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Functional Mimicry of the Acetylated C-Terminal Tail of p53 by a SUMO-1 Acetylated Domain, SAD

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Abstract

The ubiquitin-like molecule, SUMO-1, a small protein essential for a variety of biological processes, is covalently conjugated to many intracellular proteins, especially to regulatory components of the transcriptional machinery, such as histones and transcription factors. Sumoylation provides either a stimulatory or an inhibitory signal for proliferation and for transcription, but the molecular mechanisms by which SUMO-1 achieves such versatility of effects are incompletely defined. The tumor suppressor and transcription regulator p53 is a relevant SUMO-1 target. Particularly, the C-terminal tail of p53 undergoes both sumoylation and acetylation. While the effects of sumoylation are still controversial, acetylation modifies p53 interaction with chromatin embedded promoters, and enforces p53 apoptotic activity. In this study, we show that the N-terminal region of SUMO-1 might functionally mimic this activity of the p53 C-terminal tail. We found that this SUMO-1 domain possesses similarity with the C-terminal acetylatable p53 tail as well as with acetylatable domains of other transcription factors. SUMO-1 is, indeed, acetylated when conjugated to its substrates and to p53. In the acetylatable form SUMO-1 tunes the p53 response by modifying p53 transcriptional program, by promoting binding onto selected promoters and by favoring apoptosis. By contrast, when non-acetylatable, SUMO-1 enforces cell-cycle arrest and p53 binding to a different sets of genes. These data demonstrate for the first time that SUMO-1, a post-translational modification is, in turn, modified by acetylation. Further, they imply that the pleiotropy of effects by which SUMO-1 influences various cellular outcomes and the activity of p53 depends upon its acetylation state.

Protein modification by sumoylation is essential for the proper functioning of a number of biological processes, including transcription, DNA repair, and chromosomal dynamics (Heideker et al., 2009). There are three different members of the SUMO family, SUMO-1–3, each of which plays independent effects in regulating the activity of various intracellular proteins (Saitoh et al., 2000; Hay, 2005; Geiss-Friedlander and Melchior, 2007). Substrates of sumoylation are predominantly transcription factors, histones, and chromatin remodeling enzymes, particularly acetylases and deacetylases (Gill, 2005). Compared to chemical post-

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translational modifications, SUMO proteins offer a larger surface that can function as a recruitment platform for regulating the interaction of their targets with other proteins in a more complex fashion. Further, given that attachment of SUMO occurs on lysine residues, which are also recipients of other regulatory modifications, such as acetylation, methylation, and ubiquitylation, an antagonistic effect between sumoylation and other post-translational events has been proposed (Nathan et al., 2006). It is currently envisioned that through a combination of these mechanisms, SUMO moieties convey transcriptional activation or repression, and affect the subcellular localization, the stability, and protein-protein interactions of their targets.

The complexity of the mode of action of sumoylation is particularly illustrated by how SUMO-1 affects the activity of the p53 tumor suppressor. p53 is a versatile molecule that responds to various forms of stress, but most importantly to DNA damage or to DNA-replication stress due to supraphysiological levels of oncogene products (Van Dyke, 2007; Junttila and Evan, 2009; Vousden and Prives, 2009). Once activated, p53 can execute different programs, including senescence, apoptosis and cell-cycle arrest. It is well recognized that post-translational modifications affect the ability of p53 to direct cells towards these different cellular programs, and phosphorylation and acetylation play a key role in this respect (Carter and Vousden, 2009). We and others have shown that acetylation of the p53 C-terminal cluster at lysine residues K370, K372, and K373 is capable of initiating a cascade of events consisting in enhancement of the interaction of p53 with acetylases in phosphorylation of specific residues located in the p53 N-terminus, and in modifications of p53 interactions with selected pro-apoptotic promoters (Knights et al., 2006; Tang et al., 2006, 2008). Particularly, we showed that acetylation of this cluster enhances p53 binding to consensus DNA sites for which unmodified p53 has low affinity. It has been known for some time that p53 can also be sumoylated but the role of this modification has remained somewhat controversial. Initial studies reported that sumoylation stimulates p53 transcription activation function (Gostissa et al., 1999), while subsequent work has provided evidence for the contrary, showing that sumoylation represses p53-directed transcription (Schmidt and Muller, 2002). These differences can perhaps be reconciled by the observation that sometimes sumoylation of the same factor can lead to both activation and repression of transcription. For example, the effects of sumoylation on Smad4 transcription can be both positive and negative and are dependent on the promoter context or the cell type (Lin et al., 2003; Ohshima and Shimotohno, 2003; Long et al., 2004). Additionally, many of these studies have employed over-expression of various components of the SUMO pathway for studying p53 sumoylation, and consequently, the observed effects could arise from the activity of other factors targeted by SUMO, rather than from sumoylation of p53 itself. In an attempt to address this problem, a recent elegant study has employed an in vitro reconstituted assay, where SUMO-1-modified p53 was purified to near homogeneity, and its DNA binding and transcription properties were studied on one p53-regulated promoter, specifically p21 (Wu and Chiang, 2009). It was shown that sumoylation inhibits p53 binding and transcription of this promoter, and it prevents p53 acetylation by p300. Thus it was suggested that sumoylation has inhibitory activity on p53 signaling. However, evidence that SUMO plays a positive role in p53 signaling, at least in some conditions, also comes from the demonstration that the E3 ligase PIASy stimulates sumoylation and the transcriptional activity of p53, leading to either senescence or apoptosis (Bischof et al., 2006). Given the importance of sumoylation in modulating the activity of so many different proteins, including p53, it is relevant to define the molecular mechanisms that allow this modification to achieve such versatility of effects.

Compared to chemical post-translational modifications consisting of simple chemical groups, SUMO proteins are small polypeptides thus raising the intriguing possibility of whether they are, in turn, targeted by chemical modifications. Consistent with this idea,

recent work showed that SUMO-1 can be phosphorylated (Matic et al., 2008), but the functional consequences of this modification are not yet clear. A distinctive characteristic of all SUMO family members is that they are rich in lysine residues, which are acceptors of multiple post-translational modifications, particularly of acetylation. This consideration led us to ask whether SUMO proteins can be modified by acetylation. In this work, we show that SUMO-1 is acetylated at a cluster that displays similarity with the acetylated domain of several transcription factors, most noticeably with the acetylatable C-terminal region of p53. We provide evidence that acetylation is important for SUMO-1-mediated control of the transcriptional and cell growth regulatory program elicited by p53.

Thus, our data provide the first demonstration that SUMO-1 is acetylated, while offering a paradigmatic example of how post-translational modification(s) of this small regulatory molecule can either positively or negatively influence cellular outcomes and the activity of its substrates.

Results

Identification of an acetylated motif of SUMO-1 with similarity to acetylated domains of transcription factors

Although there are no obvious consensus motifs for acetylation, a certain degree of similarity between acetylation sites of different substrates does exist. This similarity has been noted by us and by others in the case of the androgen receptor, FEN-1 and GATA-1, leading to the discovery of acetylation of several of these factors (Boyes et al., 1998; Hung et al., 1999; Hasan et al., 2001; Friedrich-Heineken et al., 2003; Fu et al., 2003). When blasted against the amino acid sequences of transcription factors known to be acetylated, the N-terminus of SUMO-1 exhibited rather striking similarities with the C-terminal tail of p53 that contains the majority of acetylated residues. Particularly, K37–K39 and K45–K48 of SUMO-1 aligned with p53 lysines targeted for acetylation by p300/CBP at position K370, K372, K373, and K382–K383 (Fig. 1A, in red and indicated by arrows). In both SUMO-1 and p53 these lysines are surrounded by similarly charged amino acid residues, a characteristic that is important for substrate recognition by acetylases. This SUMO-1 region aligned with known acetylation sites of other proteins as well, such as YY1, GATA-1 and FEN-1 (Fig. 1B,C and not shown). Further, a recently developed in silico acetylation site prediction system identified K39 and K48 of SUMO-1 as potential acetylation sites with probability scores similar to those obtained for the known acetylated residues of p53 (Fig. 1D) (Li et al., 2006; PAIL, Prediction of Neacetylation of Internal Lysines).

