Role for 53BP1 tudor domain recognition of p53 dimethylated at lysine 382 in DNA damage signaling

Ioulia Kachirskaiia1,*, Xiaobing Shi1,*, Hiroshi Yamaguchi2, Kan Tanoue2, Hong Wen3, Evelyn W. Wang3, Ettore Appella2 and Or Gozani1

From the 1 Department of Biology, Stanford University, Stanford, CA 94305, 2 Laboratory of Cell Biology, National Cancer Institute, NIH, Bethesda, MD, 20892, and 3 Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305

Running title: Recognition of dimethylated p53 by the 53BP1 tudor domain

*These authors contributed equally to the work

Address correspondence to: Or Gozani, MD, PhD, Department of Biology, Stanford University, Stanford, CA 94305-5020. Phone: (650) 736-7639. Fax: (650) 725-8309. Email: ogozani@stanford.edu

Modification of histone proteins by lysine methylation is a principal chromatin regulatory mechanism (1). Recently, lysine methylation has been shown to also play a role in regulating non-histone proteins, including the tumor suppressor protein p53 (2). Here we identify a novel p53 species that is dimethylated at lysine 382 (p53K382me2) and show that the tandem tudor domain (TD) of the DNA damage response mediator 53BP1 acts as an "effector" for this mark. We demonstrate that the 53BP1(TD) recognizes p53K382me2 with selectivity relative to several other protein lysine methylation sites and saturation states. p53K382me2 levels increase with DNA damage, and recognition of this modification by 53BP1 facilitates an interaction between p53 and 53BP1. The generation of p53K382me2 promotes the accumulation of p53 protein that occurs upon DNA damage, and this increase in p53 levels requires 53BP1. Taken together, our study identifies a novel p53 modification, demonstrates a new effector function for the 53BP1(TD), and provides insight into how DNA damage signals are transduced to stabilize p53.

p53 plays a central role in directing cellular responses to DNA damage, including the most dangerous DNA lesion, double strand breaks (DSB) (3). A complex network of p53 post-translational modifications (PTMs) aids in the coordination of these activities (4). Three different lysine residues present within the C-terminal regulatory region of p53 are validated as sites of lysine methylation (5-8). Each of these methylation events either stimulates or represses p53 transcriptional activity. Yet, with multiple additional lysines in the C-terminus of p53 as potential methylation sites, and possible mono-, di-, and trimethylation states, the role of methylation in regulation of p53 and the molecular mechanisms linking different p53 methylation events to biological outcomes are just beginning to be understood.

53BP1 is a key mediator of the cell’s response to DSBs (9). Upon the induction of DSB lesions 53BP1 rapidly relocates to the sites of breaks, and is believed to promote the stabilization of additional DNA damage response factors at DSBs (9). The recognition of histone H4 dimethylated at lysine 20 (H4K20me2) by the 53BP1(TD) has been shown to be important for 53BP1 localization to DSBs – linking chromatin structure, lysine methylation and DSB signaling (10). 53BP1 might also have roles in transcription regulation. For example, a recent study reported that 53BP1 recognizes p53 dimethylated at lysine 370 through its tudor domain and modulates p53 transactivation at several target genes (7).

Here, we identify a number of novel lysine methylated p53 species, including proving the first direct evidence of endogenous p53 dimethylated at lysine 382. We show that p53K382me2 is a DNA damage-associated species, and that through its recognition by the 53BP1(TD), it is important for regulating a modular and DNA damage-dependent interaction between p53 and 53BP1. This interaction facilitates p53 stabilization in response...
to DSBs, suggesting that one mechanism by which DSB signals are transduced to activate p53 is via PTM of p53 by lysine methylation.

Materials and Methods

Constructs and reagents - Human 53BP1 cDNA and HA-53BP1 constructs were gifts from Jiri Lukas and Phillip Carpenter, respectively. 53BP1 tandem tudor domain and mutants were cloned and generated in pGEX vectors; SET8(Y334F) was generated in pcDNA and pGEX vectors using PCR mutagenesis (Stratagene). Primer sequences are available upon request. The p53K382me2 antibody was generated in rabbits immunized with the peptide: 377-TSRHKK(me2)LMFKT-387, and purified over a SulfoLink Coupling Gel (Pierce) coupled to the p53K382me2 peptide and depleted with recombinant wild-type GST-p53. Other antibodies used in this study: HRP-p53 (R&D systems); p53 (DO1; Calbiochem); SET8 (Abcam); 53BP1: mouse monoclonal (Upstate) and rabbit polyclonal (Bethyl Laboratories); Flag (M2) and tubulin (Sigma). p53 peptides bearing different modifications were synthesized at the W.M. Keck Facility at Yale.

