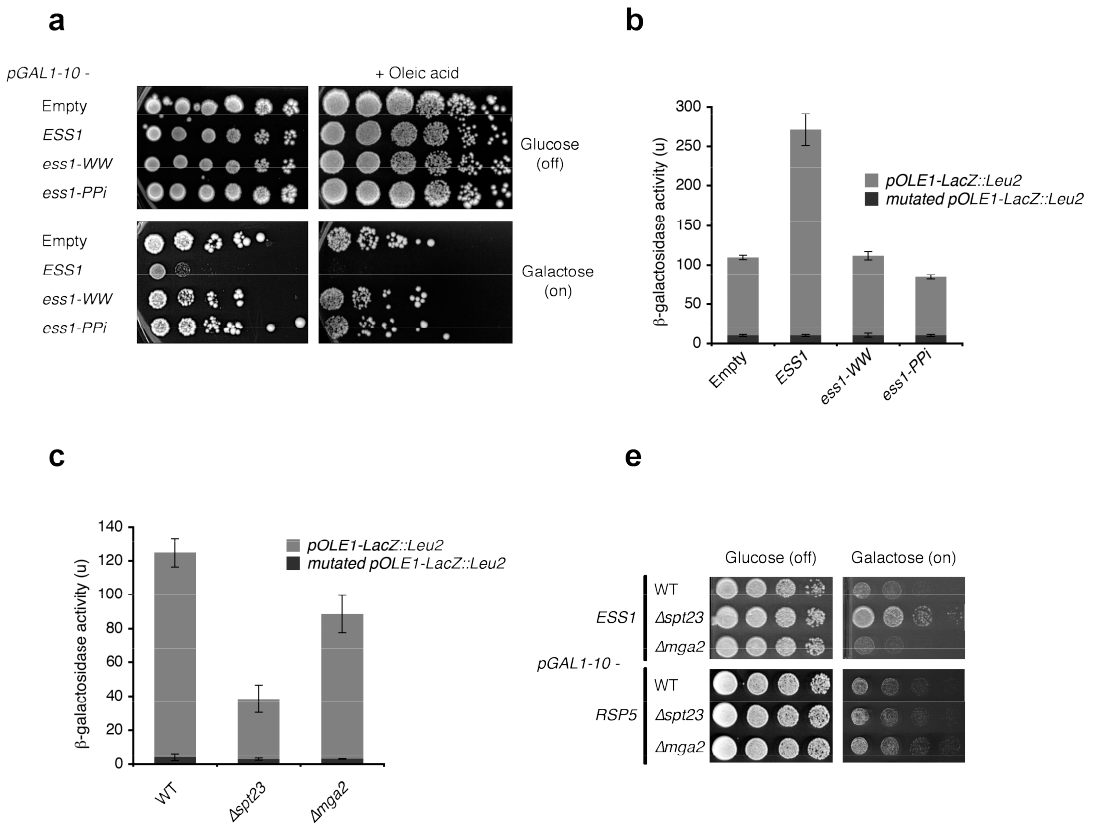

Figure 8. ESS1 activity is directly linked to *Ole1* transactivation. (a) Overexpression of full-length *ESS1* leads to cytotoxicity and hypersensitivity to oleic acid (18:1). Serial dilutions of cells expressing *ESS1*, *ess1-WW*, or the *ess1-PPIase* domain (all from *pGal1-10* promoter). Expression by the *pGAL1-10* promoter was induced by addition of galactose to the medium (lower panels). The plates on the right contain 0.2% oleic acid. **(b)** *ESS1* activity is directly correlated with *Ole1* transactivation. β-galactosidase expression (ONPG) assays with wild-type strain bearing an integrated *pOLE1-LacZ* fusion or a *mutated pOLE-LacZ* as a negative control as indicated, overexpressing either vector (Empty), *ESS1*, *ess1-WW*, or *ess1-PPi* under the *pGAL1-10* promoter. Graphs represent the mean of three independent experiments done in triplicates; standard deviations are indicated; β-galactosidase activity was normalized to the (Empty) control (set to 100%). **(c)** β-galactosidase expression (ONPG) assays comparing the *Ole1* transactivation activity of wild-type (WT), *spt23* deletion *(Δspt23)* or *mga2* deletion (*Δmga2)* strains bearing an integrated *pOLE1-LacZ* fusion or a *mutated pOLE-LacZ* as a negative control as indicated. **(d)** Cytotoxicity due to *ESS1* overexpression is suppressed in a *spt23* deletion strain (*Δspt23)* but not *Δmga2*. Serial dilutions of cells either expressing *ESS1* or *RSP5* (both from *pGAL1-10* promoter) in different deletion backgrounds comparing WT, *Δspt23* and *Δmga2*. Expression was induced by addition of galactose to the medium (right panels).

OLE1 transactivation is regulated *via* the two transcription factors Spt23 and Mga2, being functionally overlapping. Indeed, the *Δspt23* Δ*mga2* double deletion is lethal, while the *Δspt23* or *Δmga2* single deletions show no obvious phenotypes and are viable (Zhang et al., 1997). Either one of the transcription factors is enough to ensure viability but to what extent they contribute to *OLE1* transcription is still elusive. In order to analyze the contribution of Spt23 and Mga2 to the OLE pathway we measured the *OLE1* transactivation by reporter assays in either wild-type (WT), *spt23*

deletion *(Δspt23*) or *mga2* (*Δmga2*) deletion backgrounds. Interestingly, *OLE1* activation was strikingly reduced in *Δspt23* compared to *Δmga2* (Figure 8c), although Ess1 seems to bind Spt23 and Mga2 in GST pull-down assays to the same extent (Figure 7a). Furthermore, toxicity due to *ESS1* overexpression was surprisingly suppressed in a *Δspt23* while there was no suppression observed in wild-type (WT) or *Δmga2* (Figure 8d, upper panels). In contrast to this directed contribution of *ESS1* activity towards Spt23, *RSP5* seems to contribute to both transcription factors to the same extent (Figure 8d, lower panels). These results indicate that Ess1 is functionally directly linked to *OLE1* transcriptional activation mainly via the transcription factor Spt23. We therefore conclude that Ess1 is an essential new player in the OLE pathway.

4.2 Ess1 interacts with Spt23 in a phosphorylation-dependent manner *in vivo* and *in vitro*

The reversible phosphorylation of proteins on certain serine or threonine residues that immediately precede a proline (pS/T-P) appears to be an important regulatory switch for several cellular processes such as cell proliferation and differentiation (Blume-Jensen et al., 2001; Lu et al., 2002). The significance of phosphorylation dependent conformational changes becomes clear with the discovery of the unique peptidyl-prolyl *cis/trans* isomerase Pin1 in 1995 (Lu et al.), which specifically isomerizes phosphorylated S/T-Pro motifs and is a prerequisite for Pin1 substrate recognition and PPiase activity.

4.2.1 Spt23 phosphorylation is a prerequisite for Ess1 binding and activity

To uncover whether Spt23 contains a phosphorylated S/T-Pro motif, we examined phosphorylation of Spt23 *in vivo*. Indeed, by using the monoclonal antibody MPM2, which specifically recognizes phosphorylated S/T-Pro motifs (Lu et al., 1999) we observed reactivity for both Spt23 p120 and p90 forms (Figure 9a, middle panel, left lane). MPM-2 reactivity was significantly reduced by treatment with λ-phosphatase

(Figure 9a, middle panel, right lane) and phosphatase treatment also abolished *in vivo* binding of Ess1 to Spt23 (Figure 9a, lower panel). Furthermore, in *in vitro* GST pull-down assays Ess1 binds to Spt23 in a phosphorylation-stimulated manner, and binding is lost when the samples were treated with λ-phosphatase (Figure 9b). These results demonstrate that Spt23 is in fact modified by proline-directed phosphorylation and that phosphorylation is indeed a prerequisite for effective Ess1 binding to Spt23.

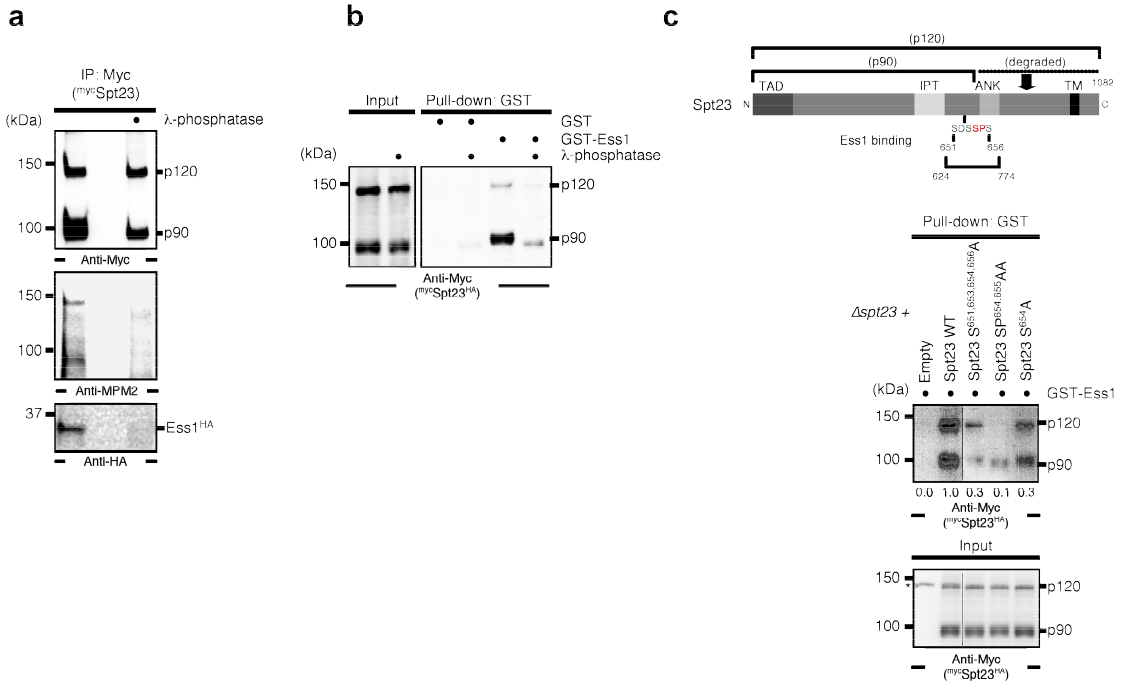

Figure 9. Spt23 is modified by proline-directed phosphorylation and Ess1 binding is stimulated by phosphorylation. **(a)** Spt23 is modified by proline-directed phosphorylation *in vivo* and a prerequisite for Ess1 binding. Lysates of cells that express myc-tagged Spt23 (*pGAL1-10* promoter) and HA-tagged Ess1 were incubated with or without λ-phosphatase, followed by myc-immunoprecipitation (IP) and immunoblotting using an anti-myc antibody (upper panel), a mitotic phosphoprotein monoclonal 2 (MPM2, Upstate) antibody, which is specific for phopho-Ser/Thr-Pro sites (middle panel), or an HA-epitope specific antibody (lower panel). Treatment with λ-phosphatase abolishes binding of Ess1 (Ess1-HA) to Spt23. **(b)** Ess1 binds to Spt23 in a phosphorylation-stimulated manner in *in vitro* GST-pull-down experiments as indicated by treatment with or without λ-phosphatase. **(c)** Schematic diagram of Spt23 showing Ess1 binding to a central region of Spt23 (aa624-774) determined by two-hybrid analysis (data not shown and Figure 5a), which contains a Ser-Pro site (SP) in the p90 part of p120. Spt23 variants with serine-to-alanine (S-A) alterations in the Ess1-binding site as the only source for Spt23 are defective in Ess1 binding as indicated by pull-down experiments using GST-tagged Ess1. $^{myc}Spt23^{HA}$ WT or variants (expressed from endogenous promoter) were transformed to a *spt23* deletions strain (*Δspt23*) and used in *in vitro* pull-down experiments.

Mapping experiments by two-hybrid assays with various Spt23 truncations and Ess1 revealed a central region of Spt23 (aa 624-774) as the major binding site for Ess1 (data not shown and Figure 9c, upper schematic diagram). Interestingly, this site is not only implicated in the processing of Spt23 p120 and close to the presumed ubiquitylation site, but also the predicted site for loop formation and proteasome entry (Piwko et al., 2006). We further noticed that the identified Ess1-binding region in Spt23 contains a typical Ser-Pro motif ($SP^{654,655}$) surrounded by several additional serines (aa 651-656), which is interestingly located in the p90 part of Spt23. To further characterize the Ess1 binding to Spt23 we conducted *in vitro* GST pull-down assays with Spt23 versions where serine and proline residues in the identified core binding region (aa 651-656) were changed to alanine. Changing the respective serine and proline to alanine ($SP^{654,655}AA$) abolished Ess1 binding, also changing all 4 serines to alanine ($SSSS^{651,653,654,656}AAAA$) reduced binding to Ess1 significantly (Figure 9c, lower panels). Changing only one serine to alanine ($S^{651}A$) showed a milder reduction in binding to Ess1, most likely due to the fact that another serine nearby is used ("phosphorylation-site hopping").

The WW protein interaction domain of Pin1-type PPIases functions as a pS/T-Pro binding module. The pS/T-Pro binding pocket within the WW domain is highly conserved and involves several critical residues, including Tyr23, Trp15, Ser16 and Arg17. Tyr23 was previously characterized to be crucial for mammalian Pin1 to recognize phosphorylated substrates (Lu et al., 1999).

To further address the functional relevance of the phosphorylation-dependent binding of Ess1 to Spt23 we performed *in vitro* GST pull-down assays. Intriguingly, changing a conserved tyrosine residue (Y23A) of GST-tagged Ess1 (Figure 10a, WW domain alignment), which lies in the phospho-recognition binding pocket of the WW domain of Ess1, abolishes the Ess1-Spt23 binding almost completely (Figure 10a). These results strongly support the conclusion that Ess1 binding to Spt23 is mediated via Ess1's WW domain and demonstrates that Spt23 is recognized by the yeast PPiase similar to most other Pin1 substrates in a phosphorylation dependent manner (Lu et al., 2007).

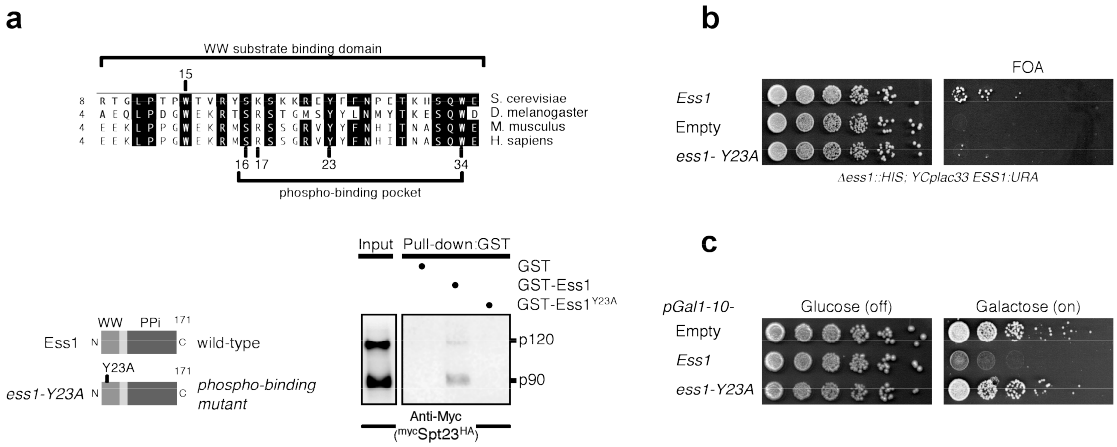

Figure 10. Ess1 WW Tyr23 is essential for recognition of Spt23, activity and viability. **(a)** Ess1 binds Spt23 p120 and predominantly p90 in GST pull-down experiments, but a variant with an alteration in the WW domain essential for phospho-dependent substrate recognition (GST-Ess1^{Y23A}; see schematic diagram above) is defective in Spt23 binding. **(b)** An Ess1 variant with an alteration in the WW domain (*ess1-Y23A*) unable to bind Spt23 is not able to restore growth of the otherwise inviable *ess1* deletion strain (*ess1Δ*; contructed as in Figure 5b). Overexpression of this variant in wild-type cells causes no cytotoxity **(c)** serial dilutions of cells overexpressing (*pGAL1-10* promoter) either empty plasmid (Empty), *Ess1*, or a variant of Ess1 with an alteration in the WW domain (*Ess1-Y23A*) that is defective in Spt23 binding. Expression was induced by addition of galactose to the plates (right panel).

Genetic support for this conclusion is derived from the finding that cells expressing this mutant variant (*ess1-Y23A)* as the only source of Ess1 are not viable (Figure 10b). Furthermore overexpression of WT Ess1 is toxic, but not of the mutant (Y23A) (Figure 10c), both results demonstrate the biological relevance of phospho-recognition mediated via Ess1's WW domain.

4.2.2 Spt23 interacts with CK2, is phosphorylated by CK2 *in vivo* and *in vitro* and promotes binding to Ess1

To identify the responsible kinase for Spt23 regulation we perfomed a two-hybrid screen (data not shown) and identified all four subunits of casein kinase 2 (CK2; Cka1, Cka2, Ckb1, Ckb2) as strong interactors of Spt23 which we confirmed by immunoprecipitation assays with ^{myc}Spt23^{HA} (expressed from the *pGAL1-10* promoter) with GFP-fusions of all four CK2 subunits (Figure 11a), control experiments were carried out in parallel using cells that do not express ^{myc}Spt23^{HA} (otherwise identical) showing no precipitation after immunoblotting using a GFP-specific antibody (Figure 11b).