The discovery of such similarities implied that this SUMO-1 motif might functionally mimic the acetylated state of similar domains of transcription factors. Hence, given the importance of SUMO-1 in transcriptional control, we next asked whether SUMO-1 is truly acetylated. Two approaches were used for this purpose. First, acetylation assays were performed in vitro using purified SUMO-1 protein and the catalytic domain of several acetyltransferases, specifically of CBP and PCAF, in the presence of ^{14}C -acetyl CoA. As shown in Figure 2A purified SUMO-1 was efficiently acetylated by CBP (lane 3), but not by PCAF (not shown). To preliminarily map the site(s) of acetylation, we tested the ability of cold synthetic peptides corresponding to specific SUMO-1 regions, to inhibit CBP-mediated acetylation of the full-length protein in a reaction containing ^{14}C -acetyl CoA. Two peptides comprising lysines at position K37–K39, and K45–K46–K48 completely blocked acetylation (Fig. 2B, lanes 3 and 4), suggesting that these residues are responsible for the majority of the SUMO-1 acetylation signal. By contrast a peptide containing lysines at position K25 and K26 only weakly abrogated CBP-mediated acetylation of full-length SUMO-1 (Fig. 2B, lane 2).

Second, to assess whether acetylation occurs *in vivo*, an epitope Flag-tagged-SUMO-1 was expressed in the human H1299 lung cancer cell line under the control of a tetracycline-regulated promoter. In this system the expression of exogenous SUMO-1 leads to a compensatory down-regulation of the endogenous protein, therefore counterbalancing over-expression (Supplementary Fig. S1A). Following treatment of cells with the class I/II deacetylase inhibitor TSA, SUMO-1 complexes were purified and the total elution material containing both free non-conjugated SUMO and sumoylated proteins, was digested with trypsin and analyzed using nano-LC/ MS/MS and multiple reaction monitoring (MRM) detection. These purification experiments enriched for SUMO-1 in a specific fashion (shown in Fig. S1B). To determine whether the same residues are acetylated *in vitro* and in cells, mass spectrometry was simultaneously performed on the CBP-directed *in vitro* acetylation reactions as well. Although it is often assumed that trypsin does not cleave N-acetylated lysine residues, it has been previously observed that tryptic digestion can result in peptides containing C-terminal acetylated lysine residues (Wang et al., 2005; Griffiths et al., 2007). Consistent with this, in cells we detected well-represented MRM transitions corresponding to tryptic peptides 26-VIGQDSSEIHFK(Ac)-37 and 26-VIGQDSSEIHFK(Ac) VK-39 and to their non-acetylated forms that undoubtedly identified acetylation of K37 (Supplementary Figs. S2 and S3). To obtain an approximate determination of the stoichiometry of acetylation at this residue, we compared the relative MRM signals between acetylated and non-acetylated peptides (Supplementary Figs. S3 and S4), an approach amply validated by others (Wang et al., 2005; Griffith et al., 2007). This analysis revealed a rough relative abundance of approximately 42% of K37 acetylation in the total amount of purified SUMO-1. Additional peptides were detected that carried individual acetylation events at K39, as well as combination of acetylation at both K37 and K39 and at K45, K46, and K48 (Table S1 and not shown). Thus, a significant fraction of SUMO-1 is acetylated at these two residues *in vivo*, although additional experiments are certainly needed to determine the absolute concentration of the acetylated peptides in cells. Less represented post-translational modifications were also identified, consisting of acetylation and palmitoylation of K24, N-terminal acetylation, and acetylation of K45, K46, and K48, (summarized in Table S1 and not shown).

From the mass spectrometric analysis performed on the *in vitro* reactions, we further determined that CBP was able to acetylate the same residues that were found acetylated *in vivo*, specifically K37, K39 and K48 (Supplementary Fig. S5A–C). Thus, we name the region comprised between amino acid K37 and K48, SUMO acetylated domain, or SAD. It is significant that these SUMO-1 residues are conserved among different family members (Fig. 2C) in spite of the only modest overall homology between SUMO-1 and SUMO-2/3 (approximately 42%). Residues surrounding K37–K39 are also well conserved, possibly identifying an acetylation motif in all SUMO proteins. In agreement with this speculation, SUMO-2 and SUMO-3 were also robustly acetylated by CBP (Fig. 2D, lanes 6–7).

As such, these results unexpectedly revealed that SUMO-1 is modified by acetylation at multiple residues, both *in vitro* and in cells.

SUMO-1 is acetylated when conjugated to its substrates

To study how acetylation influences the activity of SUMO-1, we raised a polyclonal antibody against a peptide acetylated at K37 (Ac-K37-Ab). The specificity of this antibody was first assessed in *in vitro* acetylation assays (Fig. 3A). A robust signal was seen in the Ac-K37-Ab immuno-blot only in the presence of an acetylation reaction (compare lane 3 with lane 2). As a second approach, we constructed a SUMO-1 mutant where the acetylated lysines K37–K39 and K45, K46, and K48 were substituted with the non-acetylatable, charge preserving amino acid arginine residues (SUMO-1^{K37-48R}). The rationale for employing a mutant where all acetylation sites are replaced is twofold. First, it is well known that

disruption of only one site of acetylation often results in acetylation of nearby residues. Second, due to the previously noted similarity between various acetylation clusters, antibodies raised against one single acetylated lysine can still cross-react with other sites. We have clearly observed this phenomenon when studying acetylation of p53 (our unpublished observations). In the experiments shown in Figure 3B cell extracts derived from control H1299 cells or from cells expressing SUMO-1 or SUMO-1^{K37-48R} were first immuno-precipitated with the anti-Flag antibody and then probed in immuno-blot with the Ac-K37-Ab (left part). Meanwhile the analysis of the total SUMO-1 and SUMO-1^{K37-48R} derived extracts with the anti-Flag antibody showed that SUMO-1 exists in cells mostly as a non-conjugated, free pool, and as conjugated to RanGAP (Fig. 3B, lanes 5 and 6), as also noted by others (Tatham et al., 2008). Significantly, the Ac-K37-Ab reacted with both the free- and RanGAP-bound fraction of SUMO-1, but not of SUMO-1^{K37-48R} (Fig. 3B, compare lane 2 with lane 3), further demonstrating specificity of this antibody. Of note, we found that the Ac-K37-Ab reacts very weakly with SUMO-1 in immuno-precipitation assays.

In the subsequent set of experiments we asked whether SUMO-1 acetylation is regulated by the action of deacetylases, and during the DNA damage response that requires the activity of various sumoylated proteins. Thus, SUMO-1 expressing cells were treated with either TSA or with the DNA damaging agent etoposide. As shown in Figure 3C, an acetylation signal stronger than that seen in untreated cells was detected again on free SUMO-1, in both TSA and etoposide treated cells (Fig. 3C, compare lanes 2 and 3 with lane 1), while the levels of acetylation seen on RanGAP were only modestly influenced by these treatments. To determine whether SUMO-1 is acetylated when conjugated to substrates other than RanGAP, cells were treated with hydrogen peroxide (H₂O₂), which produces an accumulation of SUMO conjugates due to inactivation of SUMO peptidases (Bossis and Melchior, 2006). In these conditions the Ac-K37-Ab reacted directly with several high molecular weight SUMO-1 substrates (Fig. 3C, lane 4).

