USP22 Antagonizes p53 Transcriptional Activation by Deubiquitinating Sirt1 to Suppress Cell Apoptosis and Is Required for Mouse Embryonic Development

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SUMMARY

The NAD-dependent histone deacetylase Sirt1 antagonizes p53 transcriptional activity to regulate cell-cycle progression and apoptosis. We have identified a ubiquitin-specific peptidase, USP22, one of the 11 death-from-cancer signature genes that are critical in controlling cell growth and death, as a positive regulator of Sirt1. USP22 interacts with and stabilizes Sirt1 by removing polyubiquitin chains conjugated onto Sirt1. The USP22-mediated stabilization of Sirt1 leads to decreasing levels of p53 acetylation and suppression of p53-mediated functions. In contrast, depletion of endogenous USP22 by RNA interference destabilizes Sirt1, inhibits Sirt1-mediated deacetylation of p53 and elevates p53-dependent apoptosis. Genetic deletion of the usp22 gene results in Sirt1 instability, elevated p53 transcriptional activity and early embryonic lethality in mice. Our study elucidates a molecular mechanism in suppression of cell apoptosis by stabilizing Sirt1 in response to DNA damage and reveals a critical physiological function of USP22 in mouse embryonic development.

INTRODUCTION

The class III histone deacetylase (HDAC) Sirt1 is the human ortholog of the yeast sir2 (silence information regulator 2) protein (Guarente, 2000; Smith et al., 2000; Tanny et al., 1999). Sirt1 contains one HDAC domain with deacetylase activity, two nuclear localization sequences, and a coiled-coil-like domain (Afshar and Murnane, 1999). Sirt1 deacetylates a variety of proteins including histones H1, H3, and H4, and may mediate heterochromatin formation (Luo et al., 2002; Pruitt et al., 2006; Vaquero et al., 2004). Besides histones, several other proteins can serve as substrates for Sirt1, including p53, FOXO, Ku70, p300, Rb, E2F1, NF-κB, AP-1, p73, and PGC-1α (Bordone and Guarente, 2005; Dai et al., 2007; Motta et al., 2004; Zhang et al., 2009). In virtue of these important targets, Sirt1 is linked to the regulation of diverse cellular processes ranging from stress responses, aging, metabolism, and immunity to cancer (Haigis and Sinclair, 2010). The activity and expression of Sirt1 is tightly regulated at many levels from broader general mechanisms, such as substrate availability and tissue and subcellular localization, to gene-specific regulatory mechanisms, such as activation of transcription factors and regulation by miRNA. Multiple transcription factors are known to regulate Sirt1 mRNA levels, including p53, FOXO3a, HIC1:CtBP complex, E2F1, and c-Myc, enabling the regulation of Sirt1 expression (Gao et al., 2012; Nemoto et al., 2004; Wang et al., 2006a; Yuan et al., 2009; Zhang et al., 2007). Interestingly, most of these transcription factors, such as p53, Foxo3, and c-Myc, are also regulated by Sirt1 via protein deacetylation, providing a feedback mechanism in the regulation of Sirt1 expression (Brunet et al., 2004; Langley et al., 2002a; Yuan et al., 2009). In addition, Sirt1 expression can also be regulated posttranscriptionally by miRNA regulation. MiR-34a and miR-199 have been recently reported to suppress the level of Sirt1 in prostate cancer cell line PC3 and cardiomyocytes (Rane et al., 2009; Yamakuchi et al., 2008).

The ubiquitin-specific peptidase 22 (USP22) is a member of the deubiquitinase family, a group of proteins comprising over 70 members in mammals. The physiological functions of USP22 are largely unknown (Lee et al., 2006). It has been identified as a death-from-cancer signature gene, i.e., a marker for predicting the likelihood of treatment failure in cancer patients (Glinsky, 2006; Sowa et al., 2009). Recent studies demonstrated that USP22 is a bona fide component of the SAGA complex (Pijnappel and Timmers, 2008; Zalmas et al., 2008; Zhang et al., 2008). USP22 reverses the polycomb-catalyzed ubiquitination of histones, including H2A and H2B, as well as nonhistone protein TRF1 (TATA box-binding
protein-related factor 1) to regulate the transcription of genes involved in cell-cycle entry and apoptosis (Atanassov and Dent, 2011; Zalmas et al., 2008).

In this study, we have identified USP22 as a specific deubiquitinase of Sirt1. USP22-mediated deubiquitination and stabilization of Sirt1 prohibits p53 transcriptional and proapoptotic functions. By generating usp22-targeted gene deletion in mice, we demonstrated that USP22 is required for early embryogenesis in mice because usp22 null mice die in utero at E10.5 of the postimplantation stage. Therefore, our studies reveal USP22 regulates cell apoptosis by stabilizing Sirt1 and suppressing p53 functions under DNA damage and during embryonic development in mice.