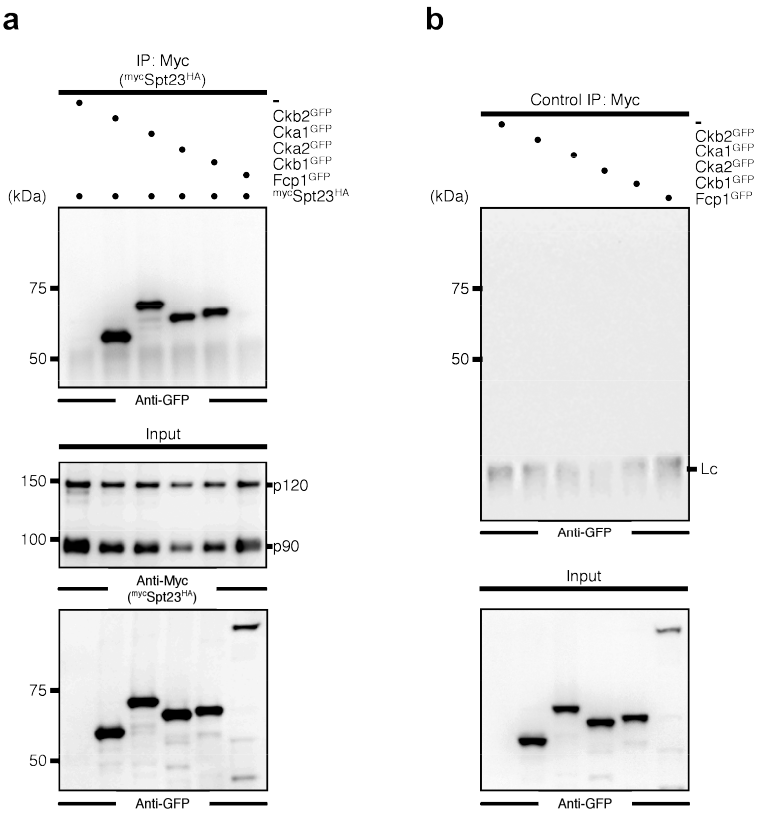

Figure 11. Spt23 binds to all four subunits of Casein Kinase 2 (CK2) *in vivo*. (a) Spt23 co-immunoprecipitates with all CK2 subunits. Lysates of cells expressing $^{myc}Spt23^{HA}$ under the control of the *pGAL1-10* promoter and GFP-tagged CK2 subunits (Cka1, Cka2, Ckb1, Ckb2), GFP-tagged Fcp1 as a negative control, or an untagged strain were subjected to myc-immunoprecipitation (IP) and immunoblotting using a GFP-specific antibody (upper panel), or an anti-myc antibody (middle panel). The lower panel shows the input identified by an anti-GFP immunoblot. **(b)** Control experiment were carried out (right panel) using cells that do not express $^{myc}Spt23^{HA}$ (otherwise identical). Immunoprecipitation using anti-myc antibodies does not precipitate CK2 in this control experiment.

Pin1 was shown to bind to pS-Pro motifs in the C-terminal domain of the large subunit of RNA polymerase II (CTD) and was discussed to stimulate CTD dephosphorylation by its phosphatase Fcp1 in the nucleus (Kops et al., 2002). We therefore included Fcp1 as a potential Spt23 phosphatase in our pull-down experiments, but GFP-tagged Fcp1 does not interact with Spt23 (Figure 11a) and therefore served as a negative control.

Furthermore, we speculated that binding of Ess1 to Spt23 might be stimulated by the protein kinase CK2. Treating cells with the specific CK2 inhibitor 4,5,6,7-tetrabromo-benzotriazole (TBB) showed that binding of Ess1 to Spt23 was indeed significantly reduced as indicated in GST pull-down experiments (Figure 12a)

In addition, isolated Spt23 forms can be phosphorylated with recombinant CK2 *in vitro* (Figure 12b). Samples were first dephosphorylated by λ-phosphatase due to residual *in vivo* phosphorylation of $^{myc}Spt23^{HA}$ immunopurified from cells, followed by an *in vitro* phosphorylation reaction with recombinant CK2 and $^{32}P\gamma ATP$. Indeed, samples pre-dephosphorylated showed stronger sensitivity in the autoradiograph compared to samples that were not dephosphorylated before CK2-phosphorylation

(Figure 12b, right panel, compare left and right lane), while cells that do not express $^{myc}Spt23^{HA}$ showed no reactivity (Figure 12b, left panel)..

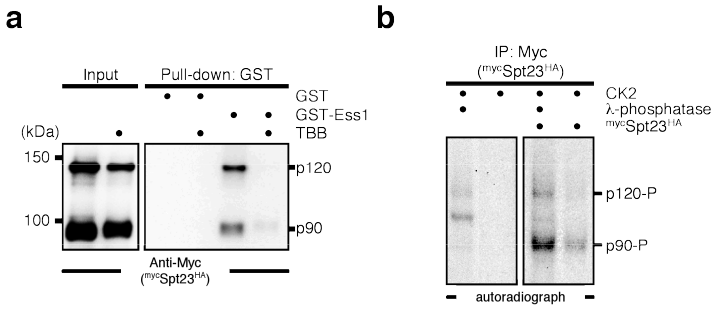

Figure 12. Ess1 binding to Spt23 is stimulated by phosphorylation mediated by Casein Kinase 2 (CK2). (a) Ess1 binding to Spt23 is lost when cells were treated with the CK2 inhibitor TBB, as indicated by GST pull-down experiments. **(b)** Isolated Spt23 forms can be phosphorylated *in vitro* by recombinant CK2. Cells not expressing (left panel) or overexpressing $^{myc}Spt23^{HA}$ (*pGal1-10* promoter*;* right panel) were subjected to myc-immunoprecipitation, due to *in vivo* phosphorylation samples were then incubated with or without λ-phosphatase followed by *in vitro* phosphorylation with recombinant CK2 and ^{32}P-γ-ATP.

Remarkably, immunopurified Spt23 forms interact with recombinant GST-tagged Ess1 in a CK2 phosphorylation-stimulated manner *in vitro*. Immunopurified $^{myc}Spt23^{HA}$ was first treated with (or without; as a negative control) λ-phosphatase to remove *in vivo* phosphorylation. After phosphatase treatment samples were left untreated or *in vitro* phosphorylated with recombinant CK2 (as above, but with non-radioactive labeled ATP). These samples were finally incubated with recombinant GST-tagged Ess1 or GST alone (as a negative control). The supernatant of the last step was then used in GST pull-down assays (with GST-Sepharose) to measure the amount of free unbound GST-Ess1 to Spt23-bound GST-Ess1 (Figure 13a, middle panel, compare left and right panels): Recombinant Ess1 was only co-immunopurified by CK2-phosphorylated Spt23, see quantification for the amount of Ess1 bound to Spt23 (Figure 13a, middle left panel) compared to Ess1 unbound to Spt23 (Figure 13a, middle right panel). By using an antibody specific for the β-subunit of CK2 we observed, that CK2 was only efficiently bound when the samples were pre-dephosphorylated (Figure 13a, lower panel).

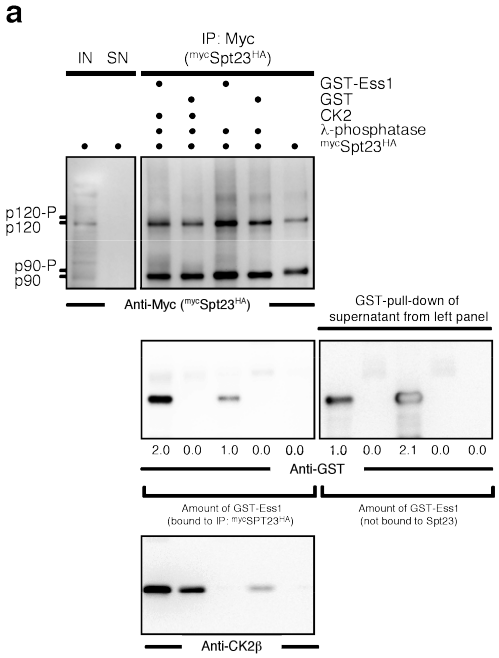

Figure 13. Spt23 interacts with Ess1 in a CK2 phosphorylation-stimulated manner in vitro. (a) Extracts of cells expressing $^{myc}Spt23^{HA}$ under the control of the *pGAL1-10* promoter were subjected to immunoprecipitation using anti-myc antibodies and incubated with or without λ-phosphatase. After phosphatase treatment, the washed samples were left untreated or phosphorylated *in vitro* with recombinant CK2. These samples were finally incubated wirt either recombinant GST-Ess1 or GST alone (control). The unbound GST-Ess1 material (supernatant of the last experiment) was subjected to a GST pull-down with GST-Sepharose to compare the amount of free to Spt23-bound GST-Ess1 material. The upper panel shows the anti-myc immunoblot of immunoprecipitated $^{myc}Spt23^{HA}$ treated with or without λ-phosphatase. The central left panel shows an anti-GST immunoblot of GST-Ess1 that co-immunoprecipitated with $^{myc}Spt23^{HA}$ (GST-Ess1 bound to Spt23) when phosphorylated by CK2. The central right panel shows unbound GST-Ess1 pulled down by GST-Sepharose from the supernatant. The lower panel shows an immunoblot using antibodies directed against the CK2β-subunit of CK2, showing that CK2 binds anti-myc immunoprecipitated, in vitro phosphorylated $^{myc}Spt23^{HA}$.

4.2.3 Phosphorylation of Ess1 on Ser16 seems to be negatively correlated with its activity and localization

Evidence suggests that PPiase activity itself is subject to physiological regulation (Lu et al., 2002). As shown before (Figure 10a-c) the WW protein interaction domain of Pin1-type PPIases functions as a pS/T-Pro binding module, interestingly Ser16 is located in the center of pS/T-Pro binding pocket. In a recent study it was demonstrated that mammalian Pin1 is modified by phosphorylation in the WW domain at Ser16. Interestingly, this phosphorylation site seems to regulate nuclear localization of Pin1 and substrate binding (Lu et al., 2002).

To examine the biological relevance of Ser16 phosphorylation of *S. cerevisiae* Pin1, we used an Ess1 shuffle-strain (*Δess1; ESS1:URA3)* in which the only source of Ess1 was expressed from the endogenous *ESS1* promoter from an autonomously replicating centromeric vector containing the *URA3* marker. This shuffle-strain was then either transformed with plasmids expressing WT Ess1 (*ESS1)* or Ess1 variants in which Ser16 was replaced by alanine (S16A) or glutamic acid (S16E) by site-directed mutagenesis (Figure 14a). These strains were assayed for survival after counterselection of the *URA3* plasmid (*ESS1:URA3)* on FOA plates. Consequently, the plasmids encoding Ess1 mutants will remain as the only source of Ess1 in these cells. Interestingly, a Ess1 phospho-deficient Ser16 variant (*ess1-S16A)* is able to rescue the otherwise lethal *ess1* deletion (after counterselection on FOA plates). By contrast, a Ess1 phospho-mimicking variant (*ess1-S16E)* failed to suppress the *Δess1* lethality (Figure 14b). Genetic support for the biological relevance of Ess1 Ser16 was obtained by the observation that overexpression of the phospho-mimicking Ser16 mutant (*ess1-S16E)* shows no growth defect when spotted on galactose containing plates, while the phospho-deficient Ser16 variant (*ess1-S16A*) shows the same toxic phenotype as overexpression of WT Ess1 (*ESS1)* (Figure 14c). These results indicate that not only the Tyr23 phospho-recognition site is crucial (Figure 10a-c), but also Ser16 seems to be crucial for Ess1 functioning properly.

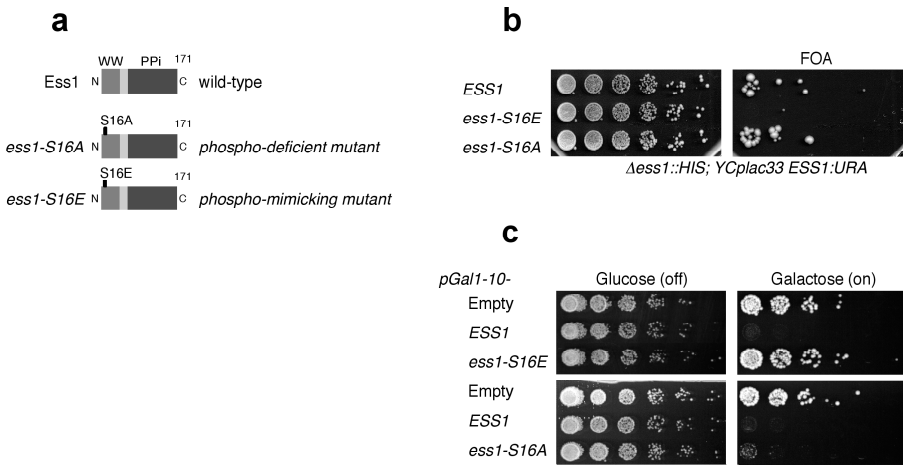

Figure 14. Ess1 Ser 16 phosphorylation is essential for Ess1 activity andviability. (a) Schematic diagram showing the Ess1 phospho-deficient Ser16 variant (*ess1-S16A*) and the phospho-mimicking Ser16 variant (*ess1-S16E*) **(b)** A phospho-mimicking Ser16 mutant (*ess1-S16E)* is not able to restore growth of the otherwise inviable *ess1* deletion strain (*ess1Δ*; contructed as in Figure 5b) while a phospho-deficient (*ess1-S16A*) variant is viable in comparison to wild-type Ess1. **(c)** Overexpression (*pGAL1-10* promoter) of the phospho-deficient Ser16 variant (S16A; lower right panel) in wild-type cells is toxic while the phospho-mimicking Ser16 variant (S16E; upper right panel) is unaffected.

Remarkably, the genetic data correlates with localization studies perfomed with GFP-tagged Ess1 and the Ser16 mutant variants of Ess1 (expressed from the *pMET25* promoter). Ess1 localization might be driven by substrate interaction *via* the WW domain as it was postulated for mammalian Pin1 (Lu et al., 2002). Although GFP was localized in both nucleus and cytoplasma, GFP-Ess1 was predominantly localized to the nuclear compartment and the ER (Figure 15a). Notably, a GFP-WW domain exhibited the same pattern as GFP-Ess1, whereas the GFP-PPIase domain was observed as a diffuse pattern in the whole cell (data not shown).

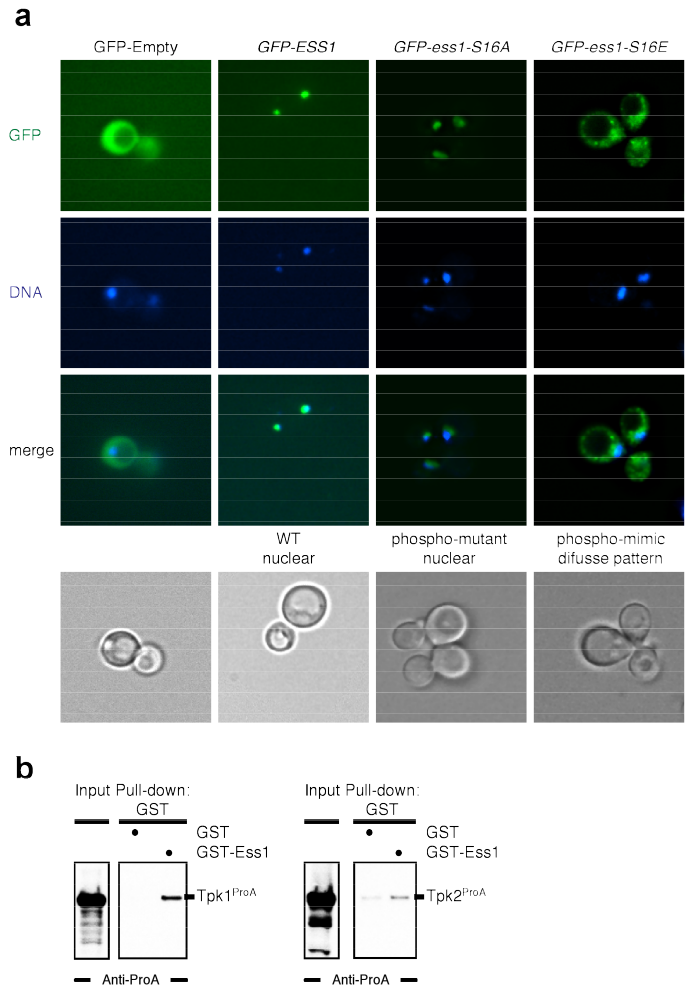

Figure 15. Ess1 Serine 16 phosphorylation regulates subcellular localization. (a) Intracellular localization of GFP-tagged Ess1 wild-type (WT) and Ess1 Ser16 variant *ess1-S16A* and *ess1-S16E* expressed from the *pMET25* promoter were investigated by confocal microscopy. Representative fluorescent images (upper panels) and transmitted light images (lower panel) are shown. **(b)** Ess1 binds Tpk1 and Tpk2 in GST pull-down experiments, lysates of cells that express chromosomally ProA-tagged Tpk1 and Tpk2 were subjected to *in vitro* pull-down assays with either GST-Ess1 or GST as a control.

These data corroborates the fact that the WW domain determines substrate specificity and thereby the subcellular loalization of Ess1, consistent with recent reports for Pin1 (Lu et al., 2002; Rippmann et al., 2000). We then further analyzed whether Ess1 localization can be regulated by phosphorylation of Ser16. The phospho-deficient Ser16 (*GFP-ess1-S16A)* mutant variant is still localized predominantly to the nuclear compartment as wild-type Ess1 (*GFP-ESS1)*. By contrast, the phospho-mimicking mutant (*GFP-ess1-S16E)* was distributed diffusely in the cell resembling a Ess1 version lacking the WW domain (Figure 15a, two panels to the right). These results demonstrate that the Ess1 phospho-mimicking (S16E) variant is not only non-functional (Figure 14b, c) but also the normal subcellular localization of Ess1 is disrupted.

Previously, it was reported (Lu et al., 2002) that Pin1 Ser16 phosphorylation might be regulated *via* cAMP (cyclic AMP)-dependent protein kinase A (PKA) *in vitro*. PKA treatment resulted in dissociation of Pin1 from MPM2 antigens, suggesting that the phosphorylation might actively disrupt the interaction between Pin1 and a substrate (Lu et al., 2002). *In vitro* pull-down assays with recombinant GST-Ess1 and the yeast PKA orthologues Tpk1 and Tpk2 indeed show binding of Ess1 to the PKA orthologues (Figure 15b).

4.3 Pin1 controls the degree of substrate ubiquitylation and thereby determines substrates fate

Regulation of protein activity often involves signaling through post-translational modifications, these either induce a structural change in the protein, thereby altering its activity, or induces the exposure of sites recognized by regulatory proteins. Pin1 induced conformational changes have profound effects on the function of many substrates (Lu et al., 2002; Lu et al., 2007). Intriguingly, Pin1 was in some cases reported to correlate with a change in substrate stability linked to the UPS (Liao et al., 2009; Lu et al., 2007; Zheng et al., 2002; Zacchi et al., 2002). Therefore it was tempting to speculate that Pin1 might regulate Spt23 stability by a S/T-Pro-directed post-phosphorylational *cis/trans* isomerization.