From the combination of these experiments we conclude that SUMO-1 is acetylated when bound to at least a set of its targets.

SUMO-1 is acetylated when conjugated to p53 in murine tumors

The p53 tumor suppressor is an important SUMO-1 target (Ferecatu et al., 2009). Although p53 sumoylation has been detected in cells as a consequence of SUMO-1 over-expression, whether this modification occurs in tumors has not been explored yet. To address this question, and also to determine whether SUMO-1 acetylation is a physiologically relevant phenomenon, we employed well established murine models of oncogene and p53-dependent tumorigenesis (Ewald et al., 1996; Tilli et al., 2003). Transgenic animals expressing SV40 large Tag develop ductal hyperplasia in the submandibular gland at around four months of age that eventually progresses to adenocarcinoma within the first year. Loss of p53 accelerates the onset of adenocarcinomas demonstrating that p53 acts as a barrier to tumor progression in these animals (Halama, Tilli, and Furth, unpublished observations). We excised and examined the submandibular tissue from an animal with suspected preneoplasia but no palpable or endured tumor on one side (PN), and one palpable tumor on the contra lateral side (MSGT1), as well as frankly malignant lesions of three other animals (MSGT2-to-4). Prototypical histological sections of these samples are shown in Figure 4A–C. Cell extracts from these tissues were prepared, and subjected to analysis with anti-SUMO-1-, anti-p53-, and anti Ac-K37-Ab antibodies. With this analysis we determined that sumoylated forms of p53 with different molecular weight (i.e., higher than the expected normal size), were detected in all these tissues (Fig. 4D, indicated by arrows). These species reacted with both the anti-SUMO and anti-p53 antibody. In the tumors where the highest levels of p53 sumoylation were detected, reactivity with the Ac-K37-Ab antibody was also seen (Fig. 4D,

lanes 3 and 4). Thus, in some mouse tumors, SUMO-1 is acetylated when conjugated to p53. We further found that p21 levels were higher in PN relatively to frankly malignant lesions, correlating with higher levels of p53 sumoylation seen in these latter, while the contrary was true for 14-3-3-sigma (Fig. 4E, compare lane 2 with lane 3 and 4). These results raise the question of how sumoylation affects p53-dependent transcription and proliferation.

SUMO-1 enhances the apoptotic activity of p53, via SAD

To study how p53 sumoylation influences proliferation, several strategies were employed. First, we expressed p53 in either naïve H1299 cells, or in the H1299 cell line harboring SUMO-1 or SUMO-1^{K37-48R} that we have described before. To achieve a homogenous level of p53 expression, a replication deficient adenovirus was used, as described by others (Tanaka et al., 2007). As shown in Figure 5A, expression of p53 alone led to an arrest of the cycle at the G1 phase in the H1299 cell line (compare part i with part iv). By contrast, 15% of SUMO-1 expressing cells underwent apoptosis in the presence of p53-but not of control adenovirus (part v vs. part ii), and this effect was partially reverted by SUMO-1^{K37-48R} whose expression resulted in more cells arrested in G1 (part vi). Similarly to SUMO-1^{K37-48R}, only 2% of apoptotic cells were seen when p53 was expressed in the parental H1299 cell line. This result suggests that sumoylation enhances the apoptotic activity of p53 and underscores the importance of lysines within SAD in this respect.

Although compelling, these experiments did not rule out that over-expression of SUMO-1 proteins indirectly affects proliferation in the presence of p53, for example, via sumoylation of other factors in turn acting as regulators of p53 activity. To overcome this problem, we constructed chimerical proteins where one moiety of SUMO-1 was attached as linear fusion to the C-terminal tail of p53. This approach generates a gain of function, stable sumoylated form of p53, which allows the study of this modification in the absence of other unpredictable events. An identical strategy was employed by others to assess the effects of p53 mono-ubiquitination or of histone H4 sumoylation (Li et al., 2003; Shiio and Eisenman, 2003). Of similar importance, since sumoylated p53 is present in lower abundance relatively to the non-sumoylated fraction, the use of these chimeric proteins now permits a definition of the biological activities of this under-represented p53 population. Two chimeras were used for these experiments, one where the last two glycine residues of SUMO-1 required for conjugation were eliminated (p53-SUMOΔGG), and a second one where these residues were left intact (p53-SUMO). To study the activity of the acetylated domain of SUMO-1, SAD, lysine K37-to-K48 were replaced with amino acid residues that destroy acetylation, arginine or alanine, to generate p53-SUMO^{K37-K48R} or p53-SUMOΔGG^{K37-K48A}. In the case of androgen receptor acetylation, alanine and arginine mutants behave identically (Fu et al., 2003; Haelens et al., 2007). p53 proteins were expressed with the tetracycline inducible system in the p53 null H1299 cell line that has been extensively used for studying p53 signaling (Knights et al., 2006; Shi et al., 2007). When we examined the cellular localization of these proteins, we found that unlike native p53 whose expression results in a diffuse nuclear staining, p53-SUMO and p53-SUMOΔGG localized in promyelocytic leukemia (PML) nuclear bodies (Supplementary Fig. S6A), where p53 was shown to co-localize together with PML in several conditions (Gostissa et al., 1999; Pearson et al., 2000; Rodriguez et al., 2001). In addition, SUMO-1 was found to be acetylated in both p53-SUMO and p53-SUMOΔGG chimeras (not shown) Thus, these results gave us confidence that p53-SUMO chimeric proteins recapitulate physiological aspects of p53 sumoylation.

The cell-cycle distribution of these cells was next studied and compared to the H1299 cell line expressing naïve p53 (Knights et al., 2006). Paralleling our previous results, a higher percentage of apoptotic cells was again seen in p53-SUMO expressing cells relatively to those harboring either naïve p53 or p53-SUMO^{K37-K48R} that arrested predominantly in G1 (Fig. 5B, compare parts ii and iii with part iv). Because sumoylated p53 co-exists with the

non-sumoylated fraction, we also explored how co-expression of these two populations affects proliferation. Expression of p53 with the adenoviral vector in cells harboring p53-SUMOΔGG (or in p53-SUMO cells, not shown) only modestly enhanced apoptosis relatively to cells expressing p53-SUMOΔGG alone (Fig. 5C, compare parts iv and v), while cells harboring p53-SUMOΔGG^{K37-K48A} arrested in G1 in the absence or in the presence of native p53 (compare parts iii and vi with part iv). Inspection of cells expressing p53-SUMOΔGG and p53-SUMOΔGG^{K37-K48A} via immuno-fluorescence, revealed the presence of apoptotic fragmented nuclei in the former but not in the latter (Fig. 6A and quantified in Fig. 6B).

To determine whether these differences rely upon discriminatory expression of apoptotic or cell-cycle regulatory genes induced by p53 proteins, we next examined the expression pattern of two of its downstream transcriptional targets, bax and p21. We found that p21 was induced by naïve p53 and by p53-SUMOΔGG^{K37-K48A}, but not by p53-SUMOΔGG (Fig. 7A, compare lane 4 and 3, with lane 2). A clearly different pattern of expression was instead observed in the case of bax, which was strongly expressed in cells harboring p53-SUMO proteins relatively to cells expressing naïve p53 (compare lanes 2–3 with lane 4). Noticeably, the pattern of expression of p21 or bax was unaffected by co-expression of naïve p53 together with p53-SUMO proteins (Fig. 7A, compare lanes 5 and 6 with lanes 2 and 3).