Protein Purification and Mass Spectrometry – Nuclear extract (NE) were prepared from Hela cells ± doxorubicin treatment (0.5 ug/ml for 4 hours) as previously described (8). To IP endogenous p53 proteins, ~10 mg of NE was incubated with 50 µl of DO1-conjugate agarose in buffer containing 20 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.01% SDS, 1% Trition X 100, 1 mM EDTA and protease inhibitors with gentle rotation at 4°C for overnight. The beads were washed 2X with the same buffer, 2X with high salt buffer (20 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% SDS, 1% Trition X 100, 2 mM EDTA), once with LiCl buffer (20 mM Tris, pH 8.0, 500 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA), and once with TE buffer (10 mM TrisHCl 8.0, 1 mM EDTA). The p53 protein bound to the beads was subject to SDS-PAGE, excised from the gels, and incubated with trypsin overnight at 37°C. Pooled supernatants containing extracted peptides were dried and resuspended in 30% acetonitrile and 0.1% TFA prior to mass spectrometry analysis. Samples were analyzed on a reflectron time-of-flight mass spectrometer, MALDI-TOF instrument (Ultraflex, Bruker Daltonics, Billerica, MA), equipped with a 337 nm nitrogen laser and delayed ion extraction capability (delay times: 30-50 ns). Ion structure information was obtained by post source decay (PSD), using the mass gate feature, to select specific m/z window for fragmentation. The mass gate resolution was 1% of the precursor mass. Data was recorded in both positive and negative ion modes at 20kV acceleration, and mass analysis of ions determined using a dual micro-channel plate detector. Detector output was collected with a 1 GHz digitizer and displayed directly on a Windows NT based computer. Ten positive ion reflectron TOF mass spectra of 1000 laser shots were accumulated and externally calibrated with commercial peptide mix (Bruker Daltonics, Billerica, MA). For analysis of in vitro methylated synthetic peptides, the synthetic peptides untreated and treated with SET8 were equilibrated with 0.1% trifluoroacetic acid (TFA), and 50% acetonitrile with 0.1% TFA, and applied to the MALDI target plate with equal volumes of the matrix α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma).

Peptide pull-down assays - Peptide pull-down assays were performed as previously described (11). Briefly, 1 µg of biotinylated peptides were incubated with 1 µg of protein in binding buffer (50 mM Tris-HCl 7.5, 300 mM NaCl, 0.1% NP-40, 1 mM PMSF plus protease inhibitors) for 4 h at 4 °C with rotation. After 1 h incubation with Strepavidin beads (Amersham) and extensive washing, bound proteins were analyzed by SDS-PAGE and Western blotting.

Histone Methyltransferase Assay - Methyltransferase assays were performed as previously described (8). Briefly, 2 µg of GST-p53 or 1 µg of p53 peptides were incubated with 1µg of recombinant HMT and 2 µCi S-adenosylmethionine (Amersham) in reaction buffer containing 50mM Tris-HCl pH 8.0, 10% glycerol, 20mM KCl, 5 mM MgCl2, 1 mM DTT and 1 mM PMSF, at 30°C for 30 min to 2hrs. The reaction mixtures were then subject to electrophoresis on SDS-PAGE, followed by either radioactivity or Western analysis. The reactions with peptides were subject to mass spectrometry analysis.

Cell Culture and Transfections - U2OS, H1299, and 293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. Cells were transfected with plasmids or
siRNA duplexes by TransIT-LT1 (Mirus) or DharmaFECT (Dharmacon), respectively, according to the manufacturers’ protocols.

**Knockdown of 53BP1** - 100 nM of control or 53BP1 siRNAs were transfected into U2OS cells using DharmaFECT transfection reagent (Dharmacon) for 48 to 96 hrs before treatments. siRNA target sequences for 53BP1 are: 5’-GAGCUGGAAGUAAUAUUU-3’ or 5’-GGACUCAGUGUUUGCUAAUU-3’. On-target plus siControl siRNA (5’-UGGUUACUGUCGACUA-3’, Dharmacon) or off-target plus SMARTpool siControl siRNA (Dharmacon) were used as controls.