4.3.1 Ess1 determines the fate of Spt23 by a *cis/trans* isomerization

To test whether a Pin1-mediated *cis/trans* isomerization of Spt23 leads to a change in Spt23 stability we followed the steady-state levels of Spt23 over time in two different temperature-sensitive (ts) mutants expressing Ess1 variants: one with alteration in the WW domain (*ess1-W15R*), the other with an amino acid substitution in the catalytic (*ess1-H164R*) domain. Indeed, we found that the steady-state level of Spt23 was drastically reduced over time in two different temperature-sensitive (ts) mutants expressing Ess1 variants after the cells were shifted to the non-permissive temperature (Figure 16a).

When we further characterized Spt23 processing in detail in an expression shut-off experiment, we observed a rapid decay of Spt23 levels in the *ess1* mutants compared to WT cells at the restrictive temperature (Figure 16b). Notably, p90, the active transcription factor, rapidly disappeared to diminutive levels over time, because the p120 precursor was quickly turned over by degradation (Figure 16b).

The quantification (Figure 16c) further reveals the drastic differences in the life-span of Spt23 p120 in the *ess1* mutants compared to WT (*ESS1*): In WT cells Spt23 p120 is efficiently processed to the active transcription factor p90, indicated by increasing p90 and decreasing p120 levels over time. By contrast, in the *ess1* mutants p120 as well as p90 levels decreased rapidly over time.

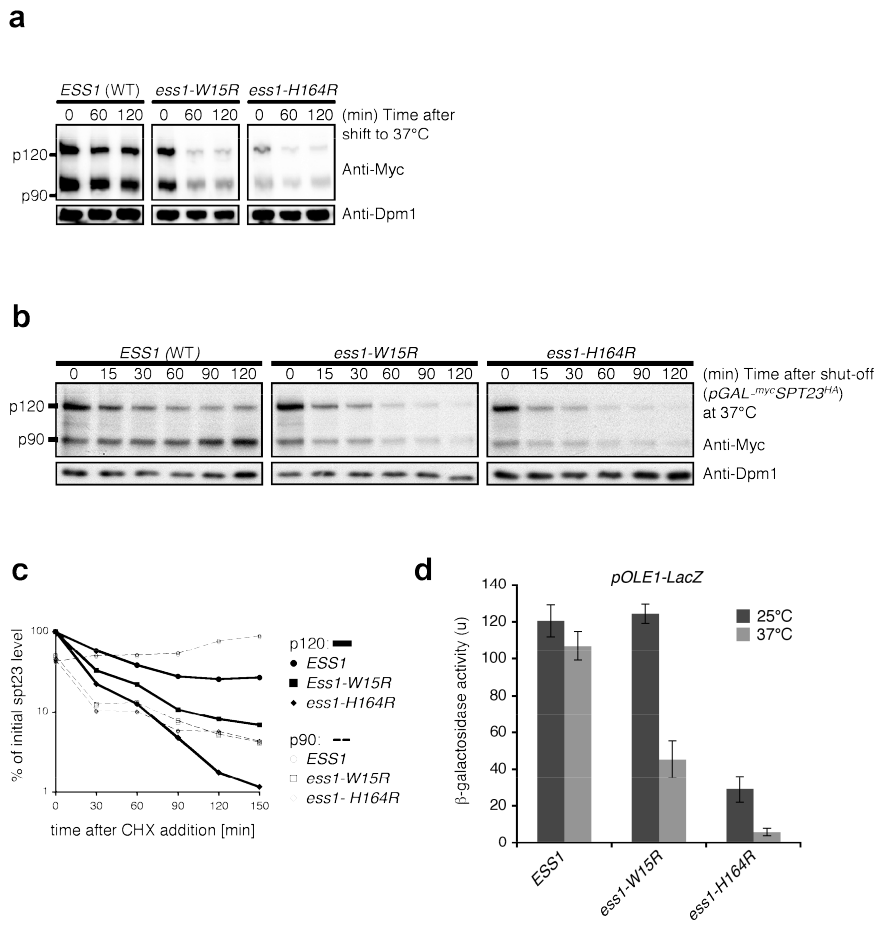

Figure 16. Ess1 determines Spt23 fate by a *cis/trans* isomerization. (a) Steady state levels of $^{myc}Spt23^{HA}$ expressed in WT, *ess1-W15R*, and *ess1-H164R* mutant cells. Samples were taken (0-120 min) after shifting to the non-permissive temperature (37°C) of the ts-mutants and analyzed by anti-myc immunoblots (upper panel). Dpm1 was used as a loading control (lower panel). **(b)** Spt23 p120 is short lived in *ess1* mutants. Expression shut-off experiments with WT, *ess1-w15r*, and *ess1-h164r* mutant cells expressing $^{myc}Spt23^{HA}$ by the *pGAL1-10* promoter. Cells were grown in a galactose-containing medium to an OD_{600} of 0.4 at 23°C and shifted for another 2 h to 37°C. After adding glucose and cycloheximide (to block translation) to the medium, samples were taken at the indicated time points, and Spt23 levels were analyzed by anti-myc immunoblots (upper panel). Dpm1 was used as loading control (lower panel). The graph **(c)** shows the quantification of the respective protein levels (100% was defined as p120 levels at time point 0). **(d)** Ess1 regulates *OLE1* transcription via Spt23. *ESS1* (WT), *ess1-W15R*, and *ess1-H164R* cells expressing β-galactosidase by the *OLE1*-promoter (*pOLE1-LacZ*) were grown either at 23°C (dark grey) or for 2 h at 37°C (light grey). Samples were withdrawn and β-galactosidase activity was measured by an ONPG (ortho-nitrophenyl-β-galactoside) assay. Graphs represent the mean of three independent experiments done in triplicates. Standard deviations are indicated. A mutated *pOLE1* promoter is inactive in this assay (right graph).

The reduction in p90 levels in the two *ess1* mutants, especially in the catalytic ts-mutant *ess1-H164R*, nicely correlated with their capacity to drive *OLE1* transcription in cells, as measured by reporter assays (Figure 16d).

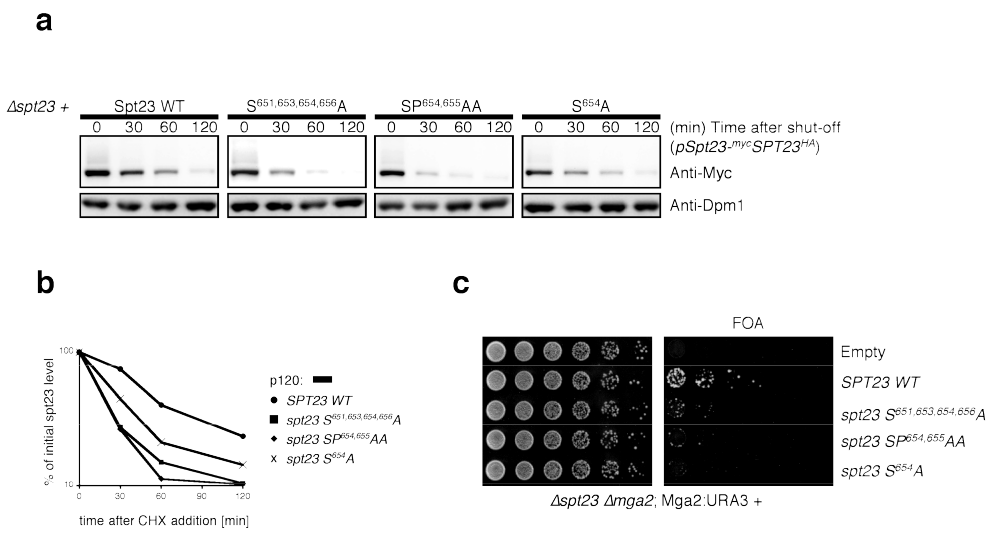

Figure 17. Spt23 variants with alterations in the Ess1 binding site are short lived. (a) Spt23 p120 is short lived when either serine (S-A), proline (P-A) or both residues (SP-AA) are mutated to alanine in the Ess1 binding site of Spt23. Expression shut-off experiments with $^{myc}Spt23^{HA}$ WT or Spt23 variants with serine-to-alanine (S-A) or proline-to-alanine (P-A) alterations expressing $^{myc}Spt23^{HA}$ under its endogenous promoter in a *Δspt23* deletion strain. Cells were grown to an OD_{600} of 0.8 in selective medium, after adding cycloheximide (to block translation) to the medium, samples were taken at the indicated time points, and Spt23 p120 levels were analyzed by anti-HA immunoblots (upper panel). Dpm1 was used as loading control (lower panel). The graph **(b)** shows the quantification of the respective protein levels (100% was defined as p120 levels at time point 0). **(c)** Spt23 variants with serine-to-alanine (S-A) or proline-to-alanine (P-A) alterations in the Ess1 binding site are unable to complement the otherwise inviable *Δspt23 Δmga2* deletion strain.

Interestingly, re-examining the Spt23 variants with mutations in the Ess1-binding site for p120 (Figure 9c, d) in fact revealed that Spt23 is short lived in these mutants (Figure 17 a,b). Changing the respective serine and proline to alanine (SP654,655AA) drastically reduced p120 levels. Also changing all 4 serines to alanine (SSSS651,653,654,656AAAA) reduced Spt23 p120 half-life significantly. Changing only one serine to alanine (S651A) showed a milder reduction in p120 levels, again in line with the binding assays (Figure 9c, d) most likely due to "phosphorylation-site hopping" (Figure 17a, b). Notably, these residues are indeed crucial for Spt23 activity as strains expressing these mutant proteins as the only source of Spt23 are not viable (Figure 17c). The explanation is most likely because of the fact that the encoded proteins are short-lived as shown above (Figure 17a, b) in expression shut-off experiments.

These results suggest that the *Δess1* null mutant is inviable because the Spt23 transcription factor precursor is completely degraded instead of being processed in these cells.

4.3.2 Pin1 acts as a binary fate switch by controlling Spt23 ubiquitylation

The activity of the peptidyl-prolyl isomerase Pin1 directly influences the stability of Spt23 and therefore the fate of the transcription factor. Hence, the question was whether Ess1 acts by controlling the ubiquitylation status of Spt23 and furthermore, if Ess1 might act as a binary fate switch by inducing a *cis/trans* conformational change in the polypeptide chain that is translated into a change in substrate ubiquitylation and thereby substrate fate.

To directly test the influence of Ess1 on Spt23 ubiquitylation, we compared the Spt23 ubiquitylation patterns of WT and *ess1-ts* mutants (*ess1-W15R*). Cells that express ^{myc}Spt23^{HA} (from the *pGAL1-10* promoter) and His-tagged ubiquitin (from the *pMET25* promoter) were shifted for 3 hours to the non-permissive temperature (37°C) of the *ess1* mutant and ubiquitin conjugates were isolated by denaturing Ni-NTA pull-down. Whereas Spt23 ubiquitylation occurs only at low levels on both p120 and p90 forms in WT cells (Hoppe et al., 2000; Rape et al., 2001), Spt23 p120 ubiquitylation was dramatically induced in the *ess1-W15R* mutant after shifting to the non-permissive temperature. This drastic induction in ubiquitylation was accompanied by rapid Spt23 decay (Figure19a, see also quantification below).

To address the involvement of the proteasome directly, we followed Spt23 p120 ubiquitylation in the *ess1-W15R* mutant in a time course experiment with or without the proteasome inhibitor MG132. After an initial shift of 2 hours to the non-permissive temperature (Figure 18b, lane 2 from left) cells were either treated with (Figure 18b, lane 6-8) or without MG132 (Figure 18b, lane 3-5) and ubiquitin conjugates were isolated by denaturing Ni-NTA pull-down. As observed before (Figure 18a) Spt23 p120 ubiquitylation is strongly induced in the *ess1-ts* mutant after 2 hours. Notably, Spt23 ubiquitylation stays high over time when the cells were treated with the proteasomal inhibitor MG132 (Figure 18b, lane 6-8), while the p120 ubiquitylation decreased over time in samples untreated for MG132, indicative of ongoing degradation of p120 in the *ess1-ts* mutant.

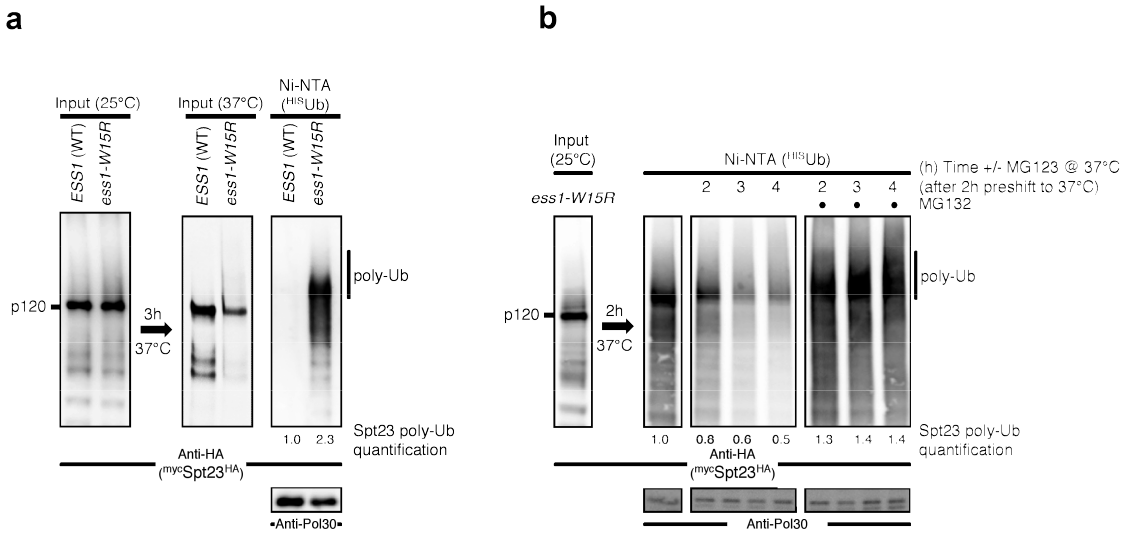

Figure 18. Ess1 controls the degree of substrate ubiquitylation. (a) Ubiquitylation of HA-tagged Spt23 protein (p120) of WT and the *ess1-W15R* mutant. Cells expressing ^{myc}SPT23^{HA} (glucose-repressible *pGAL1-10* promoter) and HIS-tagged ubiquitin (^{HIS}Ub; *pMET25* promoter) were grown to an OD_{600} of 0.4 at 23°C (Input 23°C) and shifted (arrow) to 37°C and grown for another 3h (Input 37°C). Thereafter, ^{HIS}Ub-modified proteins were isolated under denaturing conditions by Ni-NTA pull-down. Inputs and precipitates were probed with HA-specific antibodies (upper panels). To normalize pull-down efficiency, cells (10 OD_{600}) expressing HIS-tagged Pol30 were added to the samples before harvesting, and the blots were re-probed with an antibody against Pol30 (lower panel). Polyubiquitylated Spt23 p120 is indicated, the quantification below refers to the level of polyubiquitylated Spt23 p120. **(b)** Ubiquitylation time course of HA-tagged Spt23 protein (p120) from *ess1-w15r (Δpdr5* background) after addition of the proteasomal inhibitor MG132. Cells expressing ^{myc}SPT23^{HA} (*pGal1-10* promoter) and HIS-tagged ubiquitin (^{HIS}Ub; *pADH1* promoter) were grown to an OD_{600} of 0.4 at permissive temperature (25°C) and then preshifted to the permissive temperature (37°C) for 2h. Cell were then treated with or without proteasomal inhibitor MG132, samples were withdrawn at the indicated time points and subjected to ^{HIS}Ub pull-down assays under denaturing conditions (Identification of proteins as in Figure 18a). The quantification below refers to the level of polyubiquitylated Spt23 p120. Note that polyubiquitin levels of p120 are unstable most likely due to ongoing proteasomal degradation, while the p120 poyubiquitin levels are stable in cells treated with MG132.

We further addressed the question, whether this striking boost of Spt23 precursor ubiquitylation is directly regulated by Ess1 activity by gradually reducing Ess1 levels in cells and following p120 ubiquitylation. Therefore a *Δess1* strain was constructed that is rescued by a galactose-inducible Ess1 (*Δess1; pGAL1-10-ESS1)*, additionally expressing His-tagged ubiquitin (from the *pADH1* promoter). This strains was cultivated on selective plates at low galactose/raffinose (0.5% / 1.5%) levels for cell survival. This experimental setup bears the advantage that it does on rely on a temperature shift, to reduce Ess1 levels we simply change the type of carbon-source from galactose to glucose. Cells were then shifted to high glucose/raffinose medium.

We followed Spt23 p120 precursor ubiquitylation in a glucose shut-off experiment where the expression of Ess1 is gradually reduced by turning off its expression after addition of glucose to the cells. Ubiquitin conjugates were then isolated by denaturing Ni-NTA pull-down. Remarkably, after shutting off Ess1 expression, ubiquitylation of Spt23 occurs rapidly and steadily in cells: low levels of p120 ubiquitylation at the beginning with high levels of Ess1 followed by massive polyubiquitylation of p120 over time with low levels of Ess1. These data indicate that the degree of Spt23 p120 ubiquitylation is reversely correlated with Ess1 activity in cells (Figure 19a, b), Ess1 crucially controls the balance between Spt23 processing *versus* complete degradation through regulating substrate ubiquitylation.