Therefore, three conclusions can be made based upon these findings. First, even though it has been shown that in vitro p53-SUMO can form tetramers with non-sumoylated p53 (Wu and Chiang, 2009), it seems likely that the presence of non-sumoylated p53 within these tetramers would not significantly modify the ability of sumoylated p53 to influence transcription. Second, the data suggest that lysine residues within SAD might influence the modality by which p53 selects activation of its downstream target genes. Third, these lysine residues also appear essential for p53-mediated induction of apoptosis but not of cell-cycle arrest.

SUMO-1 restricts the transcriptional ability of p53 to selected target genes

Since the ability of p53 to induce apoptosis relies, in large part, on regulation of transcription, we next examined more closely the role played by sumoylation in this respect. First, we conducted canonical reporter assays by employing a p21-regulated promoter placed upstream of the luciferase gene. To exclude the possibility that observed difference(s), if any, might be due to variations in the levels of the various p53 protein, titration experiments were performed, while expression of non-conjugated SUMO-1 provided a control for possible transcriptional effects of SUMO, independently of p53. As shown in Figure 7B, in cells expressing non-conjugated SUMO-1 a modest induction of p21 reporter activity was seen, therefore luciferase levels detected in these samples were used for normalization. When compared to the background activity of SUMO-1, p53-SUMO was unable to transactivate the p21 reporter relatively to unmodified p53. This result is in full agreement with previous studies showing that purified sumoylated p53 does not support transcription of the p21/ WAF promoter in in vitro assays (Wu and Chiang, 2009). We further determined that a p53-SUMO chimera lacking part of the N-terminus and the entire C-terminal half of SUMO-1, but containing only amino acids 14–55 (p53-SAD) behaved identically to p53-SUMO in that it was similarly unable to robustly stimulate transcription. Thus, the presence of SAD is entirely sufficient to convey the inhibitory effect of SUMO-1 on p21 transcription. Even more significantly, the non-acetylatable form of SUMO, p53-SUMO^{K37-K48R} reverted this inhibition and stimulated the p21 reporter. These data imply that reversible acetylation of SADS might switch the activity of SUMO-1 from an inhibitor to an activator of transcription, at least on the p21 promoter.

Due to significant variations of genomic p53 DNA-consensus sites along with differences in chromatin density and architecture, the promoter context is a key determinant of the ability of p53 to select its downstream targets (Menendez et al., 2009). Therefore, to determine how SUMO-1 influences the large repertoire of p53 regulated genes we decided to perform gene expression micro-array analysis of cells expressing native p53 or p53-SUMO. The gene expression profiles derived from these cells were compared to those obtained in the parental H1299 cell line and in H1299 cells expressing SUMO-1, employed as controls. Somewhat surprisingly, we found that very few genes were affected by the expression of SUMO-1 alone (not shown), therefore we employed the arrays obtained in H1299 cells as the background for comparison. A complete list of genes modulated by p53-SUMO is provided as Supplementary Information, and a partial list of genes is shown in Table 1. A schematic diagram of the total number of genes regulated by p53 and p53-SUMO is shown in Figure 7C. Through this approach we determined that SUMO-1 leads to global attenuation of the transcriptional activity of p53. In fact, 1,032 genes were found modified by p53, while only 562 were influenced by p53-SUMO. Such reduction occurred predominantly at the expenses of the repressed genes: in fact, native p53 repressed 634 transcripts, while only 168 were inhibited by p53-SUMO, suggesting that, like in the case of other proteins (Ihara et al., 2005; Gómez-del Arco et al., 2005), SUMO-1 alleviates trans-repression. Further, while the global number of activated transcripts was essentially comparable between the two proteins, several genes normally up-regulated by p53 were activated much less efficiently by p53-SUMO (most noticeably p21) and vice versa, 162 genes were found up-regulated by p53-SUMO relatively to native p53 (Supplementary Fig. S7). By taking into consideration only well known and validated p53 targets, we determined that FDXR, NOXA, IGFBP3, VDR, and 14-3-3-sigma were all up-regulated in p53-SUMO-1 expressing cells relatively to p53, while other transcripts such as MDM2, PUMA, or AIP1 were under-represented (Table 1). As shown in Figure 7D, real-time PCR confirmed modulation of some of these transcripts in p53-SUMO expressing cells.

We interpret these data to suggest that SUMO-1 restricts the transcriptional activity of p53 by alleviating trans-repression, while stimulating the transactivation potential on a discrete number of genes. To then determine whether SUMO-1 acts by modifying p53 ability to interact with chromatin embedded promoters we performed chromatin immuno-precipitation assays (ChIP) on a variety of p53-regulated genes that, based on the micro-array assays, showed similar or differential modulation by p53-SUMO compared to native p53 (Fig. 8A,B). Such analysis revealed that p53-SUMO bound with stronger affinity to the promoters of several of the gene products that were found up-regulated in the micro-array platform, such as FDXR, 14-3-3-sigma, NOXA, and IGF-BP3, while DNA binding was compromised on the p21, PUMA and MDM2 promoters. Significantly, p53-SUMO^{K37-K48R} reverted the effects of p53-SUMO on several binding elements, such that it interacted less efficiently with promoters to which p53-SUMO bound more efficiently, while it enhanced binding activity on the promoters for which p53-SUMO had less affinity (Fig. 8C).

Discussion

One of the more investigated questions in the p53 field pertains to the molecular mechanisms that allow this protein to regulate the expression of its multiple downstream target genes and how this, in turn, translates into cell fate decisions (Das et al., 2008; Riley et al., 2008). Acetylation of various p53 lysines within the p53 C-terminus is important in determining the transcriptional and growth-regulatory program elicited by p53, and it does so at least in part by enhancing DNA binding to consensus sites for which unmodified p53 has low affinity that are often contained in the promoters of pro-apoptotic genes (Knights et al., 2006; Horvath et al., 2007). This ability of acetylation to influence the gene target selection process is not restricted to p53, but it appears to be a general “modus of

operandum” by which this post-translational modification regulates the function of other sequence specific transcription factors, for example MyoD and GATA-1 (Freiman and Tjian, 2003; Letting et al., 2003; Di Padova et al., 2007). Data presented in this work identify an acetylated domain within SUMO-1, SAD, with similarity to the C-terminal domain of p53 which, in the acetylatable form, elicits apoptosis and promoter dependent recruitment of p53. Therefore, we propose that SAD mimics signals normally conveyed by the acetylated state of the p53 C-terminal tail. Why so? We speculate that SUMO-1 acetylation becomes important when the activity of enzymes required for acetylation of p53 itself is limiting or, alternatively, to enable acetyltransferases to recognize p53 as a substrate for modification(s). For example, DNA damage induced p53 phosphorylation enhances its interaction with CBP and, similarly, phosphorylation of histone H2A at DNA damage sites is a signal for recognition and activation by acetylases that, in turn, modify chromatin (Lambert et al., 1998; Downs et al., 2004). It is possible that in certain conditions, such as in the absence of a wide spread DNA damage response, it is SUMO-1 acetylation that provides the signals for p53 activation. Furthermore, SUMO-1 may also physically bridge p53 with acetyltransferases. Consistent with this idea, we found that p53-SUMO has stronger affinity for CBP relatively to naïve, unmodified p53, but only when SUMO-1 is in the acetylatable state (Supplementary Fig. S8).