**Immunoprecipitation and Western Immunoblotting** - Endogenous p53 or ectopically expressed Flag-tagged p53 were IPed with agarose conjugated p53 or Flag antibodies from whole cell extracts in cell lysis buffer (50 mM TrisHCl pH 7.4, 250 mM NaCl, 0.5% Triton X100, 10% glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitors). After incubation at 4°C for overnight, the beads were washed 3X with the same buffer, and boiled in 2xLaemmlli buffer. The IPed p53 was resolved on SDS-PAGE gel and detected by αp53K382me2 and α53BP1 antibodies, or HRP-αp53 to avoid crossreactivity with IgG heavy chain.

**RT-PCR and real-time PCR** - RT-PCR and real-time PCR were performed as previously described (8). mRNA was prepared using RNeasy plus kit (Qiagen) and reverse-transcribed using First Strand Synthesis kit (Invitrogen). Quantitative Real-time RT-PCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Gene expression was calculated normalized to GAPDH levels by the comparative Cycle threshold method.

**Results**

*p53 is dimethylated at lysine 382 in vivo* – To search for novel p53 methylation marks, we performed a mass spectrometry analysis of endogenous p53 purified from HeLa nuclear extracts ± DNA damage (Fig. 1a; Supplementary Fig. 1; see Materials and Methods). In both samples we identified three peaks representing the digested peptide product containing lysine 382 (K(382)LMKF) that was either unmodified or was shifted by a mass that corresponds to one and two methyl moieties (Fig. 1a). We have previously reported the second peak as the monomethylated species, K(382me1)LMKF (8). The identity of the third peak was confirmed by MS/MS to represent dimethylated K382 species. These data demonstrate that endogenous p53 is dimethylated at lysine 382 (p53K382me2).

We also identified p53 peptides in the digests that contain lysine mono- and dimethylation events (Supplementary Fig. 1). Specifically, we have evidence for two novel methylated p53 species: K386me1 and K386me2, as well as potentially confirm by mass spectrometry the previously reported K370me2 species (6,7).

p53K382me2 is a high affinity ligand for 53BP1 tudor domain in vitro - The amino acid sequence surrounding p53K382 is highly homologous to that of histone H4K20 (Fig. 1b). 53BP1(TD) binds to H4K20me2 via the formation of contacts with the sequence HRKme2 (10). We therefore reasoned that 53BP1(TD) might likewise recognize p53K382me2 through the similar sequence HKKme2. Indeed, as shown in Figure 1c, in in vitro binding assays, recombinant 53BP1(TD) preferentially bound p53K382me2 peptides versus other p53K382 methylation states. Further, the binding affinity of 53BP1(TD) for p53K382me2 was moderately stronger than that observed for H4K20me2 and p53K370me2 (15.5 µM versus 27.2 µM and 27.0 µM, respectively), as well as multiple other histone lysine dimethylation sites and potential or reported p53 dimethylation sites (Figs. 1c-g). Many other methyl-lysine effector domains, including numerous PHD fingers and chromodomains do not bind to p53K382me2 (data not shown). Thus, in vitro, p53K382me2 is a novel and high affinity binding ligand for the tandem tudor domain of 53BP1.

Generation of p53K382me2 in vitro and in vivo - Next, a site-specific polyclonal antibody recognizing p53K382me2 was raised. This antibody is specific for dimethylated p53K382me2, and does not recognize unmethylated, monomethylated, or trimethylated p53K382 (Fig. 2a), several additional p53 dimethylation sites (Fig. 2b), and H4K20me2 (Supplementary Fig. 2). We have not yet identified the enzyme that generates p53K382me2, despite testing more than thirty lysine methyltransferase (KMT) enzymes (data not shown). Therefore, to investigate the biological
role of p53K382me2, we established a system to generate this species in vitro and in vivo. SET8/PR-Set7 adds a single methyl moiety to H4K20 (12-14) as well as p53K382 (8). Substitution of Y334 to F within SET8 (SET8(Y334F)) converts its product specificity with respect to H4K20 from a strict mono-methyltransferase to a mono- and dimethyltransferase (15). Accordingly, incubation of a p53 peptide encompassing K382 (aa 367-389) with recombinant SET8(Y334F) generated mono- and dimethylation at p53K382 (Fig. 2c; data not shown). We confirmed that SET8(Y334F) dimethylated p53K382 in the context of recombinant full-length p53 in vitro (Fig. 2d), and validated its activity in cells on endogenous p53 (Fig. 2e). Thus, the SET8(Y334F) enzyme provides a tool for modulation of p53K382 dimethylation levels in vitro and in cells.