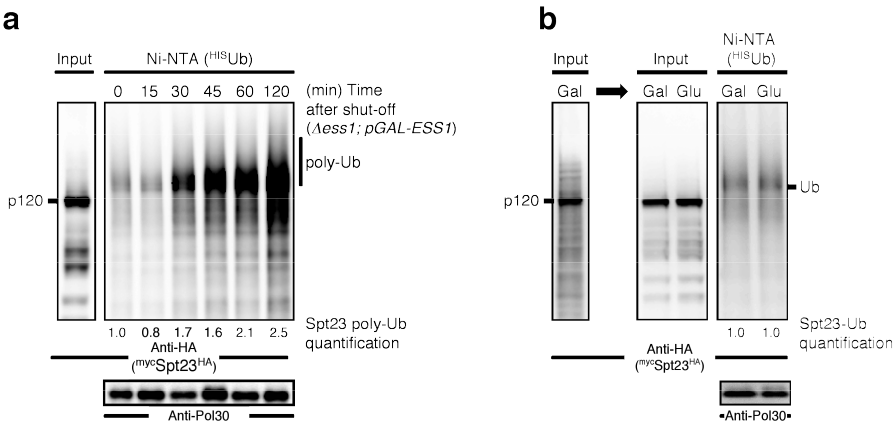

Figure 19. The degree Spt23 p120 ubiquitylation is correlated with Ess1 activity. (a) Lowering Ess1 levels results in enhanced Spt23 ubiquitylation. Null mutant of ESS1 (*Δess1*) complemented by a construct that expresses ESS1 from the GAL1-10 promoter (*pGAL-ESS1*) were pregrown to an OD_{600} of 0.5 at 30°C in a 1.5 % raffinose, 0.5 % galactose-containing medium to express *ESS1*. To turn off *ESS1* expression, cells were then shifted to a 2% glucose-containing medium and samples were withdrawn at the indicated time points. (Identification of proteins as in Figure 13a). The quantification below refers to the level of polyubiquitylated p120 Spt23. **(b)** Control experiment for Figure 19a. To exclude a side-effect of the nutritional downshift from a galactose to a glucose-containing medium, a wild-type strain expressing HIS-tagged ubiquitin and $^{myc}Spt23^{HA}$ was grown to an OD_{600} of 0.5 at 30°C in a 1.5 % raffinose, 0.5 % galactose containing medium, the cells were then shifted either to a 2 % galactose or 2 % glucose-containing medium, and samples were withdrawn and subjected to isolation of ubiquitylated proteins as in Figure 18a. The quantification below refers to the level of polyubiquitylated p120 Spt23. Note that this media shift does not induce Spt23 ubiquitylation.

4.4 Pin1 acts as a fate switch by controlling p53 ubiquitylation

The peptidyl-prolyl isomerase Pin1 is highly conserved and has been linked to important roles in numerous cellular processes including differentiation, cell proliferation, the immune response, and also Alzheimer's disease. Among the known substrates are β-catenin, c-Myc, cyclins, tau and p53, which are all involved in fate determining pathways in the cell (Lu et al., 2007; Yeh et al., 2007)). The p53 protein is widely known as the guardian or gatekeeper of the genome because of its crucial role in coordinating cellular responses to genotoxic stress (Lane, D.P., 1992; Levine, A.J., 1997). The tumor suppressor function of p53 relies on its transcriptional activity, which is modulated by several interactions with regulatory proteins, although the precise mechanism of p53 induced activation is not fully understood, they are generally thought to involve post-translational modifications, including acetylation, SUMOylation, phosphorylation and ubiquitylation. Interestingly, the prolyl isomerase Pin1 has a central role in transducing phosphorylation of p53 into conformational changes that affect p53 stability and function (Ryo et al., 2002; Zhang et al., 2002; Zacchi et al., 2002). However, less clear from these studies is how Pin1 affects p53 function and stabilization. Thus we wondered whether Pin1, in analogy to its role in the OLE pathway, might as well regulate the degree of p53 ubiquitylation and thereby the fate of p53 in cells.

4.4.1 Influence of Pin1 on p53 ubiquitylation *in vivo*

To address the question whether p53 is regulated analogous to Spt23 in the OLE pathway we investigated the influence of Pin1 on p53 ubiquitylation and stability *in vivo* in mammalian wild-type p53 containing U2-OS cells.

In early studies it was shown that the p53 steady state level was reduced upon treatment of cells with the Pin1 inhibitor juglone (5-hydroxy-1,4-naphtoquinone; Paulsen & Ljungman, 2005), but without addressing the mechanistic details of Pin1 as a PPiase and its possible impact on p53 regulation. In U2-OS cells, p53 was found to be predominantly unmodified, but a minor fraction was ubiquitylated by usually not more than four ubiquitin moieties, corresponding to mono/oligoubiquitylated p53. Taking advantage of the Pin1 inhibitor juglone, U2-OS cells were treated either with 5 μM or 10 μM juglone for 2 h or with 5 μm juglone for

different timepoints. Treating the U2-OS cells with juglone surprisingly resulted in the appearance of a diffuse population of higher molecular mass forms of p53. In contrast, the pool of mono/oligoubiquitylated species of p53 disappeared, suggesting increased polyubiquitylation of p53 by Pin1 inhibition (Figure 20a, upper panel). Notably, the induction of p53 polyubiquitylation by juglone was dose (increasing concentration) and time dependent and was in both cases accompanied by lower steady state levels of p53 as indicated by quantifications of p53 steady state (Figure 20a, lower panels).

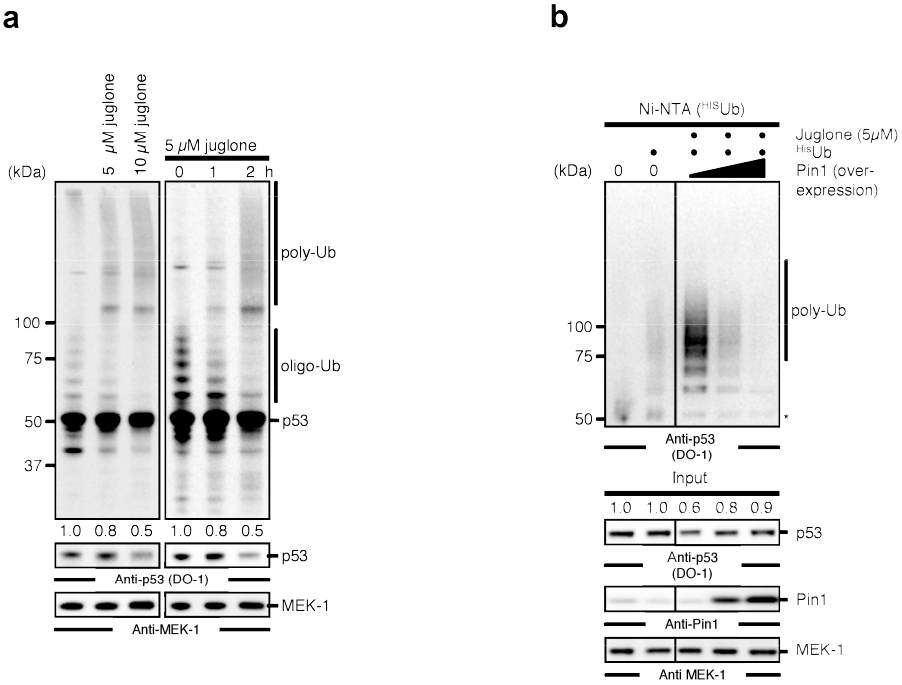

Figure 20. Pin1 controls the degree of p53 ubiquitylation *in vivo*. (a) Pin1 inhibition by juglone induces p53 polyubiquitylation and degradation. U2OS cells were either untreated or treated with 5 or 10 µM juglone for 2 h (left), or with 5 µM juglone for the time indicated (right). Cell lysates were subjected to immunoblot analysis for p53 (upper and middle panels) or MEK-1 as a control (lower panel). The middle panel shows a weaker exposure of the p53 blot to reveal p53 decay upon juglone treatment together with the quantification (initial level set as 1.0). Pools of oligo- (oligo-Ub) and polyubiquitylated (poly-Ub) p53 are indicated. **(b)** Specificity of juglone for Pin1. U2OS cells were transiently transfected with His-tagged ubiquitin (^{HIS}Ub) in combination with increasing amounts of Pin1. 72 h after transfection, cells were either left untreated or treated with 5 µM juglone for 2 h and lysates were subjected to Ni-NTA pull-down to isolate ubiquitin-conjugates. This material was analyzed by immunoblotting using an antibody specific for p53 (DO-1; first panel and p53 Input). Inputs for p53, Pin1, and MEK-1 (loading control) are shown by immunoblots. Cells not treated with juglone (left panels) express either HA-tagged (lane 1) or His-tagged ubiquitin (lane 2). (The figure represents data of one gel.) Note that Pin1 overexpression reverts the juglone-induced effects on p53 ubiquitylation and protein levels (quantification is shown above; p53 levels without juglone treatment is set as 1.0).

Juglone was described to act in a stoichiometric way on inhibiting Pin1 (Hennig et al., 1998). Hence, by increasing the amount of Pin1 by Pin1 overexpression in juglone treated cells we should be able to reverse the juglone-induced p53 polyubiquitylation and restore p53 stability. To perform this experiment, U2-OS cells were transfected with His-tagged ubiquitin (^{HIS}-Ubi, or ^{HA}Ubi as a control) in combination with increasing amounts of WT Pin1. After juglone treatment cells were harvested and lysates were subjected to denaturing Ni-NTA pull-down to isolate ubiquitin-conjugates. Importantly, the observed effect of juglone on p53 polyubiquitylation *in vivo* was indeed specific for Pin1, as it could be completely repressed by additionally overexpressing Pin1 in juglone treated cells in a dosage dependent manner (Figure 20b, upper panel). Furthermore, juglone treatment resulted in a significant decrease of celluar p53 levels which were again stabilized upon Pin1 overexpression (Figure 20b, p53 Input levels).

Beause p53 levels are controlled by nuclear degradation and monoubiquitylation of p53 serves as a signal for p53 nuclear export (Li et al., 2003; Brooks et al., 2004), we followed the fate of p53 in cells by immunofluorescence microscopy. As expected, p53 was readily detected in the nucleus under normal conditions in U2-OS cells (Figure 21a, two upper panels).

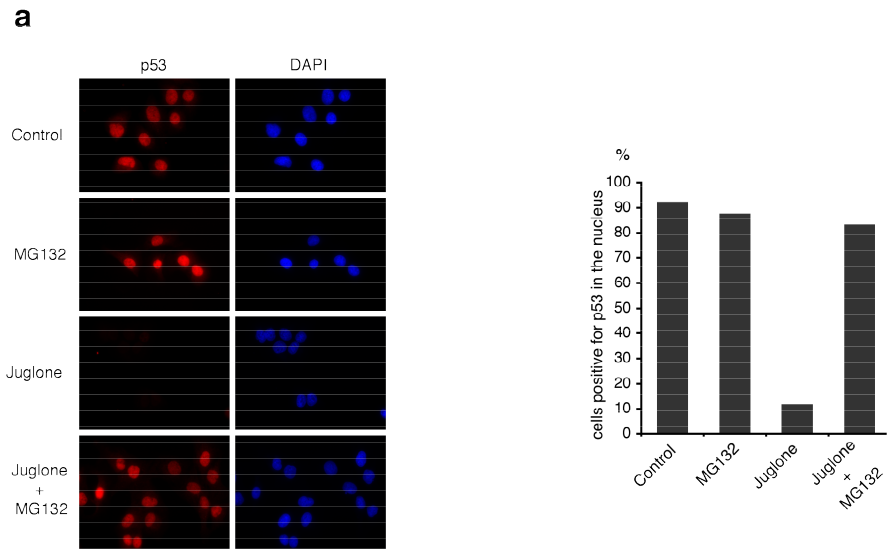

Figure 21. Pin1 controls the degree of p53 ubiquitylation *in vivo*. (a) Pin1 controls p53 levels *in vivo*. Immunofluorescence microscopy of cells using an anti-p53 antibody (DO-1) antibody. DAPI staining visualizes the nucleus. Cells were either untreated (Control), treated with the proteasome inhibitor MG132, juglone, or both. The graph shows the quantification of the data. The numbers represent p53-positive cells showing nuclear localization relative to the total number (in average 200) of cells counted.

However, upon treatment with the Pin1 inhibitor juglone, p53 disappeared dramatically, suggesting rapid degradation. Remarkably, when these cells were pretreated with the proteasome inhibitor MG132 and then the Pin1 inhibitor juglone was applied, p53 again accumulated in the nucleus, indicating that juglone-induced p53 polyubiquitylation indeed targets the protein to nuclear degradation (Figure 21a, also see quantification). In conclusion, these data demonstrate that Pin1 regulates p53 levels *in vivo* through inhibition of ubiquitin-dependent degradation in cells.

4.4.2 Influence of Pin1 on p53 ubiquitylation *in vitro*

Recent studies demonstrate a differential control of p53 activity by Mdm2: whereas low levels of Mdm2 activity induce monoubiquitylation and nuclear export of p53, high Mdm2 levels promote p53 polyubiquitylation and nuclear degradation (Li et al., 2003; Brooks et al., 2006).

We established an in vitro ubiquitylation system for p53 to directly test the influence of Pin1 on the degree of p53 polyubiquitylation. We therefore employed p53 *in vitro* ubiquitylation assays using two approaches: we either used purified, recombinant p53 (Figure 22 a-c), or p53 expressed in a coupled *in vitro* transcription/translation system (p53 IVT; Figure 23a, b). As was previously shown, polyubiquitylation of p53 *in vitro* is efficiently triggered by high levels of the p53-specific E3 ligase Mdm2 (Figure 22a; Li et al., 2003), we therefore used high Mdm2 levels in the *in vitro* ubiquitylation assays to effectively trigger p53 polyubiquitylation and then tested the direct influence of recombinant Pin1 or a Pin1 mutant in this *in vitro* system.

Remarkably, pre-incubation of p53 with recombinant Pin1 strongly inhibited this Mdm2-dependent reaction (Figure 22b). Interestingly p53 polyubiquitylation negatively correlated with the amount of Pin1 added to the reaction, whereas the addition of an inactive Pin1 mutant variant had no influence on the p53 polyubiquitylation levels (Figure 22c).

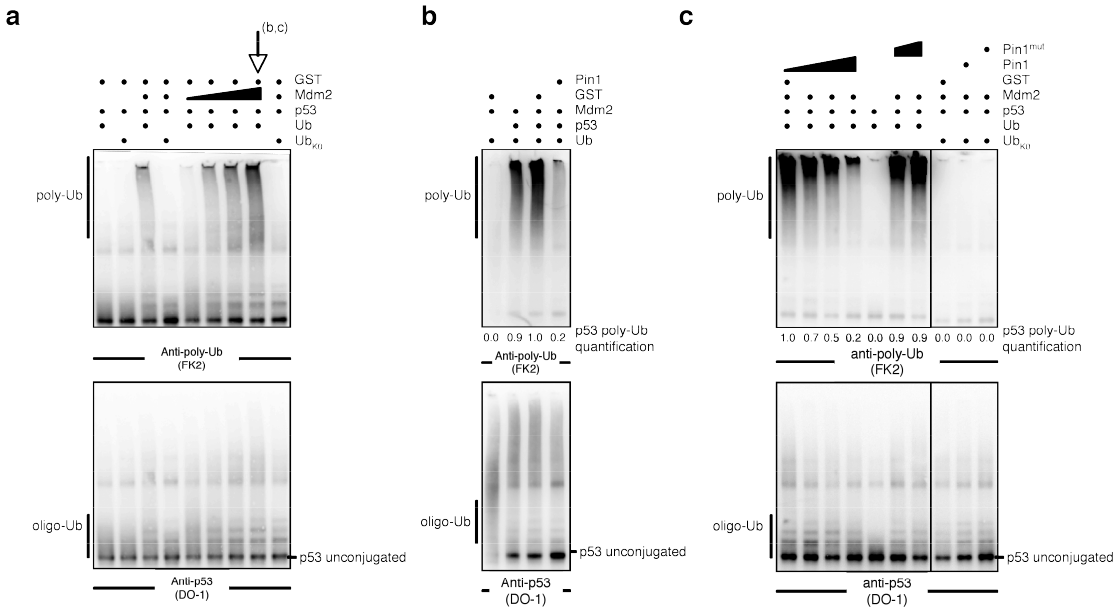

Figure 22. Pin1 controls the degree of p53 ubiquitylation *in vitro*. **(a)** Polyubiquitylation of recombinant p53 *in vitro* is triggered by high concentrations of Mdm2. Recombinant p53 was incubated with ubiquitin (Ub), mammalian E1, E2 (UbcH5), ATP, and increasing amounts of of E3 (Mdm2, lane 5 – 8, 0 - 202.5 ng) to induce p53 polyubiquitylation. A lysine-less ubiquitin variant (Ub_{K0}) that fails to form polyubiquitin chains (lanes 2, 4 and 9) was used to control for p53 polyubiquitylation. Polyubiquitylated p53 (poly-Ub) is detected by immunoblotting with FK2 antibodies (upper panels). Oligoubiquitylated p53 (Ub) was detected by DO-1 antibodies (lower panels). The high concentration of Mdm2 that leads to p53 polyubiquitylation (lane 8) was used for the experiments shown in Figure 22b, c. **(b)** Pin1 inhibits polyubiquityation of p53 in an *in vitro* assay with recombinant proteins. Recombinant p53 (lane 2-4) was either pre-incubated (1h, 4°C) with recombinant Pin1 (lane 4), or GST (lane 3), respectively and then incubated with ubiquitin (Ub), mammalian E1, E2, ATP, and high concentration of E3 (Mdm2) that induces p53 polyubiquitylation (Figure 22a, lane 8). Lane 1 represents a control reaction without p53 (recombinant GST was used instead) to show that the observed polyubiquitin conjugates (detected by the FK-2 antibody in lane 2-4 upper panel) are conjugates with p53. Polyubiquitylated p53 (poly-Ub) is detected by immunoblotting with FK2 antibodies (upper panels). Oligoubiquitylated p53 (Ub) was detected by DO-1 antibodies (lower panels). The quantification below refers to the level of polyubiquitylated p53 detected by the FK2 antibody (upper panel). **(c)** Pin1 inhibits polyubiquityation of p53 in an *in vitro* assay with recombinant proteins. Recombinant p53 was preincubated (1h, 4°C) with either GST (controls, lanes 1 and 8), increasing amounts of recombinant Pin1 (Pin1, lane 2 - 4), or a Pin1 mutant variant ($Pin1^{mut}$, lanes 6 and 7), respectively and then incubated with ubiquitin (Ub), mammalian E1, E2, ATP, and a high concentration of E3 (Mdm2) that induces p53 polyubiquitylation (Figure 22a, lane 8). A lysine-less ubiquitin variant that fails to form polyubiquitin chains (Ub_{K0}, lane 8 - 10) was used to control for p53 polyubiquitylation (right panels). Figure 20b, Lane 1 represents a control reaction without p53 (recombinant GST was used instead) to show that the observed polyubiquitin conjugates (detected by the FK-2 antibody in the upper panel) are conjugates with p53. Polyubiquitylated p53 (poly-Ub) is detected by immunoblotting with FK2 antibodies (upper panels). Oligoubiquitylated p53 (Ub) was detected by DO-1 antibodies (lower panels). The quantification below refers to the level of polyubiquitylated p53 detected by the FK2 antibody (upper panel).