The SUMO pathway has been linked to transcription repression and activation. Although in many instances sumoylation acts by inhibiting transcription, the effects of sumoylation on several of its targets can be positive and negative depending upon the factor and the promoter context, as in the case of Smad4 (Gill, 2005; Lyst and Stancheva, 2007). A switch in transcription regulation was observed in this study when luciferase reporter assays were performed on the p21 promoter. A p53-SUMO chimera repressed p21-driven transcription relative to naïve p53, while a similar chimera differing only for the presence of non-acetylatable residues within SAD stimulated luciferase activity (Fig. 6). We would assume that the reverse should be true on other p53-regulated promoters, but obviously more work is needed to fully corroborate this proposal. It is further possible that sumoylated p53 “qualitatively” modifies and expands p53 transcriptional program by allowing it to act on a different sets of genes. This is also suggested by our micro-array data, showing that 203 genes were uniquely regulated by p53-SUMO relatively to naïve p53. Furthermore, it is clear that although the list of p53-regulated genes grows to be larger and more complex, it is the combination of only a few targets activated or inhibited that might dictate cellular outcome. For example, in colorectal cancer cells the relative expression level of PUMA and p21 influences this decision, such that when p21 is low relatively to PUMA cells undergo apoptosis (Iyer et al., 2004). A similar correlation was seen here between p21 whose activation is linked to cell-cycle arrest, and bax, an important target of p53-mediated apoptosis: in cells expressing p53-SUMO p21 levels were lower relatively to cells harboring non-acetylatable p53-SUMO moieties, while bax was up-regulated by both chimeric proteins. This result, and other of our data, suggests that SUMO-1 acetylation might alter the equilibrium between pro-apoptotic and cell-cycle regulatory genes regulated by p53. The idea that SUMO-1 serves a pro-apoptotic function within p53 signaling is further corroborated by the study and the comparison of micro-array data performed in cells that undergo apoptosis versus cells that undergo cell-cycle arrest that identified SUMO-1 as one of the pro-apoptotic genes induced by p53 in the former but not in the latter (Kannan et al., 2001).

In keeping with the importance attributed by this study to SUMO-1 acetylation, a key question is therefore whether the acetylation state of SUMO-1 itself is modulated by the specific landscape of various promoters, for example, by the presence therein of acetylases or deacetylases. Or rather, whether it is SUMO-1 which, depending upon its acetylation state, recruits these enzymes to create an environment permissive or restrictive for

transcription activation. The answer to this question will require the generation of additional antibodies directed against acetylated forms of SUMO-1 that are suitable for chromatin immuno-precipitation assays. This is apparently not an easy task to accomplish, due to the hydrophobic nature of the region where the acetylated SUMO-1 residues reside. We generated two antibodies directed against acetylated peptides of SUMO-1 within this region, and they both showed only modest activity in immuno-precipitation assays. Nevertheless, our data show that in the acetylatable form SAD promotes the interaction of p53-SUMO with CBP (Supplementary Fig. S8), arguing that acetylation-dependent recruitment of this protein might contribute to SUMO-1-mediated control of p53 transcription. Further, molecular modeling simulations of acetylation in the structure of SUMO-1 (Fig. 9) reveal that the loss of positive charge upon acetylation while causing only a minimal disturbance to the local region of SUMO-1, generates a slightly differently oriented interaction interface that might function for protein–protein adhesion and signaling. Thus, it seems likely that acetylation modifies SUMO-1 interactions with regulatory proteins once bound to p53 or to others of its substrates.

In conclusion, our study reveals that SUMO-1 is capable of undergoing a post-translational modification, acetylation that is well known to control the activity of many intracellular proteins and of transcription factors. In keeping with our finding that SUMO-1 is acetylated when bound to several others of its substrates there are many questions that can now be asked. For example, does acetylation compete with—or substitute for—acetylation of histones and of other transcription factors? How many other residues of SUMO are acetylated, and what are the enzymes that synchronize deacetylation with intracellular signals? Future studies should shed light on these important issues.

Materials and Methods

Proteins and antibodies

Purified SUMO-1, SUMO-2, and SUMO-3 were purchased from Biomol-Enzo Life Sciences (#UW9190, UW9200, UW9210, respectively). CBP protein was also from Biomol. The anti-SUMO antibodies were from Zymed/Invitrogen (monoclonal); or Santa Cruz Biotech. (scbt) (monoclonal D11). The anti-Flag antibodies were from Sigma Aldrich (M2 monoclonal; anti-rabbit polyclonal). The p53 antibody employed for chromatin immuno-precipitation assays was from Santa Cruz (FL393). A polyclonal antibody was raised against peptide sequences SEIHF{K-Ac}VKMTTHLKK and purified by immuno-affinity column.

Plasmids

The p53-SUMO-1 chimerical proteins (p53-SUMO and p53-ΔGG) and the corresponding lysine mutants were produced by amplifying SUMO-1 domain from an existing plasmid expressing full-length SUMO-1 by PCR followed by site directed mutagenesis. The PCR products were cloned into the pcDNA4/TO-Flag-p53 vector whose construction has been previously described (Knights et al., 2006). For the generation of non-conjugated SUMO-1, an N-terminal Flag epitope was added to SUMO-1 by PCR amplification of SUMO-1 using the following primer: N-term Flag-SUMO, 5'-AAAGGATCCGCATGGATTATAAAGATGATGATGATAAGGGATCTGACCAGGAGGCA-3'. The resulting PCR product was then cloned into pcDNA4/TO. Mutagenesis of the lysine residues in SUMO-1 was conducted with the ExSite PCR-Based Site-Directed Mutagenesis Kit Stratagene. The vector expressing SUMO-1 was a kind gift from Dr. G. Del Sal.

Acetylation assays

Acetylation reactions were typically assembled in a 30 μ l volume, containing HAT buffer (1 $\times$ 25mM Tris-HCl, pH 7.9; 50mM NaCl, 1mM DTT), 6 μ l of 14 C-acetyl-coenzyme A (Amerhsam biosciences) or cold acetyl-coenzyme A (lithium salt, from Roche), 1–2 μ g CBP, 0.5–1 μ g of SUMO proteins.

Cell lines, transfections, and generation of the H1299 tetracycline inducible cells

Tetracycline inducible cell lines were generated with the T-REx system (Invitrogen), as described previously (Knights et al., 2006). After selection with proper antibiotics, polyclonal cell cultures were isolated and first examined for p53-expression levels by employing both immuno-fluorescence and immuno-blot assays. Typically three-to-five clones showing homogenous levels of expression (i.e., more than 80% of cells expressing p53 proteins) were pooled together and used for further studies.

Cell-cycle analysis

Flow cytometry was performed as described previously (i.e., Knights et al., 2006).

Immuno-blots, immuno-precipitations and treatments

Unless otherwise indicated cell extracts were prepared in RIPA buffer (0.1% SDS; 50mM Tris-Cl, pH 6.8; 150mM NaCl; 0.5% Nonidet P-40; 2mM EDTA) supplemented with protease a protease inhibitor mixture, with TSA (500 μ M) and with 10 nM NEM as an inhibitor of de-sumoylation. Treatment with etoposide was performed at 100 μ M overnight. When probed with the anti-SUMO or anti-acetyl-K37 antibody in direct immuno-blot, membranes were treated with 6M Guanidine for 30 min, followed by incubation in blocking solution. The Ac-K37 antibody was used to a 1:500 dilution in direct immuno-blots on in vitro acetylation reactions (i.e., Fig. 3A) and to a 1:250 dilution for the experiments in cells and in murine tumors. In experiments where we used tumor samples from animals, PDF membranes were denatured in 6M guanidine HCl, then renatured, and subjected to immuno-blot analysis with the anti-SUMO or the anti Ac-K37 specific antibodies. For H₂O₂ treatment cells were first trypsinized and collected in 10 ml of growth media. H₂O₂ was added drop by drop at a final concentration of 100 μ M. Cells were then incubated at 37°C for 20 min, washed twice with PBS and lysed in RIPA buffer.