Methylation of p53K382 modulates interaction between p53 and 53BP1 - 53BP1 was originally identified in a two-hybrid screen as a p53-interacting protein (16). Subsequently, the 53BP1 BRCT tandem repeats were shown to bind the DNA-binding domain of p53, though the physiologic context in which this interaction functions remains elusive (9). To determine whether 53BP1(TD) recognition of p53K382me2 can augment the baseline p53-53BP1 interaction, we performed co-immunoprecipitation (co-IP) experiments. As shown in Figure 3a, the ability of Flag-p53 to co-IP HA-53BP1 was enhanced when levels of p53 dimethylation at p53K382 were increased via co-expression with SET8(Y334F). Substitution of p53K382 to R abolished this SET8(Y334F)-mediated increase, whereas the corresponding substitution at p53K372 did not (Fig. 3b). Thus, the enhanced interaction of p53 with 53BP1 following p53 dimethylation by SET8(Y334F) specifically requires K382. We note that the affinity of 53BP1(TD) for p53K382me1 is 10-fold lower than for p53K382me2 (Fig. 1g), and therefore it is unlikely that the generation of this species by SET8(Y334F) has a major impact on the p53-53BP1 interaction.

Key residues within 53BP1 tudor domain are required for binding methylated p53 - Due to homology in the amino acid sequence surrounding p53K382 and H4K20, we turned to the crystal structure of the 53BP1(TD)-H4K20me2 peptide complex to obtain insight into the molecular basis of p53K382me2-recognition by the 53BP1(TD) (see Figure 1b) (10). Mutation of residues within the first tudor domain of the 53BP1(TD) that constitute the binding cage accommodating the dimethyllysine of H4K20 (W1495A, Y1502L, D1521A), as well as the residues that contact a histidine residue two amino acids N-terminal of the methylated lysine (L1547A, M1584A), abolished or severely compromised the interaction with p53K382me2 (Fig. 3c) (10).

An intact 53BP1(TD) is critical for optimal binding to K382-methylated p53 in the context of full-length 53BP1, as introduction of a mutation within the methyllysine binding pocket of 53BP1 (W1495A) impaired its ability to be co-IPed with p53 (Fig. 3d). Previous structural analysis of the 53BP1(TD) verified that these mutations do not disrupt folding (10,17). Taken together, these results indicate that recognition of p53K382me2 by the tandem tudor domain of 53BP1 can modulate the p53-53BP1 interaction in cells.

Interaction between endogenous p53 and 53BP1 increases with DNA damage – Both p53 and 53BP1 are key mediators of cellular responses to DSBs (9), raising the possibility that p53K382me2 generation, as well as the p53K382me2-53BP1(TD) interaction might be linked to genotoxic stress. Consistent with this notion, in U2OS cells the endogenous levels of p53K382me2 increased in response to the DSB-inducing drug necrozinostatin (NCS) relative to control treatment (Fig. 3e). To determine if the endogenous interaction of p53 and 53BP1 paralleled the DNA damage-dependent increase in p53K382me2, p53 IPs were performed from U2OS cells with and without NCS treatment. In the absence of DNA damage, little endogenous 53BP1 was present in the p53 IP, but after DNA damage 53BP1 was readily detected co-IPed with p53 (Fig. 3f, compare lanes 1 and 3). Moreover, this DNA damage-dependent interaction was augmented by expression of SET8(Y334F) (Fig. 3f, compare lanes 3 and 4). Thus, endogenous p53K382me2 levels increase with DNA damage and possibly augment the physiological interaction between p53 and 53BP1.

Role for p53K382me2-53BP1 interaction in mediating p53 stability upon DNA damage - To dissect potential roles for p53K382me2 and p53K382me2-53BP1 interactions in DNA damage responses, we first asked whether 53BP1 acts as a
transcriptional co-activator of p53. Treatment of U2OS cells with short interfering RNA (siRNA) targeting 53BP1 (Fig. 4a) did not decrease DNA damage-dependent induction of p21 or a number of additional p53 target genes (Fig. 4b; Supplementary Fig. 3; data not shown). These results suggest that the 53BP1-p53K382me2 interaction likely modulates a p53 function distinct from its transactivation activity.