Due to the fact that Pin1 is a phosphorylation driven peptidyl-prolyl *cis/trans* isomerase we conducted further p53 *in vitro* ubiquitylation assays with p53 expressed in a coupled *in vitro* transcription/translation system (p53 IVT), in such a system all necessary p53 modifications can take place (Figure 23a). Notably, also in this setup recombinant Pin1 efficiently inhibited the Mdm2-dependent polyubiquitylation reaction in a dose-dependent manner (Figure 23b). By contrast, addition of an inactive Pin1 variant had again no influence on p53 ubiquitylation levels. To test the reactivity of the FK2 antibody to specifically detect p53 polyubiquitylation, all *in vitro* ubiquitylation reactions were additionally performed using a lysine-less ubiquitin variant that fails to form polyubiquitin chains (Ub_{K0}) and indeed FK2 reactivity was only observed with WT ubiquitin.

These findings strongly suggest that Mdm2 and Pin1 exert opposing effects on p53 polyubiquitylation and indicate that the two proteins are possibly rivals in cells.

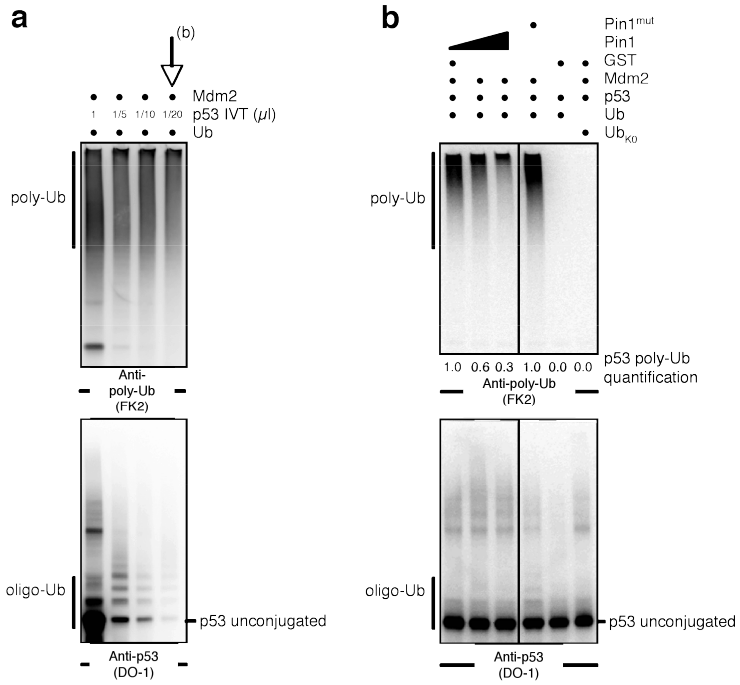

Figure 23. Pin1 controls the degree of p53-IVT ubiquitylation *in vitro*. (a) Titration of the amount of p53 expressed in a coupled transcription-translation system (p53 IVT) used for *in vitro* ubiquitylation assays. High amounts of E3 (Mdm2, lane 1-4) was incubated with ubiquitin (Ub), mammalian E1, E2 (UbcH5), ATP, and decreasing amounts of p53 IVT to induce p53 IVT polyubiquitylation. Polyubiquitylated p53 (poly-Ub) is detected by immunoblotting with FK2 antibodies (upper panels). Oligoubiquitylated p53 (Ub) was detected by DO-1 antibodies (lower panels). The concentration of p53 IVT that leads to p53 mono- and robust polyubiquitylation (lane 4) was used for the experiments shown in Figure 23b.

Figure 23 continued

(b) *In vitro* ubiquitylation of p53 expressed in a coupled transcription-translation system. *In vitro* translated p53 (IVT p53; see Figure 23a) was preincubated (1 h, 4°C) either with GST, increasing amounts of recombinant Pin1 wild-type or Pin1 mutant (same as in Figure 22c). Ubiquitylation reaction was done with E1, E2, high E3 and ATP as in Figure 20c. Pools of oligo- (Ub) and polyubiquitylated (Poly-Ub) p53 (*in vitro* translated) are indicated. Shown are immunoblots developed with FK2 antibodies specific for polyubiquitylated proteins (top panel) and anti-p53 antibodies (DO-1; lower panel). The quantification refers to the level of polyubiquitylated p53 detected by the FK2 antibody. A lysine-less ubiquitin variant that fails to form polyubiquitin chains (Ub_{K0}) was used to control for p53 polyubiquitylation (right panel, last lane).

5 Discussion

The diversity and functional repertoire of an organism's proteome is extended greatly by means of covalent post-translational modifications. Since the discovery of ubiquitin in the mid-1970s, the covalent attachment of ubiquitin to protein substrates is the classical and so far best understood example of targeting proteins for degradation. The canonical view of the ubiquitylation cascade is, however, oversimplified. The ability to form a polyubiquitin chain depends on the type of the ligase (e.g. whether it acts processively) or even on the substrate. However, the switch from mono/oligoubiquitylation to polyubiquitylation (termed here polyubiquitylation switching) is much more complex and stems from the existence of a large number of enzymes and auxiliary factors that finally catalyze the polyubiquitylation reaction resulting in either proteolytic or non-proteolytic function.

The most important finding of this study is that the peptidyl-prolyl *cis/trans* isomerase Pin1 regulates the degree of substrate ubiquitylation. Pin1 acts directly at the heart of the ubiquitylation reaction and represents a completely different mechanism of polyubiquitylation switching. Pin1 most likely acts by translating the induced structural change ("kink") in a substrate into a change of E3 ligase affinities and therefore presents a so far unrecognized regulatory mechanism in the ubiquitin pathway.

5.1 Peptidyl-prolyl *cis/trans* isomerases

In folded proteins, peptidyl-prolyl peptides have the unique property of existing in two distinct isomers, *cis* or *trans*. This property provides a potential backbone switch in the polypeptide chain and can be controlled by *cis/trans* isomerization. This intrinsically slow conversion is catalyzed by a ubiquitous class of enzymes termed peptidyl-prolyl *cis/trans* isomerases (PPIases), which can be divided into three major families: the cyclophilins and the FK506-binding proteins (FKBPs), also called conventional PPIases and additionally a recently identified third family of PPIases, which can be further subdivided into two subfamilies based on their substrate specificity, parvulin-type and Pin1-type PPiases. Although structurally unrelated in their primary sequences and three-dimensional structures, all PPIases are able to

catalyze a similar reaction (Lu et al., 1999). Furthermore, a role for PPIases in folding of newly synthesized proteins was implied and indeed chaperone-like activity has been associated with several PPIases (Kruse et al., 1995; Schmid, 1995).

Isomerization of S/T-Pro bonds between *cis* and *trans* can alter the local, and even the global protein structure. The structural difference between the two isoforms (*cis/trans*) also constitutes a fundamental molecular change that might be translated into different functional states, or distinguish between intermolecular binding partners (Figure 24). In fact peptidyl-prolyl *cis/trans* isomerases form a special class of enzymes in many respects, because unlike most other post-translational modifications *cis/trans* isomerization is a non-covalent post-translational modification.

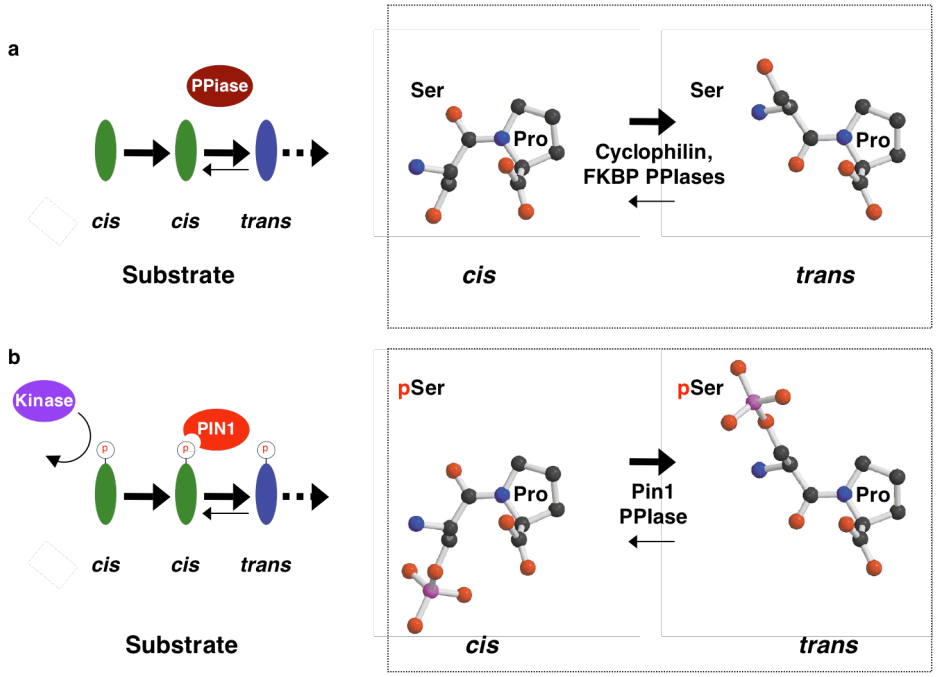

Figure 24. The conformation and regulation of proline-directed *cis/trans* isomerization. Proline is unique among amino acids in its ability to adopt either the *cis* or *trans* state, due to its five-membered ring in the peptide backbone. Peptidyl-prolyl isomerases catalyze the intrinsically rate-limiting process Based on substrate specificity, PPIases can be divided into **(a)** phosphorylation-indipendent, Cyclophilins and FKPBs and **(b)** phosphorylation-dependent enzymes, Pin1 and Pin1-type enzymes.

The reversible phosphorylation of proteins on Ser/Thr residues immediately preceding Pro (S/T-Pro) is a major regulatory mechanism. Remarkably, phosphorylation of S/T-Pro motifs not only further slows the uncatalyzed proline conversion from *cis* to *trans*, it furthermore renders the peptide bond resistant to the catalytic action of the two conventional PPIases (cyclophilins and FKBPs) and parvulin-type PPIases. The Pin1-type PPIases are so far the only known PPIases

that are specific for phosphorylated S/T-Pro bonds (Yaffe et al., 1997; Ranganathan et al., 1997; Lu et al., 2002).

The conventional PPIases, cyclophilins and FKBPs, play a fundamental role in the regulation of several cellular processes including the immune-response. Surprisingly, all known members of the cyclophilins and FKBP family can be entirely disrupted without any significant phenotype. In fact, evidence for the biological importance of the enzymatic activity in these PPIases remains elusive (Lu et al., 1996; Dolinsky et al., 1997).

By contrast, Pin1 and its homologs have a well-defined function in phosphorylation signaling by specifically targeting phosphorylated S/T-Pro bonds, an activity that cannot be effectively catalyzed by conventional or parvulin-type PPIases. Furthermore, the requirement of substrate phosphorylation for Pin1-type PPIases isomerization reveals an additional level of regulation and specificity, coupling the isomerization activity to the tightly controlled activities of proline-directed kinases (and phosphatases). This phosphorylation specificity implies that Pin1 plays a unique role in the regulation of signaling cascades that function via proline-directed phosphorylation (Figure 24).

Notably, Pin1 was reported to be subject of post-translational modification itself. Pin1 PPIase catalytic activity is modulated via phosphorylation at S16, which lies in the center of the phspho-binding pocket of its WW domain and prevents Pin1 interaction with its substrate (Lu et al., 2002) and demonstrates an additional mechanism for regulation of the phosphorylation-specific isomerase Pin1.

5.2 Pin1 acts a fate switch in the UPS: mono/oligoubiquitylation *versus* polyubiquitylation

Polyubiquitin-chain assembly is usually a processive reaction that requires the canonical ubiquitin conjugation machinery E1, E2, E3. However, the process of polyubiquitylation switching (mono/oligoubiquitin to polyubiquitin) is mediated by a variety of dedicated factors that mediate ubiquitin chain elongation. Enzymes possessing the specific activity to catalyze polyubiquitin-chains in collaboration with E1, E2 and E3 had been coined E4 enzymes (Hoppe et al., 2005). It seems likely that these different mechanisms to switch from mono/oligoubiquitylation to polyubiquitylation are exploited under distinct physiological settings and must be

therefore tightly regulated. Certain substrates (e.g. Spt23 or p53) are mono/oligoubiquitylated by an archetypical E3, which might alone only have a low affinity or processivity and therefore catalyze a mono/oligoubuquitin reaction (e.g. Rsp5 or Mdm2). In an additional reaction the E4 enzyme, which serves as a processivity factor, then polyubiquitylates the substrate with a K48-linked chain, which subsequenty results in substrate degradation. Another strategy for a polyubiquitylation switch can be found in the regulation of PCNA (proliferating-cell nuclear antigen), PCNA associates with various DNA polymerases and functions as a sliding clamp, stimulating accurate and processive DNA synthesis. In addition, PCNA appears to function as a platform for accessory factors. Here the switch from mono- to polyubiquitylation involves two ubiquitin-conjugating enzymes, Rad6 and Ubc13/Mms2, and two ubiquitin ligases, the RING-finger proteins Rad18 and Rad5. Upon DNA-damage, PCNA is monoubiquitylated by a Rad6/Rad18 heterodimer. By contrast, PCNA polyubiquitylation requires in addition the heterodimeric E2 Ubc13/Mms2 and the E3 ligase Rad5, which leads to a K63-linked polyubiquitin chain (Hoege et al., 2002). Another variation of this theme is found with the ubiquitin ligase APC/C, which involves a specific E2 for monoubiquitylation but another E2 for polyubiquitin chain elongation (Rodrigo-Brenni et al., 2007).

This study describes a completely different mechanism of polyubiquitylation switching, which involves the highly conserved peptidyl-prolyl isomerase (PPIase) Pin1. Among the PPIases, Pin1 is special as its association with substrates involves a highly conserved WW protein-interaction domain that recognizes preferentially phosphorylated serine-proline or threonine-proline motifs (pS/T-Pro motifs; “proline-directed phosphorylation”). Due to this special control, Pin1 often functions as a regulated binary switch in fate-determining pathways (Lu et al., 2007; Yeh et al., 2007). Notably, Pin1 has been linked to differentiation, cell proliferation, the immune response, and also Alzheimer’s disease (Takahashi et al., 2008; Lu et al., 2007; Shaw, P.E., 2007). Among the known substrates are celebrities like p53, Cdc25, c-Myc, cyclins, and tau. Pin1-induced conformational changes are translated into a variety of responses, including substrate degradation. Indeed, Pin1 is often linked to the UPS, but how Pin1 controls protein stability remained unclear.

By studying two cellular key regulators, the mammalian tumor suppressor p53 and the NFκB-related transcription factor Spt23 of yeast, this study shows that the activity of Pin1 crucially influences the degree of substrate polyubiquitylation (Figure 18-23). This work demonstrates that in these two cases, high Pin1 activity supports

weak substrate ubiquitylation largely in the form of mono/oligoubiquitylation. By contrast, low Pin1 activity stimulates massive induction of polyubiquitylation and leads to subsequent proteasome dependent degradation (Figure 18-23), illustrating the fundamental importance of a polyubiquitylation switch for signal transduction.

5.2.1 Pin1 controls the degree of Spt23 ubiquitylation in the OLE pathway

Recent studies have shown that Spt23 (and its relative Mga2) is synthesized as inactive, ER-membrane-anchored transcription factors of *OLE1*, encoding fatty acid desaturase. Upon fatty acid restriction, Spt23 becomes activated by proteasomal processing, which eliminates the C-terminal membrane anchor of Spt23 by ERAD. The N-terminal, processed form then translocates into the nucleus and drives *OLE1* transcription (Hoppe et al., 2000; Rape et al., 2001). Efficient proteasomal processing requires low levels of Spt23 ubiquitylation in the form of mono/oligoubiquitylation catalyzed by the WW domain containing HECT E3 ligase Rsp5. Binding of Rsp5 to Spt23 involves one of the enzyme's three WW domains (Hoppe et al., 2000).

Intriguingly, Pin1 (termed Ess1 in yeast) using its WW domain binds apparently to the same region in Spt23 (Figure 7). However, in contrast to Rsp5, Pin1 binding strictly depends on the phosphorylation of pS-Pro sites of Spt23 (Figure 9, 10 and 25). In addition, CK2 was found as a strong binding partner of Spt23 *in vivo* (Figure 11) and it could be demonstrated that binding of Ess1 to Spt23 is stimulated by CK2-mediated phosphorylation of the transcription factor (Figure 11-13), concluding that Spt23 phosphorylation is an essential prerequisite for Ess1 binding and PPIase activity.

Mammalian Pin1 was reported to be subject of post-translational modification by S16-directed phosphorylation. Furthermore, the phosphorylation status was linked to Pin1 activity and localization, but the biological relevance of this modification was largely elusive (Lu et al., 2002). This study demonstrates that also the phosphorylation status of yeast Ess1 S16 is directly linked to the PPIase activity and additionally subcelluar localization of Ess1 in the cell(Figure 14 and 15). Remarkably, the phosphorylation status of Ess1 S16 is directly linked to viability in *S. cerevisiae* (Figure 14b).

Hence, post-translational modification of Ess1 by S16 phosphorylation might represent an additional mechanism, critical for regulating WW domain S/T-Pro binding activity and Ess1 PPIase, which is especially interesting with regard to a putative additional regulatory function in the OLE pathway.