Purification of SUMO-1 for tryptic digestion and mass spectrometric analysis

Pilot experiments were first conducted to optimize the purification and digestion procedure for detection of acetylation by mass spectrometry. We found that when SUMO-1 was extracted from SDS gels, it was difficult to retrieve sufficient amounts of peptides spanning within the N-terminus of SUMO-1 to detect acetylation, likely due to the poor efficiency of digestion in these conditions. Therefore, we used the entire purification reaction from the Flag IP from cells, or the soluble in vitro reaction derived from the CBP-directed acetylation. In the former case, 50 dishes of H1299 (15 cm) cells were grown to confluence, treated with TSA (500 μ M) and induced with tetracycline for 24–36 h. Cell extracts were prepared in RIPA buffer complemented with inhibitors as described previously, preabsorbed on agarose beads, and then subjected to immuno-precipitation with 1.5 ml of anti-Flag-M2 immuno-affinity column, overnight. Beads were washed seven times in 10 volumes of RIPA buffer, then for 1 h with 15 volumes of RIPA buffer. Immuno-precipitates were subjected to a final wash with buffer containing 25mM HEPES pH 7.5; 100mM KCl; 10% glycerol, 0.4mM EDTA, 10mM BME, and eluted in the same buffer containing the flag peptide at 400 μ g/ml. Three subsequent elutions in 0.5 (v/v) of elution buffer were carried out and pulled together. For mass spectrometry analysis, samples were dried under vacuum and resuspended in 25mM ammonia bicarbonate, reduced by incubation at 60°C for 1 h with

5mM TCEP (2, carboxy ethyl phosphine) and alkylated with 10mM MMTS (methyl methane thio sulfonate) for 10 min at room temperature. One microgram of sequencing grade trypsin was used for digestion (Promega) that was carried out at 37°C for 18 h. Following desalting with C18 reverse phase spin columns (Nest Group) the eluted peptides were dried and resuspended in 20µl of solvent A (98% water, 2% acetonitrile, and 0.1% formic acid) and finally analyzed with nano-LCMS/MS or nano-LC MRMMS/MS. Additional methods for the mass spectrometric analysis are provided in the Supplementary Materials and Methods file.

Molecular simulation of acetylation in the structure of SUMO-1

The methods for molecular modeling of acetylation are described in detail in the Supplementary Materials and Methods file.

Micro-array analysis

Cells were harvested for RNA extraction and 7µg of total RNA was used for cDNA and biotinylated cRNA synthesis. Expression profiling analysis was performed using the HG-U133A 2.0 human Affymetrix high-density oligonucleotide micro-array. Each gene-chip was used for a single hybridization with RNA isolated from one cell line. Each sample was run in a duplicate or in triplicate. Expression profiling was performed as described previously (Knights et al., 2006). We used two normalization processes: one for chip-chip comparisons (scaling factors), and one for gene-gene comparison (normalization to the average of the naïve signal intensities for each gene). The scaling factor determinations were done using default Affymetrix algorithms (MAS 5) with a target intensity of chip sector fluorescence to 800. The use of Affymetrix MAS 5.0 signal intensity values, together with a “present call” noise filter achieves an excellent signal/noise balance relative to other probe set analysis methods (dchip, RMA). Data analyses were limited to probe sets that showed 1 or more “present” (P “calls”) in the eight gene-chip profiles in our complete dataset. Data were analyzed using the GeneSpring software (Silicon Genetics).

Chromatin immunoprecipitation assays

ChIP assays were performed as described previously. Briefly, 2×10^7 H1299, or H1299 expressing p53, p53-SUMO, or p53-SUMO^{K45R} cells were grown in the absence or presence of tetracycline and subsequently exposed to 1% formaldehyde-PBS solution for 13 min at room temperature. The extracts were sonicated after lysis to obtain DNA fragments of lengths comprised between 300 and 800 bp. Chromatin solutions were precipitated overnight with rotation using a rabbit polyclonal anti-p53 antibody (FL393). On the following day, protein A agarose beads that had been previously blocked with salmon sperm DNA and BSA were added to each reaction to precipitate DNA-p53 complexes. These were washed and then incubated at 65°C overnight in parallel with “input” samples to reverse the cross-linking. DNA was isolated with the Qiagen-PCR purification kit. The precipitated DNA was then subjected to PCR reactions for 30–35 cycles. Quantification of ChIP assays was performed as follows. The mean relative intensity of input and experimental ChIP PCR bands was determined using the histogram feature of Adobe Photoshop 7.0. Input intensities were normalized to correct for minor differences in the total amount of DNA contained in the samples. Experimental band intensities were then adjusted against their corresponding normalized input signal. Fold change represents the comparison of normalized p53 or p53-SUMO band intensity compared to normalized H1299 band intensity. The sequence of the primers used for amplification is provided in the Supplementary Materials and Methods file.

Quantitative real-time PCR (qRT-PCR)

Parental H1299 cells or H1299 cells expressing p53, p53-SUMO or Flag-SUMO were treated with tetracycline for 48 h followed by extraction of total RNA. Reverse transcriptase PCR (SuperScript III first-strand synthesis system, Invitrogen) was performed with random hexamer primers and 2mg of RNA to obtain sufficient cDNA for analysis. cDNA samples were then mixed with gene specific primers (generated by SciEd Central, Scientific & Educational Software) and iQ SYBR green super mix (BioRad) according to the manufacturer's instructions. Samples were then analyzed using an ICycler iQ thermocycler (BioRad) and software version 3.1.7. Each cDNA was analyzed in duplicate. Cycle counts were normalized against B-actin and reported relative to H1299 cells using the DDCT method.

Mouse models

Mice carrying a transgene composed of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) linked to sequences encoding the tetracycline responsive reverse transactivator (tTA) for "tet-off" gene regulation and a transgene composed of the tetracycline operator (tet-op) promoter linked to sequences encoding the Simian Virus 40 T Antigen (TA_g) on a C57Bl/6 background (Ewald et al., 1996; Tilli et al., 2003) were maintained on regular mouse chow and euthanized in accordance with institutional and federal guidelines approved by the Georgetown University Animal Care and Use Committee. Submandibular salivary gland preneoplastic and tumor tissue was isolated at the time of necropsy. One half was formalin fixed and processed for hematoxylin and eosin section staining; the other half was flash frozen and stored at -20°C until used for biochemical experiments.

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Fig. 1. Similarity of the N-terminus of SUMO-1 with acetylated domains of transcription factors. A: The p53 C-terminal region from amino acids 300 to 384 was blasted against SUMO-1 sequences by using the L ALIGN or ALIGN program at the Gene-stream server (<http://xylian.igh.cnrs.fr/>). Known sites of acetylation in p53 are highlighted in red. Arrows, at the top or the bottom, indicate the relevant lysines in p53 or SUMO-1 that display identity. B,C: Alignment of the N-terminal region of SUMO-1 with known acetylated domains of YY1 and GATA-1. In both A and B, amino acids that show identity are denoted by double dots, while those with similarity are denoted by single dots. Known acetylated residues in YY1 and GATA-1 are indicated at the bottom of each part. D: The region of SUMO-1 comprised between amino acids 28 and 58 was analyzed for the presence of potential acetylation motifs, and compared to p53. The Prediction of Neacetylation of Internal Lysines (PAIL) program was employed for such analysis (<http://bioinformatics.lcd-ustc.org/pail/>). Searches were performed in high stringency conditions and by using a high threshold to yield higher specificity performance (threshold of 0.5). Sites of acetylation in p53 and SUMO and their relative scores, as identified by PAIL, are highlighted in red.