Regulation of p53 protein levels is critical for proper p53-mediated responses. The stability of p53 was previously shown to be compromised in 53BP1 knock-down cells (18), and 53BP1 was identified in an RNAi-based screen as a gene necessary for the toxicity associated with the MDM2-inhibitor Nutlin-3 (19). We therefore postulated that recognition of p53K382me2 by the tudor domain of 53BP1 might positively regulate the accumulation of p53 protein that accompanies DSB lesion formation. To investigate this possibility, total p53 protein levels present in U2OS cells were determined at baseline and in response to NCS treatment, under conditions in which p53K382me2 levels were normal or elevated, respectively. We observed that expression of SET8(Y334F) increased p53 protein levels relative to control at both baseline and genotoxic stress conditions (Fig. 4c). This increase was not due to enhanced p53 mRNA synthesis; rather, SET8(Y334F) elicited a small decrease in p53 mRNA levels relative to control (Fig. 4d). Next, we tested the role of 53BP1 in SET8(Y334F)-mediated augmentation of p53 protein levels. Consistent with previous studies, depletion of 53BP1 by siRNA led to a decrease in p53 protein levels (Fig. 4e, compare lanes 2 and 6) (19). Furthermore, while SET8(Y334F) expression increased p53 protein levels in control siRNA treated cells, it failed to do so in cells lacking 53BP1 (Fig. 4e, compare lanes 4 and 8). Notably, no decrease in p53 mRNA levels was observed in 53BP1 knock-down cells (Supplementary Fig. 4).

In summary, we have identified a novel DNA damage-associated p53 species that is dimethylated at K382. This modification is recognized by the tandem tudor domain of 53BP1, and the interaction may promote stabilization of p53. Homooligomerization of 53BP1 is important for its DNA repair activity – we propose that this allows 53BP1 to serve as an adaptor at DSB sites, with one 53BP1 molecule bound to H4K20me2 and a paired second 53BP1 molecule free to dock dimethylated p53 or potentially other methylated nuclear proteins (Supplementary Figure 5) (9). The DNA damage-triggered rapid accumulation of 53BP1 at DSB sites, in conjunction with the assembly of additional p53-regulatory factors such as ATM and Chk2, may cooperate to create a high-affinity site for p53. We hypothesize that this can facilitate accumulation of p53 by sequestering it away from proteins that target it for degradation as well as promoting p53-activating PTMs. In this context, there is evidence that p53 localizes to sites of double-strand breaks (20). We have also observed increased occupancy of p53 at a defined DSB (data not shown). The identification of the KMT that generates p53K382me2 will greatly facilitate the uncovering of the molecular function(s) associated with this DNA damage linked mark.

In our purification of endogenous p53, we identified potentially three p53 methylated species in addition to p53K382me2: p53K370me2, p53K386me1, and p53K386me2 (Supplementary Figure 1). The function of K386 mono- and dimethylation is unknown, and we have not confirmed their existence by independent methods or identified a KMT enzyme that acts on this residue (data not shown). The existence of p53K370me2 has been demonstrated by the use of a state-specific antibodies (7). In this study, removal of the p53K370me2 mark by the lysine demethylase LSD1 was shown to repress p53 transactivation by preventing the interaction between between p53 and 53BP1 – which was shown to be critically dependent on 53BP1(TD) recognition of p53K370me2. Here, we also found that the 53BP1(TD) binds to p53K370me2, albeit with slightly lower affinity than p53K382me2 (see Fig. 1). Indeed, we observe binding of the 53BP1(TD) to several dimethylated p53 peptides, suggesting that many different lysine dimethylation events on p53 can potentially
promote a modular protein-protein interaction between p53 and 53BP1. In the case of p53K370me2, recognition of 53BP1 was shown to be required for transcriptional activation by p53 of its target genes (7). In contrast, binding to p53K382me2 by 53BP1 does not appear to play a role in p53 transactivation (Fig. 4). How the recognition of different methylation events on p53 by the same effector protein (53BP1) can result in alternative physiologic outcomes remains an open question. Based on the many different potential methylation sites on p53 and the observation that at least two different 53BP1 recognition events can lead to different physiologic outcomes, we propose that lysine methylation-mediated funneling of p53 to particular functions likely depends on additional modifications and binding partners that co-occur with one modification (e.g. K382me2) versus another (e.g. K370me2).