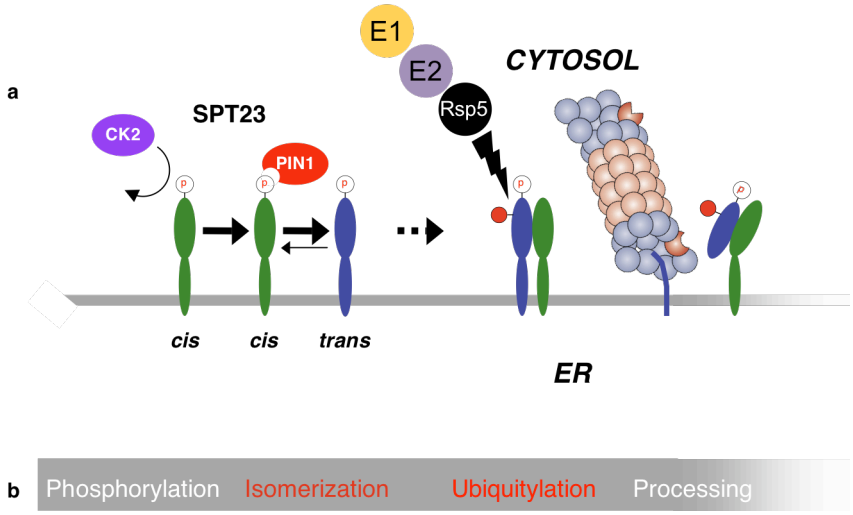

Figure 25. Proline-directed Spt23 phosphorylation mediates specificity for Pin1-mediated *cis/trans* isomerization. (a,b) Proline-directed phosphorylation of Spt23 (S/T-Pro) stimulated by CK2 is a prerequisite for Pin1-mediated *cis/trans* isomerization of the Spt23 precursor and thereby connects proline-directed phosphorylation and ubiquitylation and results in subsequent processing by the 26S proteasome (see also Figure 4).

Pin1 deletion in *S. cerevisiae* is lethal, but interestingly, cells survive if unsaturated fatty acids are provided exogenously or *OLE1* was expressed (Figure 5 and 7). Conversely, Pin1 overexpression results in a disproportionate up-regulation of Ole1 activity and is toxic for cells. Notably, this growth defect was even exacerbated by addition of unsaturated fatty acids to the medium (Figure 8). Pin1 loss in yeast led to complete Spt23 p120 precursor degradation (Figure 14) and Pin1 activity was directly correlated with p120 stability (Figure 14). In addition, Spt23 precursor stability could be directly linked to the Ess1 binding site in Spt23 (Figure 14 and 15).

The reason for the lethality of yeast Pin1 mutants is that Pin1 loss triggers massive Spt23 precursor polyubiquitylation, leading to its complete elimination by ERAD (Figure 14; Figure 16 and 17). Thus, Pin1 acts on Spt23 directly at the level of substrate ubiquitylation: high (normal) Pin1 activity activates the OLE pathway through mono/oligoubiquitylation and proteasomal processing; low Pin1 activity triggers Spt23 polyubiquitylation followed by ERAD (Figure 25 and 26).

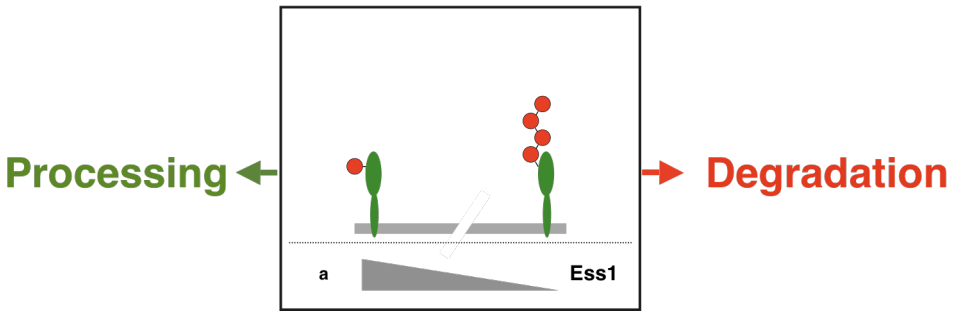

Figure 26. Simplified scheme of Spt23 ubiquitylation and the opposing activities of the peptidyl-prolyl *cis/trans isomerase* Pin1. (a) Pin1 acts as a binary switch directly at the level of substrate ubiquitylation: high (normal) Pin1 activity activates the OLE pathway through mono/oligoubiquitylation and proteasomal processing, whereas in contrast low Pin1 activity triggers Spt23 polyubiquitylation and degradation.

Membrane homeostasis and regulation of membrane composition of eukaryotic membranes is highly dynamic. Membrane fluidity in *S. cerevisiae* is largely controlled by the activity of the essential Δ9-fatty acid desaturase enzyme Ole1, which is a key enzyme in lipid and membrane synthesis pathways regulating the amount of unsaturated fatty acids in the cell. The importance of the homeostasis of fatty acid pools and the functional integrity of the cellular vesicular systems is reflected by the fact that low levels of unsaturated fatty acids result in a severe impairments of the cellular membrane systems. How a change in membrane fluidity is sensed and transduced to regulate the transcription factor Spt23 that activates Ole1 in the OLE pathway is still enigmatic. The discovery that Pin1 acts by controlling the degree of substrate ubiquitylation and is thereby directly involved in the decision between proteasomal processing (RUP) *versus* proteasomal degradation reveals a so far unrecognized role of this PPIase in the OLE pathway and an interesting new level of complexity (Figure 25 and 26). However, it seems unlikely that CK2-mediated phosphorylation of Spt23 and subsequent Pin1-induced *cis/trans* isomerization is directly triggered by a change in membrane homeostasis. Interestingly, mRNA levels of both Spt23 and Mga2 were shown to be upregulated by the unfolded protein response (UPR) and Spt23 precursor processing is induced upon heat stress (Travers et al., 2000). Additionally, considering the drastic changes in membrane fluidity and composition in response to e.g. cell growth suggests that it is more likely, that the membrane-bound Spt23 precursor p120 or a membrane-associated protein functions as the sensor of membrane composition. A change in membrane fluidity or composition might induce a proline-directed (S/T-Pro) phosphorylation in Spt23, which thereby triggers an association of Spt23 with the peptidyl-prolyl isomerase

Ess1 and subsequently with the processing machinery. The special requirement of substrate phosphorylation for Ess1-mediated *cis/trans* isomerization uncovers an additional level of regulation and specificity, coupling the isomerization activity to the tightly controlled activities of proline-directed kinases (Figure 9-13 and Figure 25) and furthermore upstream signaling.

5.2.2 Pin1 acts by regulating the degree of p53 ubiquitylation

The influence of Pin1 on p53 ubiquitylation lies at the heart of the regulation of the tumor suppressor p53. The ubiquitin ligase Mdm2 controls p53 stabilization and is one of the best characterized ubiquitin ligases. Mdm2 catalyzes normally mono/oligoubiquitylation of p53, apparently because it quickly dissociates from the substrate after adding a single ubiquitin molecule to it (Lai et al., 2001). However, *in vitro* and *in vivo*, high levels of Mdm2 have been shown to trigger strong p53 polyubiquitylation (Figure 22 and 23; Li et al., 2003; Brooks et al., 2004). Pin1 was previously shown to bind p53 and to have an influence on p53 stability (Zacchi et al., 2002; Zheng et al., 2002; Mantovani et al., 2004). However, how Pin1 controls p53 turnover was still largely enigmatic.

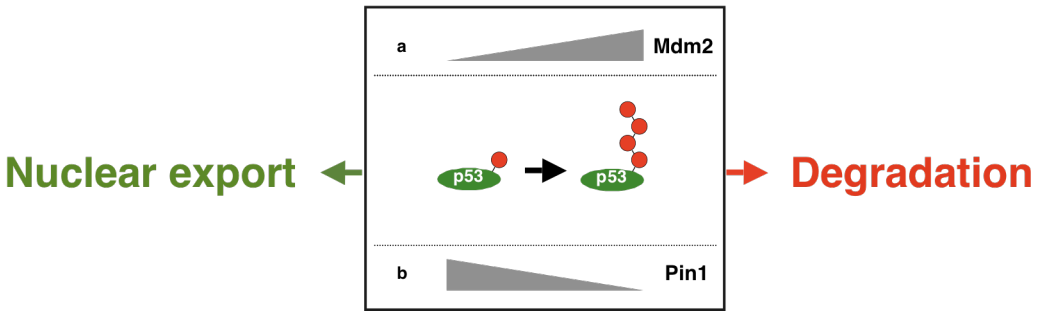

Figure 27. Simplified scheme of p53 ubiquitylation and the opposing activities of the ubiquitin ligase Mdm2 and Pin1. (a) Low Mdm2 levels induce mono/oligoubiquitylation of p53 whereas increasing Mdm2 levels (rising slope) promote robust p53 polyubiquitylation. **(b)** Pin1 activity, by contrast, reduces p53 polyubiquitylation, but lowering Pin1 levels (descending slope) stimulates p53 polyubiquitylation and nuclear degradation.

Intriguingly, the data presented here show that inhibition of Pin1 (e.g. by juglone) results in robust p53 polyubiquitylation *in vivo* (Figure 20), which subsequently leads to nuclear proteasomal degradation (Figure 21). The consequences of the Pin1-controlled polyubiquitylation switch are particularly striking: mono/oligoubiquitylation leads to p53 nuclear export, whereas p53 polyubiquitytation triggers its rapid degradation (Figure 21; Brooks et al., 2004).

The direct influence of Pin1 on p53 polyubiquitylation was independently confirmed by employing two p53 *in vitro* ubiquitylation systems either with purified, recombinant p53 (Figure 22), or p53 expressed in a coupled in vitro transcription/translation system (Figure 23). As noted previously (Li et al., 2003), Mdm2 can efficiently trigger p53 polyubiquitylation if present at high levels. Remarkably, in experimental setups Pin1 strongly inhibited this Mdm2-dependent reaction in a dose-dependent manner, whereas an inactive Pin1 variant had no influence (Figure 22 and 23).

Hence, Mdm2 and Pin1 are both fundamental for balancing p53 levels, yet in clearly opposing ways (Figure 21-23 and 27). These findings indicate that Mdm2 and Pin1 do indeed exert opposing effects on p53 polyubiquitylation and furthermore suggest that the two proteins may antagonize each other's function in cells.

5.2.3 Mechanism of Pin1-induced polyubiquitylation switching

Previous work has shown that switching mono/oligoubiquitylation to polyubiquitylation can be achieved by a dedicated E4 enzyme, that assembles polyubiquitin chains on a pre-existing ubiquitin modification, or by specific pairs of E3 ubiquitin ligases, which together catalyse polyubiquitylation. This work demonstrates that Pin1, through its PPIase activity, can also decisively regulate the ubiquitylation status of a substrate. Thus in contrast to the regulation by E4 enzymes, Pin1 seems to act earlier, directly at the level of the E3. Notably, due to the fact that substrate phosphorylation (pS/T-Pro) is a prerequisite for Pin1 binding, the switch from mono/oligoubiquitylation to polyubiquitylation has the additional advantage of being highly specific and precisely regulated.

How Pin1 influences polyubiquitylation is currently unclear due to the lack of structural data. Interestingly, in the case of the Spt23 the same E3 ligase (Rsp5) seems to either monoubiquitylate Spt23 leading to processing of the precursor, or in

the absence of Pin1, promotes polyubiquitylation and degradation of the same substrate. This suggests that the action of the PPIase Pin1 might most likely change the affinity or processivity of the E3 ligase. Notably, Spt23 might represent a special case of regulation: both Rsp5 and Ess1 have WW substrate-interaction domains and not only target Spt23 *via* this domain (Figure 7b); in fact binding seems to be partially competitive (Figure 7c). By contrast, the RING E3 ligase Mdm2 does not belong to the family of WW domain-containing E3s, and therefore binding of Mdm2 and Pin1 are most likely not competitive reactions. Hence, it seems more plausible that peptidyl-prolyl *cis/trans* isomerization in a substrate introduces a structural change that might directly influence ubiquitin ligase affinities.

Given the fundamental importance of p53 for tumor biology, it will be crucial to test how Pin1 collaborates with other p53-directed E3s. Mdmx for example has been described to enhance p53 ubiquitylation by altering the substrate preference of the Mdm2 ubiquitin ligase (Okamoto et al., 2009) or p53's interplay with the acetyltransferase p300, which acts as an E4 enzyme in the cytosol to promote p53 polyubiquitylation (Dingding et al., 2009).

Hence, future objectives are to understand how Pin1-mediated *cis/trans* isomerization leads to a change in E3 affinities or processivity. One way to test this hypothesis is to directly measure E3 ligase affinities in *in vitro* experiments in the presence or absence of Pin1 or Pin1 mutant variants (e.g. in surface plasmon resonance (SPR) experiments). Furthermore, by changing the affinities of the E3 ligase towards a substrate by either enhancing or decreasing the binding properties (e.g. screening for E3 ligase mutant variants) one might be able to circumvent the requirement for Pin1 *cis/trans* isomerization. Finally, changing the substrate polypeptide-backbone to "lock" a substrate in a particular conformation (*cis* or *trans*) would be an interesting tool to study E3 binding properties and ubiquitylation processivity.

5.2.4 General implications of Pin1-mediated *cis/trans* isomerization in regulatory pathways

Pin1 overexpression was observed in several types of cancer where it functions to promote tumorigenesis induced by oncogenes (e.g. Ras and Neu), additionally Pin1 overexpression shows a correlation with poor clinical prognosis (Lu et al., 2003; Finn

and Lu, 2008). Consequently, Pin1-mediated phosphorylation-dependent isomerization represents a unique regulatory mechanism in cell signaling and therefore Pin1 was discussed as a novel diagnostic marker (Finn and Lu, 2008). The fact that Pin1 is predominantly overexpressed in several human tumors including breast, colorectal and prostate cancer (Wulf et al., 2001; Ayala et al., 2003; Kim et al., 2005) makes it a potential therapeutic target for cancer treatment.

Pin1 is known to act on numerous substrates, in particular regulatory proteins in fate determining pathways. When considering for example the p53 gene family, recent data now links the p53 sibling p73 with Pin1 in apoptosis (Mantovani et al., 2004). Interestingly, also in this case the Pin1-p73 interaction is phosphorylation-dependent. Additionally loss of Pin1 seems to destabilize p73, which strongly suggests that p73 might be regulated by a similar principle by Pin1 as it does for Spt23 and p53 by regulating the degree of ubiquitylation. Pin1 may even function to assemble and stabilize a transcriptional complex involving all members of the p53 family, including p53, p63 and p73 together with other cofactors. Intriguingly, also p63 has several conserved S/T-Pro sites and future experiments may hopefully explore this exciting prospect, especially if p63, as the third member of the p53 family might be a Pin1 substrate as well.

As Pin1-mediated *cis/trans* isomerization is a phosphorylation-dependent regulatory mechanism, Pin1 is also involved in cell cycle regulation. Pin1 targets key proteins such as cyclin D1 or Cdc25, which is a mitotic inducer phosphatase that controls the activation of Cdc2/cyclin B protein kinase and entry into mitosis in eukaryotic cells. Phosphorylation-induced isomerization of Cdc25 by Pin1 appears to mediate a conformational change within the regulatory domain of Cdc25, affecting its catalytic activity (Hutchins et al., 2004). Due to the important role that Cdc25 plays in response to genotoxic stress, particularly in cancer cells defective in p53 regulation, it will be crucial to review the regulation of Cdc25 with regard to Pin1's new role as a fate switch in the UPS.

Cancer and Alzheimer's disease (AD) are two common disorders for which the final pathophysiological mechanism is not yet clearly defined. Interestingly, Pin1 is downregulated in degenerative neurons of Alzheimer's disease (AD) patients and Pin1 deletion in mice leads to age-dependent neurodegeneration (Lu et al., 2004). Pin1 was described as the molecular partner for tau and amyloid precursor protein (APP), the key factors of Alzheimer's disease (AD). Pin1 binds to and isomerizes a pThr-Pro motif in amyloid precursor protein (APP) to promote non-amyloidogenic

APP processing and to reduce Aβ production. Pin1 was shown to interact with hyperphosphorylated tau protein and regulates its activity to bind microtubules. Remarkably, S/T-Pro directed phosphorylation of tau has been directly linked to tau stability (Lim et al., 2008), how and whether tau stability might be linked to the UPS *via* a Pin1-mediated change in the degree of substrate ubiquitylation is so far unclear.

Although Pin1 is known to influence other events (for example phosphorylation) as well, we propose that Pin1-induced *cis/trans* isomerization is often translated into a change in ubiquitylation status. Hence, it will be important to review other known Pin1 substrates and ask whether this polyubiquitylation switch is a recurring theme and central for cellular regulation.

6 Materials and Experimental Methods

The subsequent microbiological, molecular biological and biochemical methods are based on standard techniques (Ausubel et al., 1994; Sambrock et al., 1989) or on the manufacturer's instructions. For all methods described, de-ionized sterile water, sterile solutions and sterile flasks were used. Unless otherwise mentioned, chemicals and reagents were obtained from Amersham-Pharmacia, Applied Biosystems, Biomol, Biorad, BostonBiochem, Difco, Fluka, Invitrogen, Kodak, Merck, New England Bioloabs, Promega, Roth, Roche, Riedel de Haen, Serva, or Sigma.

6.1 Computational analysis

For database searches (sequence search and comparison, literature research) electronic services provided by Saccharomyces Genome Database (http://www.yeastgenome.org/) and National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) were used. DNA and protein sequence analyses (DNA restriction enzyme maps, DNA sequencing analyses, DNA primer design, protein sequence comparison) were done with the DNA-Star software (DNA Star Inc.). Western-Blot films were scanned on an AGFA T1200 and on a Mikrotek ScanMaker i900. For quantification of immunoblots, the chemiluminescence signals were detected by a CCD camera (LAS 3000, Fujifilm) and processed with Image Gauge V4.23 (Fujifilm), and Adobe Photoshop (Adobe Systems Inc.). For the presentation of texts, tables, graphs and figures, the Microsoft Office software package (Microsoft Corp.) was used.

6.2 Microbiological and genetic techniques

6.2.1 *E. coli* techniques

• *E. coli* strains used for cloning and protein expression

- BL21 (DE3*) E.coli* B F^- *dcm ompT* hsdS($r_{B-}m_{B-}$) galλ (DE3)
- BL21 Codon Plus (DE3) -RIL*: E.coli* B F^- *ompT* hsdS($r_{B-}m_{B-}$) *dcm*+ Tet^r galλ (DE3) endA Hte [*argU ileY leuW* Cam^r]
- XL1-Blue MR Δ(*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*

• *E. coli* plasmids for protein expression

Plasmids of the pET System from Novagen (pet28a/b/c(+)) and of the pGEX (pGEX4T1/2/3) from Amersham were used in this study.