Fig. 2.

Identification of an acetylated domain of SUMO-1 conserved among SUMO family members. **A:** Purified SUMO-1 was subjected to an in vitro acetylation reaction in the absence (lane 2) or presence (lane 3) of CBP, and of ^{14}C -acetyl-coenzyme A. Lane 1 contains CBP in the absence of SUMO-1. Reactions were assembled in 30 μl volume (see the Materials and Methods Section), and developed with autoradiography. One of 20 of the reaction mixtures were run independently and proteins were visualized with Coomassie Brilliant Blue staining (lanes 4–6). The position of SUMO-1 and CBP is indicated by arrows. **B:** Peptide competition experiments. The following acetylated peptides were used: K25-K27:YIK(Ac) LK(Ac) VIG (lane 2); peptide K37–K39:HFK(Ac)VK(Ac)MTT (lane 3); peptide K45–K46–K48: HLK(Ac)K(Ac)LK(Ac)ES (lane 3). Lane 1 contains the acetylation reaction with a control peptide (QIRGRERFEM) and lane 5 contains the acetylation reaction without peptide(s). Peptides were incubated in a 10-fold excess relatively to full-length SUMO-1. **C:** Identification of lysines residues acetylated in vitro and in vivo by mass spectrometry (see Supplemental Data, Figs. S2–S5 for further details), and alignment of acetylated residues in SUMO-1 with SUMO-2 and SUMO-3. Acetylated lysines of SUMO-1 are indicated by arrows and bolded in red, and conserved residues are shown. Based on the results of mass spectrometry we name the domain comprised between amino acid K37 to K48 *SUMO-1 Acetylated Domain*, or *SAD*. **D:** Acetylation of SUMO

family members. Acetylation reactions were carried out as described in A. SUMO-1 (lanes 2, 5) SUMO-2 (lanes 3, 6), or SUMO-3 (lanes 4, 7) were subjected to an acetylation reaction in absence (lanes 2–4) or presence (lanes 5–7; indicated by +) of CBP. Lane 1 contains an acetylation reaction with CBP without SUMO proteins.

Fig. 3.

SUMO-1 is acetylated when bound to its substrates. **A:** A polyclonal antibody raised against a SUMO-1 peptide SEIHF{Lys-Ac} VKMTTTLKKC acetylated at K37 was raised and purified by peptide affinity purification. The specificity of this antibody was tested first in an in vitro acetylation reaction similar to that described in Figure 2A. SUMO-1 was incubated in the presence (lane 3) or absence (lane 2) of CBP and cold acetyl-coenzyme A. Lane 1 contains the acetylation reaction with CBP alone without SUMO-1. Samples were subjected to SDS electrophoresis, blotted on a PDF membrane, and probed with Ac-K37-Ab in immunoblot (upper part). After chemi-luminescence, the membrane was stained with Coomassie Brilliant Blue to reveal SUMO-1 (lower part). **B:** Acetylation of SUMO-1 in vivo. Control H1299 cells (lanes 1 and 4) or H1299 expressing naïve SUMO-1 (lanes 2 and 5) or a SUMO-1 harboring lysine to arginine replacements at position K37–K39 and K45–K46–K48 (SUMO^{K37-K48R}, lanes 3 and 6) were induced with tetracycline. Cell extracts were absorbed on the anti-Flag M2 immuno-affinity column, eluted, and run in on 4–20% gradient gel. Following electrophoresis and transfer, membranes were immuno-blotted with the Ac-K37-Ab (left part, lanes 1–3). Forty micrograms of total cell extracts were probed with the anti-Flag antibody indirect immuno-blot (right part, lanes 4–6). The position of free SUMO-1 and of SUMO-1 conjugated to RanGAP is indicated by arrows. **C:** Modulation of SUMO-1 acetylation by deacetylases and by DNA damaging agents. H1299 cells expressing SUMO-1 were left untreated (lanes 1 and 5) or they were treated with 500 nM TSA (lanes 2 and 6), with 1 μ M etoposide (lanes 3 and 7), or with 100 mM H₂O₂ (lanes 4 and 8; see the Materials and Methods Section for details on H₂O₂ treatment). Cell extracts were prepared in RIPA buffer, immuno-precipitated with the anti-Flag antibody and processed in immuno-blot with the Ac-K37-Ab as described in B (lanes 1–4). Total cell extracts are shown in the right part. The position of SUMO-1, RanGAP and of SUMO-1 conjugates is indicated. Full arrows indicate non-specific reactivity of the Ac-K37-Ab with heavy and light chains of the anti-Flag immuno-precipitation.

Fig. 4. SUMO-1 is acetylated when bound to p53 in murine tumors. A–C: Histopathology of a salivary gland preneoplastic dysplasia (PN) and a murine salivary adenocarcinoma (MSGT1-2). Open arrows indicate preneoplastic tissue and dark arrows indicate adenocarcinoma tissue. D: Cell extracts prepared from PN and from murine salivary gland tumors (MSGT1–MSGT4) were immunoprecipitated with the anti-SUMO-1 monoclonal antibody and subsequently immuno-blotted with either anti-SUMO-1 (upper part), anti-p53 goat polyclonal antibody (middle part), or Ac-K37-Ab (lower part). The position of sumoylated and acetylated p53 is indicated by arrows, and the position of these species relatively to the molecular weight reference marker is also shown. E: The total p53 levels as well as the expression levels of the p53 downstream targets p21 and 14-3-3-sigma along with the loading control actin, were determined by direct immuno-blot. Lane 5 is only apparently separate from the other samples, as it was run in the same immuno-blot, but a blank lane was cut in between.

Fig. 5.

SAD is required for the apoptotic activity of sumoylated p53, but not for the cell-cycle arrest function. A: Naïve H1299 cells (parts i and iv), or H1299 expressing SUMO-1 (parts ii and v) or SUMO-1^{K37-K48R} (parts iii and vi) were infected with adenovirus control or with the adenovirus expressing p53 (parts i through iii and iv through vi, respectively), as indicated at the top of each part. Seventy-two hours after infection, cells were harvested and their cell-cycle profile was analyzed (see the Materials and Methods Section). B,C: Analysis of the cell-cycle profile of H1299 cells harboring p53-SUMO chimeric proteins indicated at the top of each part. Cells in C were infected with control- or p53-expressing adenoviruses as described in A. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 6.

A: H1299 cells expressing p53-SUMO Δ GG (upper parts) or p53-SUMO Δ GG^{K37-K48A} were plated onto cover-slips, induced with tetracycline for 4 days and processed in immunofluorescence. Cells were stained with the anti-p53 polyclonal antibody (left green parts) or with DAPI (middle blue parts), or merged (right parts). Z-stack images were acquired at 1- μ m intervals, and best-focused areas are shown. Arrows in the DAPI staining point to apoptotic fragmented nuclei. B: Quantification of experiments shown in D. H1299 control cells, or cells expressing p53-SUMO Δ GG or p53-SUMO Δ GG^{K37-K48A} were processed for immunofluorescence as described in D. Apoptotic and fragmented nuclei were counted from a triplicate experiment and plotted. Bars represent standard deviations.