Taken together, our study sheds light on potential molecular mechanisms by which DSB signals are transduced to activate p53, begins to dissect how lysine dimethylation contributes to a p53 post-translational code that modulates distinct p53 functions, and highlights the notion that lysine methylation on non-histone proteins is likely a general molecular paradigm utilized to regulate diverse nuclear processes.

References:


**Footnotes**

We thank P. Carpenter for HA-53BP1 and J. Lukas for 53BP1 cDNA. We also thank C. Anderson for helpful comments and K. Walters for technical assistance. This work was supported in part by grants from the NIH (O.G.) and the Intramural Research Program of the NIH (E.A., H.Y., S.R.M.). O.G. is a recipient of a Burroughs Wellcome Career Award in Biomedical Sciences and a Searle Scholar Award.

Abbreviations used are: 53BP1, p53 Binding Protein 1; 53BP1(TD), 53BP1 Tandem Tudor Domain; DSB, double strand break; KMT, lysine methyltransferase; PTM, post-translational modification; NCS, neocarzinostatin; WCE, whole cell extract; IP, immunoprecipitate.
Figure Legends

Figure 1. 53BP1\((TD)\) binds specifically to p53K382me2 in vitro. a, Identification of endogenous p53 dimethylated at lysine 382 by mass spectrometry analysis. Unmethylated, monomethylated and dimethylated K382 within the KLMFK peptide obtained from trypsinized purified endogenous p53 from HeLa nuclear extract (NE) (left panel: no DNA damage; right panel: 4 hr doxorubicin (dox)). b, Alignment of amino acid sequences surrounding p53K382 (aa 367-389) and H4K20 (aa 17-22). Blue residues: binding site for 53BP1\((TD)\) (10); Red residues: other lysines in p53 C-terminus. Asterisks indicates methylation sites. c-e, 53BP1\((TD)\) preferentially binds p53K382me2 peptides. Western analysis of pull-downs with the indicated biotinylated peptides and GST-53BP1\((TD)\) or GST control. aa, amino acids. f-g, The binding affinities (IC\(_{50}\)) of p53 and H4 peptides for 53BP1\((TD)\) (shown in (g)), were determined by competition assays (with the indicated non-labeled peptides) using fluorescence anisotropy change of fluorescein-labeled H4K20me2 of known affinity.

Figure 2. In vitro and in vivo generation of p53K382me2. a-b, Specific recognition of p53K382me2 by an αp53K382me2 antibody. Dot blot analyses of the indicated biotinylated peptides (a, p53K382me0-3; b, p53 aa 367-389 w/indicated dimethylated lysines) with p53K382me2 antibody. Blots were probed with HRP-conjugated streptavidin to control for peptide loading. c, p53 is mono- and di- methylated by SET8\(_{\text{Y334F}}\) at K382. Mass spectrometry analysis of p53 peptide (aa 367-389) before (left panel) and after (right panel) SET8\(_{\text{Y334F}}\) methyltransferase reaction. Masses of peptides are indicated. d, SET8\(_{\text{Y334F}}\) but not wild-type SET8 generates p53K382me2. Western blot analysis with αp53K382me2 of methyltransferase assays on recombinant p53 protein. Total p53 levels detected with DO-1 antibody demonstrate equal loading. e, SET8\(_{\text{Y334F}}\) dimethylates endogenous p53 at K382 in vivo. Western blot analysis with the indicated antibodies (anti-p53-HRP for p53 levels) of p53 (DO-1) IPs from 293T cells expressing SET8\(_{\text{Y334F}}\).