- pGEX4T1-ESS1
- pGEX4T1-ESS1 WW
- pGEX4T1-ESS1 Y23A
- pGEX4T1-GST
- pET28-Pin1
- pET28-Pin1 mutant

• *E. coli* media

LB-medium / (plates):
1% Trypton (Difco)
0.5% yeast extract (Difco)
1% NaCl
(1.5% agar)
sterilized by autoclaving

• Cultivation and storage of *E. coli*

E. coli liquid cultures were grown in LB media at 37°C (or 22°C for protein expression experiments), with shaking at 200 rpm. Solid cultures were grown on LB agar plates at 37°C. Selection of transformed bacteria was done by supplementation of LB Medium or LB Plates with 50 μg/ml Ampicillin or 30 μg/ml Kanamycin. Density of liquid cultures was determined by measuring the absorbance at a wavelength of 600 nm (OD600) with a standard spectrophotometer (Eppendorf). Cultures on solid media were stored at 4°C up to 7 days. For long-term storage, stationary cultures were frozen in 15% (v/v) glycerol solutions at –80°C.

• Preparation of chemically competent bacteria

For the preparation of highly competent cells the method of Inoue et al. (1990) was followed, bacteria from frozen stock or single colonies from plates were used for inoculation of a 20 ml starter culture in SOB Medium (2% bacto-tryptone, 0.5% yeast extract, 0.05% NaCl and after components were dissolved addition of 1% 250 mM KCl, 5 N NaOH ad pH 7.0) and cultured for 4-6h 37°C shaking. Then three flasks with 500 ml of SOB Medium were inoculated with 1, 2 and 5 ml of the starter culture and cultured overnight at 18°C. The cultures were chilled in ice-cold water after they reached an OD600 of 0.55 for 20 min. The cells were harvested by centrifugation (2500g, 10 min, 4°C) and the pellet resuspended in 100 ml pre-chilled Inoue Buffer (55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, 10 mM PIPES pH 6.7) and again harvested by centrifugation. Cells were resuspended in 40 ml Inoue Buffer and 3 ml DMSO were added, the mixture was incubated on ice for 10 min. Aliquots of 100 μl were frozen in pre-chilled tubes in liquid nitrogen and afterwards stored at -80°C until use.

• Transformation of plasmid DNA into bacteria cells

Competent cells were thawn on ice and then mixed with 1-4 μl of dissolved plasmid DNA and incubated on ice for 30 min. A 42°C heat shock was performed for 1 min, followed by a 1 min incubation on ice. For recovery, 1 ml pre-warmed LB medium without antibiotics was added, and cells were incubated on a shaker (200 rpm) at 37°C for 1h. Transformed cells were selected by plating the cell suspension on antibiotic-containing LB agar plates and incubating the plates over-night at 37°C.

• Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated by alkaline lysis and binding to an anion-exchange column using kits from Qiagen, Macherey&Nagel and Bioneer according to the manufacturer's protocol. DNA was quantified with a Nanodrop device (peqlab) with 1 OD250 = 50 μg DNA.

6.2.2 *S. cerevisiae* techniques

• ***S. cerevisiae*** **strains**

Name	Relevant genotype	Reference
DF5	*trp1-1 ura3-52 his3Δ200 leu2-3,11 lys2-801*	Hoppe et al., 2000
YDS109	*ess1::HIS3MX, pESS1(ESS1 promoter)-ESS1: URA3*	this study
YDS066	*ess1::HIS3MX, pESS1-ESS1-W15R*	this study
YDS067	*ess1::HIS3MX, pESS1-ESS1-H164R*	this study
YDS124	*rsp5::HIS3MX, pRSP5(RSP5 promoter)-RSP5:URA3*	this study
YDS119	*DF5, pGAL1-10-mycSPT23HA::LEU2*	this study
YDS122	*YDS066, pGAL1-10-mycSPT23HA::LEU2*	this study
YDS120	YDS067, *pGAL1-10-mycSPT23HA::LEU2*	this study
YDS054	*ess1::HIS3MX, pADH1-OLE1::LEU2*	this study
YDS113	*DF5, mutated pOLE1-lacZ::LEU2*	this study
YDS114	*DF5, pOLE1-lacZ::LEU2*	this study
YDS117	*YDS066, mutated pOLE1-lacZ::LEU2*	this study
YDS118	*YDS066, pOLE1-lacZ::LEU2*	this study
YDS115	*YDS067, mutated pOLE1-lacZ::LEU2*	this study
YDS116	*YDS067, pOLE1-lacZ::LEU2*	this study
DF5	*pGAL1-10-mycSPT23HA::URA3*	Hoppe et al., 2000
DF5	*spt23::HISG, mga2::LEU2, pMga2-MGA2:URA3*	this study
DF5	*spt23::URA3*	this study
DF5	*spt23::NAT*	this study
YDS161	*YDS066, pGAL1-10-mycSPT23HA::URA3*	this study
YDS160	*YDS067, pGAL1-10-mycSPT23HA::URA3*	this study
YDS409•	*EY0986, CKA1-GFP::HIS3MX*	Huh et al., 2003
YDS410•	*EY0986, CKA2-GFP::HIS3MX*	Huh et al., 2003
YDS411•	*EY0986, CKB1-GFP::HIS3MX*	Huh et al., 2003
YDS408•	*EY0986, CKB2-GFP::HIS3MX*	Huh et al., 2003

All strains are isogenic to DF5 apart from the following exceptions:
• These strains are based on the S288C derivative strain EY0986, obtained from Invitrogen.

• ***S. cerevisiae*** **vectors**

The following vectors were used in this study:
- Centromeric plasmids (CEN): pYCplac33, pYCplac22, pYCplac111, pUG36 (URA3, CEN, MET25 promoter) (Niedenthal et al., 1996), pRS416 (Sirkorski and Hieter, 1989).
- Highcopy 2*μ* plasmids: pYEplac195, pYEplac112, pYEplac181
- Integrative plasmids: pYIplac211, pYIplac204, pYIplac128 (Gietz and Sugino, 1988)
- Yeast-Two-Hybrid vectors: pGBT9, pGAD424 (Bartel et al., 1993), pGAD-C1-3, pGBD-C1-3 (James et al., 1996)
- Integrative plamids suitable for lacZ fusions: Yip356, Yip357, Yip367 (Kinney and Lusty, 1986).

• *S. cerevisiae* plasmids

ESS1 including its promoter and terminator was cloned (YCplac22, YCPlac33, YCplac111; with different markers), and the *ess1-W15R*, *ess1-H164R*, *ess1-Y23A* mutants were generated by site-directed mutagenesis. For overexpression, the corresponding DNA was cloned into YCplac22 under the control of the *pGAL1-10* promoter, or for the competitive two-hybrid assay, the *pADH1* promoter. For fluorescence analysis of GFP-tagged ESS1, ESS1-WW, ESS1-PPiase and mutants, the corresponding DNA was subcloned into pUG36 under the control of the *pMET25* promoter. For two-hybrid analysis, *ESS1* and *ess1-WW* were cloned into the pGAD vector. To construct GST-Ess1 and Gst-Ess1^{Y23A}, Ess1 sequences corresponding to the full-length proteins were subcloned into pGEX4T-1 (GST) for expression in bacteria. ^{myc}SPT23^{HA} under the control of its own promoter and terminator was subcloned to YCplac22 and used to generate the Spt23 SP mutants used in this study by site-directed mutagenesis. ^{myc}SPT23^{HA} was subcloned into pUG36ΔGFP and expressed under the control of the *pMET25* promoter. His6- and HA-epitope tagged ubiquitin were described previously and subcloned into YEplac195, YEplac181 and YEplac112 either under the control of the *pMET25* or *pADH1* promoter. For *Ole1* transactivation assays, the corresponding wild-type or mutated *Ole1* promoter was subcloned into Yip356, Yip357 or Yip367, the *pGAL1-10* promoter was subloned upstream of *lacZ* into YIplac211.

• *S. cerevisiae* media & solutions

- YPD / YPGal [plates]:
1% (10 g/l) yeast extract (Difco)
2% (20 g/l) bacto-peptone (Difco)
2% (20 g/l) D-(+)-glucose or galactose
[2% (20 g/l) agar]
sterilized by autoclaving

- YPD G418/NAT [plates]: After autoclaving, YPD medium with 2% agar was cooled to 50°C, and G418 (geneticine disulphate; Sigma) to 200 mg/l or NAT (nourseothricin, HKI Jena) to 100mg/l was added.

- SC-media [plates]:
0.67% (6.7 g/l) yeast nitrogen base (Difco)
0.2% (2 g/l) drop out amino acid mix (according to the requirements)
2% (20 g/l) carbon source (glucose, raffinose, or galactose)
[2% (20 g/l) agar]

- SC 5-FOA [plates]:
0.67% (6.7 g/l) yeast nitrogen base (Difco)
0.2% (2 g/l) drop out amino acid mix (according to the requirements)
3% (30 g/l) adenine
3% (30 g/l) uracil
2% (20 g/l) glucose
2% (20 g/l) agar
After autoclaving, the mixture was cooled to 50°C, and 5'FOA was added to the final concentration of 1 g/l.

- OA supplemented plates, oleic acid containing plates: After autoclaving, YPD or SC-medium with 2% agar was cooled to 50°C, and a premixed of oleic acid (Sigma) and NP40 (Fluka) was added in equal amounts with a final concentration of 0.2% (v/v).

• ***S. cerevisiae*** **cultivation and storage**

Unless otherwise mentioned liquid cultures were incolulated with single yeast colonies from freshly streaked plates and grown overnight. In principle, the main culture was inoculated with a starter culture at a dilution of 1:100 – 1:1000 and grown until the culture reached the mid-log phase growth (1-3x10^7 cells/ml). Liquid cultures were grown at 30°C (temperature sensitive strains at 23°C), in an incubator with shaking at 150 - 250 rpm. Density of liquid cultures was determined by measuring the absorbance at a wavelength of OD600 with a standard spectrophotometer (Eppendorf). Cultures on solid media were stored at 4°C up to 1 - 2 months. For long-term storage, stationary cultures were frozen in 15% (v/v) glycerol solutions at –80°C.

• **Preparation and transformation of competent** ***S. cerevisiae***

Preparation of competent yeast cells and transformation was based on standard protocols described in Knop et al. (1999).

• **Genomic integration by homologous recombination**

Chromosomal gene deletions or insertions of epitope tags were performed by a PCR strategy according to Knop et al. (1999) and Longtine et al. (1998).

• **Analyses of protein-protein interactions with the Two-Hybrid System**

The two proteins of interest for interaction were fused in frame to the DNA-binding and, respectively, the activation domain of the Gal4 transcription factor. The expression constructs of the fusion proteins were transformed in PJ69-7A cells (James et al., 1996). An interaction between the two proteins results in the reconstitution of the Gal4 transcription activator. Hence, the expression of reporter genes under the control of Gal4 (i.e. HIS3, ADE2) is turned on, and cells can grow on the respective selection media.

Competitive protein-protein interactions were established in a Yeast-Three-Hybrid approach. In addtion to the Yeast-Two-Hybrid System, a third protein was cloned in frame under the control of the *pADH1* promoter as a competitor and transformed in PJ69-7A Yeast-Two-Hybrid strain created before (see above), as a control an empty plasmid with the *ADH1* promoter was transformed.

• **Phenotype analyses by growth tests**

Characterization of the yeast growth phenotype is based on the comparison of growth rates upon various conditions (e.g. different temperature or presence/absence of 18:1) of different yeast strains cells spotted in equal amounts on a solid medium. Yeast cultures grown to mid-log growth phase or collected from fresh plates were diluted with sterile water to an OD600 of 0.2, serial dilutions were prepared in the 96-well microtiter plate (1:5 or 1:10). 5 μl of each cells suspension were spotted on respective plates with a pin-stamp (48 or 96) and the plates were incubated at different temperatures.

Moreover, this method allows the characterization of mutants of an essential gene, which are otherwise unable to support viability. In this case, cells expressing the wild-type gene only from a centromeric plasmids with the URA3 auxotrophy marker were transformed with different mutant alleles, either ts-allels or binding-mutants and plated on 5-FOA-containing plates. This drug counter-selects the URA3-expressing plasmids, and cells can survive only if the mutant alleles can confer viability.

6.3 Tissue culture methods

• Cell lines
U2-OS, human osteosarcoma, chromosomally highly altered, with chromosome counts in the hyper-triploid range.

• Mammalian expression vectors
- pcDNA3.1/GeneStorm (Invitrogen)
- pcMV-Tag2/3/4 (Stratagene)

• Plasmids for protein expression in mammalian cells
HIS6- and HA-epitope tagged ubiquitin were described previously (Treier et al., 1994). Pin1 wt and Pin1 mutant were a gift from Prof. O. Stemmann.

• Cultivation and storage of mammalian cells
U2-OS cells were maintained and propagated at 37°C, 7.5% CO_2 in DMEM (SIGMA) supplemented with 10% FCS. For long term storage, cell cultures were frozen in liquid nitrogen as follows, cells were grown to a confluence of 80%, trypsinized, centrifuged and resuspended in 10% DMSO in fetal bovine serum. The cell suspension was distributed in cryo-vials (Nalgene) and transferred to pre-cooled isopropanol-filled cryo-containers (Nalgene) and frozen at –80°C. After two days, the cryo-vials were transferred to liquid nitrogen.

• Transfection of mammalian cells
Transient transfection of U2-OS cells was perfomed by electroporation using the Nucleofector kit according to the manufacturer's protocol (Amaxa).

• Immunofluorescence microscopy
Immunofluorescence was perfomed according to standard protocols after fixation in 3.7% paraformaldehyde (PFA). Images were acquired on a Leica DM RXA microscope equipped with a Hamatsu ORCA-ER digital camera, image acquisition was carried out using Openlab software.

6.4 Molecular biology methods

• Enzymatic manipulation of DNA
Restriction enzymes were employed for sequence-specific cleavage of DNA according to standard protocols (Sambrock et al., 1989) and the instructions of the manufacturer (New England Biolabs). For the digestion of 1-5 μg 2-10 units of restriction enzyme were usually used. Reaction samples were incubated at the recommended temperature for 2 h. To avoid the re-circularization of linearized vectors, the 5' end of vector DNA was dephosphorylated by incubation with 5-10 units of Shrimp alkaline Phosphatase (NEB) at 37°C for 30 min. PCR products were digested overnight with 5-20 U of restriction enzyme. Digested DNA was subjected to gel electrophoresis, the bands were visualized by ethidium bromide staining, the favored DNA fragments were sliced out with a sterile razor blade and subsequently purified from the gel matrix using a standard extraction kit (Qiagen, Macherey&Nagel, Bioneer).

• Ligation of DNA fragments
Ligation of cohesive end DNA was carried out using T4 DNA Ligase (NEB) according to the manufacturer's instructions. Approximately 50-100 ng vector DNA were incubated with a 6-10 fold molar excess of insert in a reaction volume of 20 μl. The samples were incubated overnight at 16°C and subsequently transformed into chemically competent cells.

• Separation of DNA fragments by gel electrophoresis
Agarose gel electrophoresis was performed with Tris-borate buffered solutions (0.5-fold TBE: 45 mM Tris-borate, 1 mM EDTA [pH 8.0]). DNA was mixed with loading buffer (5-fold concentrated: 25% glycerol, 0.25% SDS, 25 mM EDTA [pH 8.0], Orange G in H_2O) and the mixtures transferred into the slots of the gel (0.8-1.6% agarose in TBE, mixed with 0.005% ethidium bromide after dissolution of the agarose). Electrophoresis was performed in minigels for 30-60 min at 5-15 V/cm.

• DNA sequencing
DNA sequencing was carried out by a modified Sanger dideoxy terminator cycle sequencing chemistry using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham) with 0.1 – 1 µg DNA and 10 pmol primers, the ABI BigDye kit version 3.1, on a ABI 3730 48-capillary sequencer and 36 cm capillaries which was operated by the MPI of Biochemistry Core Facility.

• Polymerase chain reaction (PCR)
PCR was performed according to standard protocols. Amplification was performed using Phusion Polymerase (NEB), taq Polymerase or Vent DNA polymerases (NEB) in a total volume of 50 µl. Amplification was carried out with 25 cycles of melting (95°C for 30 s), annealing (55°C for 30 s) and amplification (temperature [68-72°C] and time was chosen according to the used polymerase [15-60 s per 1 kb of DNA]).

• DNA site-directed mutagenesis
The method used to generate point mutations, was a PCR-based strategy based on the QuickChange protocol (Stratagene). Two complementary oligonucleotide primers containing the mutated site in the middle, flanked by 15 bases of the target sequence overhang on each side were used. The melting temperature of the primer was calculated by Tm = 81.5 + [0.41(GC)] – [675/N] - [% of mismatch]. PCR amplification was performed using Phusion polymerase (NEB) in a total volume of 50 µl. Amplification was carried out with 20 cycles of melting (95°C for 15 s), annealing (Tm - 5°C for 30 s) and amplification (72°C for 20 s per 1 kb of DNA). PCR products were subsequently digested with 10 U DpnI for 1-3 h, heat inactivated and transformed into bacteria.

6.5 Protein biochemistry techniques

• Measuring protein concentrations
The Bradford assay (BioRad protein assay: BioRad) was used to determine protein concentrations. Concentrations were determined in an OD590 nm reading window of 0.1 to 2 using standard spectrophotometers (Eppendorf).

• SDS polyacrylamide gel electrophoresis (SDS-PAGE)
For the experiments presented in this study, proteins were resolved using a Bis-Tris-buffered gel system. 4-12% gradient Bis-Tris polyacrylamide gels were used, either bought from Invitrogen or self-poured using the Mighty Small gel system (Hoefer). Aequeos solutions of proteins or cell lysates were either mixed with 5-fold SDS sample buffer (312 mM Tris [pH 6.8], 50% glycerol, 10% SDS, 25% ß-mercaptoethanol, 0.01% bromophenolblue) or HU sample buffer (8 M Urea, 5 % SDS, 1 mM EDTA, 1.5 % DTT, 1 % Bromphenolblue, 200 mM Tris-HCl pH 6.8). Samples were boiled in SDS sample buffer (95°C, 5 min) or for 10 min in HU sample buffer and transferred to the slots of the gel. Precision Plus Protein All Blue Standards (BioRad) was used as a marker for relative molecular size of proteins.

• Immuoblot
Proteins resolved by SDS-PAGE were transferred onto PVDF membranes (Millipore) using a tankblot device (Hoefer). Transfer was carried out in a cold room for 1.5 - 3 h in transfer buffer (25 mM Tris, 192 mM Glycin, 0.01% SDS and 20% methanol) with a constant voltage of 70 volt. The membrane was subsequently stained with Ponceau S if necessary, destained and washed with TBST (25 mM Tris [pH 7.5], 137 mM NaCl, 2.6 mM KCl, 0.05% Tween-20). The membrane was blocked afterwards using 2.5% w/v ECL Advance™ blocking reaent (GE Healthcare) in TBST (30 min, RT) on a rotating platform (50-100 rpm).