Fig. 7.

A: SUMO-1 restricts p53-dependent transcription. A: Naïve H1299 (lanes 1 and 4), or cells harboring p53-SUMO Δ GG (lanes 2 and 5) or p53-SUMO Δ GG^{K37-K48A} (lanes 3 and 6), were infected with control-or p53-expressing adenoviruses (lanes 1–3 and lanes 4–6, respectively). Cell extracts derived from these cells were probed in direct immuno-blot with the anti-p53 polyclonal antibody, the p21 monoclonal antibody, the bax monoclonal antibody or the anti-actin antibody, as indicated at the side of each part. The position of p53-SUMO proteins and of naïve p53 is indicated by arrows. B: Luciferase reporter assays were performed to assess how SUMO-1 influences the transcriptional activity of p53. The following vectors were employed for these experiments: the vector expressing naïve p53; or a p53 chimeric protein containing aminoacid residues 14-to-55 of SUMO (p53-SAD); or SUMO^{K37-K48R}; or a vector expressing SUMO-1. Titration experiments were performed and

plasmids were co-transfected at the concentrations indicated at the left of the part in the p53 null H1299 cell line with the luciferase reporter driven by the regulatory elements of the p21 promoter containing p53 DNA-binding sites. Luciferase activity was determined 24–36 h after transfection. The type of p53 expressing vector is indicated at the bottom of each part. Because transfection of SUMO-1 alone resulted in background transcriptional activity, values obtained in cells transfected with this vector were used for normalization, and considered as 1. At the bottom of the parts average fold activation after normalization is shown, for each p53 protein expressed at intermediate concentration of each plasmid (0.125 μ g). C: Comparison of gene regulation between p53 and the p53-SUMO. Arrays were conducted as described in the Materials and Methods Section. A: Venn diagram representation of the total number of genes regulated by p53 and p53-SUMO, relatively to H1299 cells. Pie charts represent the number of genes either activated or repressed. D: Quantitative real-time PCR confirms gene targets activated by p53-SUMO as identified in the micro-array platform. cDNA generated from RNA extracted from parental H1299 cells or H1299 cells expressing p53 or p53-SUMO was used for analysis by SYBR green quantitative real-time PCR. Values were normalized against β -actin and fold activation was determined relatively to the parental H1299 sample. Each sample was performed in duplicate and the error bars represent the standard deviation.

Fig. 8.

SUMO and SAD redistribute p53 on chromatin embedded p53-regulated promoters. A: Extracts derived from H1299 parental cells, or cells expressing p53, or p53-SUMO were subjected to chromatin immuno-precipitation with the anti-p53 polyclonal antibody. The DNA samples derived from each anti-p53 immuno-precipitation were amplified with pairs of primers directed to various p53-responsive target genes, as indicated on the right side of each part. To normalize for DNA content, the DNA was amplified with the indicated primers prior to the anti-p53 immuno-precipitation (indicated as input at the top of each part). B: Analysis of ChIP assays by densitometry. ChIP assays from cell lines indicated at the top of each part, were conducted as described before. The primer pairs employed for the amplification reactions are indicated at the bottom of the part. To quantify these experiments, densitometry of amplified bands was performed by using Adobe Photoshop. Readings were generated relatively to the signal obtained in H1299 cells and normalized against the input signal. Numbers represent average fold induction relatively to the H1299 control, normalized against the input from two PCR reactions. C: Chromatin immuno-precipitation assays were performed in naïve H1299 cells or cells expressing p53, p53-SUMO and SUMO^{K37-K48R} by using primer pairs that amplify the p53-regulated promoters as indicated at the left side of each part. All images shown were derived from the same gel, but in some cases lanes were cut in between samples.

Fig. 9.

Molecular modeling of acetylation on the structure of SUMO-1. The X-ray crystal structure of SUMO-1 (PDB:1TGZ) was used for these simulations. Left part (A), SUMO-1 is shown before acetylation, right part(B), after acetylation. The SUMO-1 structure with K37, K39, and K46 K48 is highlighted by ball and stick model, h-bond/salt-bridge shown by dotted line. The influence of acetylation of K37, K39, K46, and K48 of SUMO-1 was investigated. Molecular dynamics simulations demonstrated that inherent conformational changes are caused by lysine acetylation at all these residues. Acetylation of K48 alone tends to stabilize the structure, however, in the case of surface residues such as K37, K39, and K46, the loss of positive charge upon acetylation causes the breakage of the inherent salt bridge interactions with the neighboring negatively charged residues.

TABLE 1

Genes influenced by native p53 and p53-SUMO

Name	Symbol	p53 fold change	P-value	p53-SUMO fold change	P-value
Neurofilament, light polypeptide 68 kDa	NEFL	3.68	0.025	45.41	0.016
Cytoplasmic FMR1 interacting protein 2	CYFIP2	39.57	0.012	31.76	0.026
Growth differentiation factor 15*	GDF15	204.64	0.005	26.45	0.006
Ferredoxin reductase*	FDXR	22.41	0.011	19.79	0.002
Membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1	MTCBP-1	2.46	0.009	18.26	0.006
Tumor protein p53 inducible protein 3*	TP53I3	42.58	0.004	16.90	0.005
Insulin-like growth factor binding protein 3*	IGFBP3	-1.46	0.023	15.43	0.041
Stratifin (2)*	SFN (14-3-3σ)	4.16	0.041	14.49	0.013
Cyclin-dependent kinase inhibitor 1A (p21)*	CDKN1A (Cip1)	28.00	0.008	13.23	0.018
Polo-like kinase 3 (Drosophila)	PLK3	11.08	0.035	12.53	0.027
Mdm2, transformed 3T3 cell double minute 2 (2)*	MDM2	18.61	0.002	11.18	0.033
Carboxypeptidase M	CPM	24.10	0.011	10.04	0.008
Protein phosphatase 1 D magnesium-dependent, delta isoform*	PPM1D (Wip1)	4.23	0.004	7.03	0.014
DEAD (Asp-Glu-Ala-Asp) box polypeptide 43	DDX43	-1.01	0.029	7.00	0.023
Ras-related associated with diabetes	RRAD	15.15	0.019	6.84	0.005
BCL2-associated X protein*	BAX	5.99	0.001	5.54	0.039
Phorbol-12-myristate-13-acetate-induced protein 1 (2)*	PMAIP1 (Noxa)	1.59	0.078	5.22	0.036
Apoptotic peptidase activating factor*	APAF1	1.84	0.044	4.17	0.048
Vitamin D (1,25-dihydroxyvitamin D3) receptor (2)*	VDR	1.87	0.042	3.86	0.026
p53 target zinc finger protein*	WIG1	6.58	0.015	3.46	0.016
p53-inducible cell-survival factor*	P53CSV	3.57	0.015	3.13	0.036

Gene expression values were compared between parental H1299 cells and H1299 cells expressing p53 or p53-SUMO, by employing the criteria for selection outlined in the Materials and Methods Section. A selected list of genes activated or repressed twofold or greater relatively to parental H1299 cells, is shown (the complete list will be provided as Supplementary Information). The *P*-values were obtained by comparing the signal values of H1299 to those of either p53 or p53-SUMO using a paired, two-tailed, *t*-test. In bold: newly identified targets. Genes followed by a (#) indicate the number of gene spots responsive on the gene-chip with the corresponding fold an average of the values. Known p53 target genes are denoted by red asterisks; known p53 target genes strongly activated by native p53 or p53-SUMO are outlined in red and blue, respectively.