Figure 3. p53K382me2 levels increase with DNA damage to facilitate the interaction between p53 and 53BP1 in vivo. a, Ectopic SET8\(_{\text{Y334F}}\) augments 53BP1-p53 interaction. Western blot analysis with the indicated antibodies of WCE and Flag-IPs from 293T cells expressing the indicated proteins. Anti-tubulin of WCE is shown to control for loading. b, p53K382 mutation abolishes the methylation-dependent increase of the p53-53BP1 interaction. Western blot analysis as in (a) in cells expressing Flag-tagged wt or mutant p53 as indicated. c, Conservation of the molecular basis of 53BP1\((TD)\) recognition of H4K20me2 and p53K382me2. The indicated GST-53BP1 tandem tudor domain mutants were tested for binding to p53K382me2 as in Figure 1c. d, An intact tandem tudor domain is required for robust 53BP1 recognition of p53 in cells. Western blot analysis as in (a) in cells expressing the indicated proteins. e, Elevation of endogenous p53K382me2 levels upon DNA damage. Endogenous p53 levels present in αp53K382me2 IPs from U2OS cells ± 45 nM NCS. Total p53 present in the WCE (input) is shown. f, Endogenous association between p53 and 53BP1 is augmented by DNA damage and dimethylation at p53K382. Western analysis of p53-bound proteins in co-IPs from U2OS cells ± 45 nM NCS and ± SET8\(_{\text{Y334F}}\) expression.

Figure 4. 53BP1 recognition of p53K382me2 couples DNA damage to p53 accumulation. a, 53BP1 siRNA treatment knocks-down endogenous 53BP1 protein levels. Western analysis of 53BP1 expression in U2OS treated with control or 53BP1 siRNAs. b, 53BP1 knock-down does not impact p53 induction of p21. Real-time PCR analysis of relative mRNA levels of p21 in U2OS cells treated with control or 53BP1 siRNAs as in (a), ± 45 nM NCS. c, SET8\(_{\text{Y334F}}\) augments p53 levels at baseline and in response to DNA damage. Western analysis of total p53 ± SET8\(_{\text{Y334F}}\) expression and ± 45 nM NCS in U2OS cells. d, SET8\(_{\text{Y334F}}\) expression does not increase p53 mRNA levels. Real-time PCR analysis of relative p53 mRNA levels in U2OS cells treated as in (c). e, 53BP1 protein is required for SET8\(_{\text{Y334F}}\)-dependent p53 accumulation. Western analysis of U2OS cells treated with control or 53BP1 siRNA, ± SET8\(_{\text{Y334F}}\) and ± 45 nM NCS. Knock-down was confirmed with anti-53BP1 immunoblot.
Figure 1

(a) and (b) show the mass spectrometry (MS) spectra of proteins under different conditions. + dox indicates the presence of doxycycline, while - dox indicates its absence. The MS peaks at m/z 667, 681, and 695 correspond to different modifications of the K(382) residue.

(b) The amino acid sequence of p53 (367-389) and H4 (17-22) is shown with highlighted residues.

(c) and (d) show Western blot analyses of 53BP1 (TD) with various conditions.

(e) Additional Western blot analyses with different conditions are shown.

(f) A graph shows the relative binding of various peptides as a function of peptide concentration. The graph includes data points for different peptides and IC₅₀ values.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 (aa 367-389)</td>
<td>&gt; 600</td>
</tr>
<tr>
<td>p53K382me1</td>
<td>169.3 ± 15.9</td>
</tr>
<tr>
<td>p53K382me2</td>
<td>15.5 ± 1.7</td>
</tr>
<tr>
<td>p53K382me3</td>
<td>234.2 ± 17.8</td>
</tr>
<tr>
<td>p53K372me2</td>
<td>27.0 ± 1.8</td>
</tr>
<tr>
<td>p53K372me2</td>
<td>31.6 ± 6.9</td>
</tr>
<tr>
<td>H4K20me2</td>
<td>27.2 ± 3.6</td>
</tr>
</tbody>
</table>
Figure 4

(a) Western blot analysis showing the levels of 53BP1 and tubulin in control siRNA and 53BP1 siRNA conditions.

(b) Graph showing the p21 mRNA levels normalized to control. The bars represent vehicle (black) and NCS (white) conditions.

(c) Western blot analysis showing the levels of p53 and tubulin under different conditions: SET8(Y334F), NCS, and control.

(d) Graph showing the p53 mRNA levels normalized to control. The bars represent vehicle (black) and NCS (white) conditions.

(e) Western blot analysis showing the levels of p53, actin, and 53BP1 under different conditions: SET8(Y334F), NCS, and control.
Role for 53BP1 tudor domain recognition of p53 dimethylated at lysine 382 in DNA damage signaling
Ioulia Kachirksaia, Xiaobing Shi, Hiroshi Yamaguchi, Kan Tanoue, Hong Wen, Evelyn W. Wang, Ettore Appella and Or Gozani

J. Biol. Chem. published online October 7, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M806020200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2008/10/07/M806020200.DC1