Primary and secondary antibody were hybridized in 2.5 ECL Advance™ blocking reaent (GE Healthcare) in TBST overnight followed by extensive washing of the membrane with TBST (6 times, 10 min, RT). Secondary HRP-conjugated antibodies were applied in combination with chemi-luminiscence detection (ECL-Plus/ECL-Advanced Westernblot Detection Kit, GE Healthcare) on conventional films (Kodak) or LAS3000 CCD cameras (Fuji).

• Immunoblot reprobing
Membranes were stripped to reprobe by incubation in stripping buffer (63.5 mM Tris [pH 6.8], 2% SDS, 100 mM DTT) for 45 min at 65°C.

• ONPG Assay
ONPG (o-nitrophenyl-beta-D-galacto-pyranoside) assay of beta-galactosidase activity in yeast was perfomed based on standard protocols described by Gietz et al. (1997).

6.5.1 Preparation of cell lysates

• Preparation of native yeast extracts
Native protein extracts were employed in binding studies. Yeast cells of mid-log phase cultures were harvested by centrifugation and washed once with ice-cold PBS. Occasionally, cell pellets were frozen in liquid nitrogen and afterwards stored at –20°C. Pellets were resuspended in 250-500 µl lysis buffer (PBS, 0.125 mM MgCl2) supplemented with 1 mM PefaBloc (Roche), protease inhibitors (complete inhibitor set, Roche), and if needed phosphatase inhibitors (200 mM Na_3VO_4, 20 mM NaF, EDTA). After addition of zirkonia/silica beads (∅ 500 µm, ROTH) cells were lysed by vortexing 4-6 times for 2 min at 4°C, using a bead beater device (Retsch). The cell lysate was recovered by piggyback centrifugation after the bottom and the lid of the tube was perforated with a needle. Triton X-100 was added to the lysates (to a final concentration of 0.1-0.4%) to avoid unspecific binding. To extract integral membrane proteins (Spt23 p120) 0.2% dodecyl maltoside (Sigma) was added to the lysates and the lysate was incubated for 30 min at 4°C on a rotating wheel for solubilization.
Cell debris was removed by centrifugation (770 g, 5 min, 4 °C). The whole cell extract contains the soluble fraction (cytosolic and nuclear proteins) as well as microsomes (ER, nuclear envelope and other organelles). The cytosolic fraction is partially contaminated with proteins from the lumen of organelles, which upon the lysis with zirconia/silica bead are partially disrupted. For separation of microsomes from the soluble phase, whole cell extracts were fractionated by high spin centrifugation (20000 g, 1 h, 4 °C).

• Preparation of denatured yeast extracts & expression shut-off experiments
Yeast cells were lysed under denaturing conditions in order to avoid de-conjugation of post-translational modifications during lysis. Usually, mid-log phase growing cells from 1 ml of a yeast culture of 1 OD600 were harvested by centrifugation, resuspended in 1 ml ice-cold water and lysed by incubation with 150 µl 1.85 M NaOH/ 7.5% β-mercaptoethanol for 15 min on ice. Proteins were precipitated by addition of 150 µl 55% trichloroacetic acid (TCA) followed by incubation on ice for 10 min and centrifugation at 20000 g for 20 min at 4°C. The pellet was resuspended in 50 µl HU sample buffer.

Stability of ^{myc}SPT23^{HA} expressed from the *pGAL1-10* promoter was determined by cycloheximide chase after *pGAL*-promoter shut-off essentially as published (Richly et al., 2005; Piwko et al., 2006). Stability of the ^{myc}Spt23^{HA} SP mutants expressed from its own promoter was determined in a *Δspt23::URA3* background by cycloheximide chase (Richly et al., 2005; Piwko et al., 2006).

• Preparation of native extracts from mammalian cells

Lysates from U2-OS mammalian cells were prepared by either scraping cells from plates using a rubber policeman or by trypsination. Cells were then centrifuged (300 g, 3 min, RT) and washed once in PBS. Cells were resuspended in 100-200 µl of IP buffer (50 mM HEPES [pH 7.2], 150 mM NaCl, 2 mM EDTA, 0.8% Triton X-100) supplemented with 1 mM PefaBloc (Roche), complete protease inhibitors (Roche), 20 mM NaF, 200 mM Na_3Vo_4 phosphatase inhibitors, MG132 and additionally 1 mM NEM if ubiquitylation should be preserved. Suspensions were incubated on ice for 30 min. Cell debris was removed by centrifugation (10 min, 16000g, 4°C). Hereafter an appropriate amount of the supernatant representing the cell lysate (input) was mixed with 2xSDS loading buffer.

• TCA precipitation of proteins

Proteins in solution were precipitated by addition of an equal volume of 10% TCA and incubation on ice for 10 min. Precipitated material was pelleted by centrifugation (16000 g, 4°C, 10 min). Pellets were washed with acetone (-20°C), dried and resuspended in SDS loading buffer.

6.5.2 Protein purification and binding experiments

• Purification of recombinant protein from *E. coli*

GST- as well as His6-fusion proteins were expressed in *E. coli* BL21(DE3)/RIL. *E. coli* cultures were inoculated and shaken at 37°C in LB with the appropriate antibiotic. Cells were harvested when OD600 reached 0.4, cultures were induced by adding 1 mM IPTG and further shaken overnight (RT).

GST fusion proteins were purified as follows, cells from 1 l bacterial culture were resuspended in 30 ml *E. coli* lysis buffer (PBS containing, 0.125 mM MgCl2, complete protease inhibitors (Roche), Pefabloc (Roche), 0.5 mM DTT and 8% glycerol) and lysed by high pressure in an cell disruptor (Emulsiflex C5). For clearing, the lysate was incubated with Triton X-100 added to the final concentration of 1%, for 30 min at 4°C on a wheel, the lysate was subsequently centrifuged for 30 min at 20000g at 4°C. The supernatant was afterwards incubated with Gluthathion Sepharose (Amersham) for 3 h at 4°C. The column was washed with 25 column volumes GST washing buffer 1 (20 mM Tris [pH 7.5], 500 mM NaCl, 2 mM EDTA, 1% Triton-X-100, 1 mM DTT, 8% glycerol), 10 column volumes of GST washing buffer 2 (PBS containing 0.125 mM $MgCl_2$, 0.1% Triton-X-100, 0.5 mM DTT, 8% glycerol) and 10 column volumes of GST washing buffer 3 (PBS containing 0.125 mM $MgCl_2$, 1 mM DTT, 8% glycerol). Bound proteins were eluted with 5 column volumes GST elution buffer (PBS containing 10 mM glutathione, 8% glycerol), dialyzed against appropriate buffers, concentrated glycerol (10% end-concentration was added prior to freezing in liquid nitrogen.

In the case of His6-fusion proteins, pellets were resuspended in NiNTA buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 20 mM imidazole, 2 mM PMSF, Complete protease inhibitors (Roche), 8% glycerol) and lysed by high pressure in an cell disruptor (Emulsiflex C5). Cleared lysates were applied to NiNTA agarose columns (Qiagen), columns washed with 50 column volumes of NiNTA buffer and bound proteins eluted using 5 column volumes of NiNTA elution buffer (50 mM NaHPO [pH 8.0], 150 mM NaCl, 250 mM imidazole).

• Immunoprecipitation from native yeast extracts

For immunoprecipitation experiments native yeast extracts were prepared and to 400-800 µl of cell lysate 20-40 µl of antibody-agarose slurry were added (for HA, myc). Coupled antibodies were incubated 2-4 h or overnight in cell lysates (4°C) on a rotating wheel Afterwards beads were washed five times with IP buffer including detergent and once without detergent, bound proteins were eluted by boiling the beads in HU sample buffer.

• GST pull-down assay from native yeast extract

For GST pull-down assays, 20 μg of GST or GST-fusion proteins in equal molar amounts were bound to GST Sepharose 1 h (4°C) in 200 μl lysis buffer, subsequently the beads were incubated with yeast native lysate for 2-4 h at 4 °C. Afterwards the beads were washed five times with the lysis buffer with detergent and once without detergent, bound proteins were eluted in HU sample buffer (10 min, 65°C).

• NiNTA Chromatography from denatured yeast extracts

Yeast cells transformed with His tagged ubiquitin expressed from the *pADH1* or *pMET25* promoter were harvested and 200 OD600 cells were lysed in 5 ml 1.85 M NaOH / 7.5% β-mercaptoethanol for 20 min on ice. The proteins were precipitated by addition of 5 ml 55% TCA and incubation for 20 min on ice. Subsequently, a centrifugation for 15 min at 3000g was performed, and the protein pellet was washed two times with ice-cold acetone to wash away all remaining TCA. The pellet was afterwards solubilized in buffer A (6 M guanidinium hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, 20 mM imidazole, pH 8) containing 0.05% Tween-20. Insoluble aggregates were removed by centrifugation for 30 min at 13000g and the protein solution was incubated overnight with 50 μl NiNTA Magnetic Agarose Beads (Qiagen). The beads were afterwards washed three times with buffer A containing 0.05% Tween-20, then four times with buffer C (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 6.3) with 0.05% Tween-20 and once with buffer C without Tween-20. Bound proteins were eluted by incubation with 30 μl 1% SDS at 65°C, dried in a SpeedVac (Eppendorf), solubilized in 10 μl water and 25 μl HU sample buffer (10 min, 65°C) and subjected to SDS-PAGE and immunoblotting

• NiNTA Chromatography from denatured extracts of mammalian cells

U2-OS cells were transiently transfected with either His or HA-tagged Ubiquitin (negative control) and harvested, pellets were washed once in PBS. Cells were lysed in 6 M guanidine-HCl, 0.1 M NaH_2PO_4, 0.1% Tween 20, 0.01 M Tris [pH 8.0]. DNA was sheared by mild sonification (2 x 20 sec 20%). An aliquot of the lysate was kept for TCA precipitation (input). The remaining lysate was incubated with 20 μl magnetic NiNTA agarose beads 20 μl (Qiagen) for 3 - 5 h or overnight (RT). Beads were washed 5 times with 8 M urea, 0.1 M NaH_2PO_4, 0.1% Tween 20, 0.01 M Tris [pH 8.0], twice with PBS, 0.01% Tween 20 and one with PBS. TCA precipitates (input) were washed twice with ice-cold acetone and boiled in loading buffer (2x), Bound proteins were eluted by boiling in loading buffer (2x) and subjected to SDS-PAGE and immunoblotting.

• Antibodies

The following antibodies were used: mouse monoclonal anti-p53 (DO-1, Santa Cruz), mouse monoclonal FK2 (MBL), mouse monoclonal anti-Pin1 (clone G8, Santa Cruz), mouse monoclonal anti-MEK-1 (H8, Santa Cruz), mouse monoclonal anti-CK2β (clone 6D5, Santa Cruz), mouse monoclonal anti-myc (clone 9E10, Santa Cruz), rabbit polyclonal anti-myc (clone A-14, Santa Cruz), mouse monoclonal anti-HA (clone F-7, Santa Cruz), mouse monoclonal anti-Dpm1 (5C5-A7, Molecular Probes), mouse monoclonal MPM2 (Upstate), mouse monoclonal anti-GST (Clone 3G10, GeneTex).

6.5.3 *In vitro* reactions

• *In vitro* dephosphorylation assay

In vitro dephosphorylation assays using recombinant λ-phosphatase (NEB) were perfomed on yeast native extracts with buffers omitting phosphatase inhibitors and immuopurified Spt23 (from 100 – 200 OD600 culture) according to the manufacturer's protocol (NEB).

• *In vitro* phosphorylation assay

In vitro phosphorylation assays using recombinant CK2 (NEB) were perfomed on immuopurified Spt23 according to the manufacturer's protocol (NEB). The assay was either carried out hot with ^{32}P-γ-ATP or cold with ATP. To preserve phosphorylation 20 mM NaF and 200 mM Na_3VO_4 were added afterwards.

• *In vitro* phosphorylation dependent pull-down assay

Extracts (from 100 – 200 OD600 culture) of cells expressing ^{myc}Spt23^{HA} under the control of the *pGAL1-10* promoter were subjected to immunoprecipitation using anti-myc antibodies and incubated with or without λ-phosphatase. After phosphatase treatment, the washed samples were left untreated or phosphorylated *in vitro* with recombinant CK2 (NEB). These samples were finally assayed in GST pull-down experiments for binding to either recombinant GST-Ess1 or GST alone (control). The unbound GST-Ess1 material (supernatant of the last experiment) was subjected to a GST pull-down to compare the amount of free to Spt23-bound GST-Ess1 material.

• *In vitro* ubiquitylation

In vitro ubiquitylation assays were performed as described previously (Li et al., 2003), however, with some modifications. For a standard reaction, 4.5 ng of recombinant His6-p53 (p53) was mixed with the other purified recombinant components (Boston Biochem), these include E1 (20 ng), E2 (UbcH5c) (100 ng), E3 (His6-Mdm2) (4.5 to 202.5 ng) and 5 μg of ubiquitin, in 14 μl reaction buffer (50 mM HEPES pH 8.0, 50 mM NaCl, 0.5 mM DTT, 4 mM Mg-ATP, pH 8.0). Where indicated either GST (500 ng) or increasing amounts of Pin1 or a Pin1 mutant variant (Pin1mut) (0 - 351 ng) with single amino acid replacements in the WW and PPIase-domain were preincubated with p53 at 4°C for 1h before the other purified recombinant components were added. A lysine-less ubiquitin variant that fails to form polyubiquitin chains (UbK0) was used as a control for p53 polyubiquitylation. The ubiquitylation reactions were stopped after 60-120 min at 37°C by addition of loading buffer, and subsequently resolved by 4-12% gradient gels for western blot analysis. Polyubiquitylated p53 (poly-Ub) is detected by immunoblotting using an antibody specific for polyubiquitin conjugates (FK2), and oligoubiquitylated p53 (oligo-Ub) was detected by DO-1 antibody.

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8 Abbreviations

μ	micro (x10-6)
2μ	multi-copy vectors
3-AT	3-aminotriazole
AAA	ATPases associated with various cellular activities
AD	Gal4 activation domain
ANK	Ankyrin repeat domain
APC/C	Anaphase promoting complex/cyclosome
APS	Ammonium peroxodisulphate
ARS	Autonomous replicating sequence
ATP	Adenosine 5-triphosphate
BD	Gal4 DNA binding domain
bp	Base pairs
BSA	Bovine serum albumin
CCD	Camera charged-coupled device camera
cDNA	Complimentary DNA
CEN	Centromeric (low copy vectors)
CHX	Cycloheximide
CK2	Casein kinase 2
C-terminal	Carboxyl-terminal
C-terminus	Carboxyl terminus
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMF	N,N-dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleoside triphosphate
DTT	Dithiothreitol
DUB	Deubiquitylating
E1	Ubiquitin activation enzyme
E2	Ubiquitin conjugation enzyme
E3	Ubiquitin ligase
E4	Polyubiquitylation factor
EDTA	Ethylenediaminetetraacidic acid
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
F	Farad
FCS	Fetal calf serum
g	Gram
g	Gravitational constant (6.6742×10^{-11} N m^2 kg^{-2})
G418	Geniticin
Gal	Galactosidase
GFP	Green fluorescent protein
GRR	gGycine-rich region
GST	Gutathion S-transferase
h	Hours
HECT	Homologous to E6AP C-terminus
HEPES	N-(2-hydroxyethyl)-piperazin-N!-(2-ethansulfonate)
IκB	Inhibitor of κB
Ig	Immunoglobulin
IKK	Inhibitor of κB Kinase
IP	Immunoprecipitation
IPT	Immunoglobulin-like/plexins/transcription factors
IVT	Coupled in vitro transcription/translation
Juglone	5-hydroxy-1,4-naphthoquinone

k	Kilo ($x10^3$)
kan	Kanamycine
kb	Kilo base pairs
kDa	Kilo dalton
LB	Luria-Bertani
M	Molar
m	Milli ($x10^{-3}$)
MAT	Mating type
MG132	Proteasome inhibitor Z-Leu-Leu-Leu-al
MHC	Major histocompatibility complex
min	Minutes
MOPS	3-N-Morpholinopropane sulfonic acid
mRNA	Messenger RNA
MW	Molecular weight
n	Nano ($x10^{-9}$)
NAT	Nourseothricin
NEM	N-ethylmaleimide ADP adenosine 5-diphosphate
NEM	N-ethyl maleimide
NFκB	Nuclear factor κB
NiNTA	Nickel-nitrilo triacetate
NLS	Nuclear localization sequence
NP40	Nonyl phenoxylpolyethoxylethanol
N-terminal	Amino-terminal
N-terminus	Amino terminus
OD	Optical density
ONPG	o-Nitrophenyl-β-D-galactopyranosid
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PefaBloc	4 -(2- Aminoethyl)-benzylsulfonylfluorid Hydrochlorid
PEG	Polyethylene glycol
PKA	Protein kinase A
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene fluoride
RING	Really interesting new gene
RIP	Regulated intramembrane proteolysis
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
RUD	Ubiquitin/proteasome-dependent degradation
RUP	Ubiquitin/proteasome-dependent processing
s	Seconds
S	Sedimentation coefficient (Svedberg)
SC	Synthetic complete medium
SCF	Skp1-Cul1-F-box complex
SDS	Sodium dodecylsulfate
TAD	Transactivation domain
TBB	CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole
TBE	Tris/borate/EDTA buffer
TBS	Tris-buffered saline
TCA	Trichloro acidic acid
TEMED	N,N,N',N'-tetramethylethylenediamin
TM	Transmembrane
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
Ub	Ubiquitin
UBA	Ubiquitin associated

UBC	Ubiquitin conjugating enzyme
UCH	Ubiquitin C-terminal Hydrolase
UFA	Unsaturated fatty acid
UFD	Ubiquitin fusion degradation
UIM	Ubiquitin interacting motif
UPS	Ubiquitin/proteasome system
UV	Ultraviolet light
V	Volt
v/v	Volume per volume
w/v	Weight per volume
WT	Wild-type
YPD	Yeast bactopeptone dextrose medium
Ω	Ohm

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10 Curriculum vitae

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