RESULTS

Identifying USP22 as a Sirt1-Interacting Protein

Using a proteomic approach and analyzing proteins coimmunoprecipitated specifically with FLAG-tagged Sirt1 from HEK293 cells, we identified 16 Sirt1-interacting proteins (Figures S1A and S1B), which are involved in a variety of cellular functions including transcriptional regulation, protein degradation, signaling transduction, and RNA splicing (Figure 1A). To confirm that the interactions between Sirt1 and its putative partners were specific, we attempted to verify the associations by coimmunoprecipitation followed by western blotting analysis. We selectively tested the interactions of Sirt1 with the rest of the seven candidates were confirmed in transiently transfected HEK293 cells (Figures 1B and S1C–S1I), suggesting that these proteins are true Sirt1 interactors. Together with the fact that USP22, HSP70, and STAT3 are previously reported Sirt1 interactors (Bernier et al., 2011; Erion et al., 2009; Nie et al., 2009; Sestito et al., 2011; Sowa et al., 2009; Westerheide et al., 2009), these results indicate that our proteomic approach yielded a highly specific and reliable identification of Sirt1-binding proteins from HEK293 cells.

USP22 is of interest since it has been reported as one of the cancer stem cell genes that are associated with epigenetic regulation of gene transcription, but the molecular mechanisms underlying USP22 in cancer promotion are not known. Since Sirt1 is a suppressor for p53 functions, it is possible that USP22 promotes cell proliferation and suppresses apoptosis.
through regulation of Sirt1 to antagonize p53 function. To test this hypothesis, we first determined whether endogenous Sirt1 interacts with USP22 in the colon cancer HCT116 cell line. Indeed, Sirt1 was detected in the anti-USP22 but not normal rabbit IgG immunoprecipitates from the cell lysate (Figure 1C). Therefore, USP22 protein interacts with Sirt1.

The deubiquitinase USP22 comprises of an N-terminal zinc finger domain followed by a C19 ubiquitin-specific peptidase domain. To map the region within USP22 that mediates its interaction with Sirt1, truncated mutants of USP22 were generated (Figure 1D). The zinc finger-containing N terminus of USP22 binds Sirt1. In contrast, the C terminus ubiquitin-specific peptidase domain did not interact with Sirt1, suggesting that the N terminus of USP22 mediates their interaction (Figure 1D). Furthermore, we found that Sirt1 N terminus interacted with USP22, and this interaction was enhanced by including the HDAC domain of Sirt1. However, the HDAC domain together with the C terminus of Sirt1 did not interact with USP22 (Figure 1E), suggesting that the region between Sirt1 N terminus and its HDAC domain is important for Sirt1 interaction with USP22.

**USP22 Suppresses Sirt1 Ubiquitination**

USP22 contains a carboxyl-terminal ubiquitin hydrolase domain that defines the C-19 class of peptidases. Therefore, USP22 may regulate Sirt1 function via its deubiquitinase activity. To test this hypothesis, we first investigated whether Sirt1 is ubiquitinated in cells using methods as described (Li et al., 2007). In fact, ladders with higher molecular weight were detected in anti-HA immunoprecipitates using anti-FLAG antibodies (Figure 2A), indicating that Sirt1 is polyubiquitinated. Coexpression of USP22 significantly inhibited ubiquitin-conjugation to Sirt1 (Figure 2A). Conversely, knockdown of endogenous USP22 by small interfering RNA dramatically enhanced polyubiquitination.
of endogenous Sirt1 in HCT116 cells (Figures 2B and S2A), and led to a decrease in Sirt1 protein expression levels (Figure 2B). The ubiquitinated protein is not likely from Sirt1-binding partners because it was not affected by denaturing Sirt1-ubiquitin conjugates in the presence of 1% SDS ubiquitination lysis buffer (Figure S2D). The in vitro deubiquitination assay using a GST-USP22 fusion protein shows that incubation of ubiquitinated Sirt1 with GST-USP22 but not GST or GST-USP22C185A mutant control inhibited Sirt1 ubiquitination in vitro (Figures S2B and S2C). Furthermore, other unrelated deubiquitinase USP14 (Figure S2C) or USP15 (Figure 2C) failed to suppress Sirt1 ubiquitination. Together with our findings that Sirt1 interacts with USP22, these results indicate that USP22 functions as a bona fide deubiquitinase specifically for Sirt1 and appears to regulate Sirt1 protein stability.

The ubiquitin-specific peptidase activity of USP22 is required to deubiquitinate Sirt1, as the catalytically inactive USP22 (USP22/C185A) mutant failed to inhibit Sirt1 ubiquitination (Figures 2D and 2E) without affecting USP22 interaction with Sirt1 (Figure 2F). In addition, mutation of the cysteine residues in the N-terminal zinc finger of USP22, which presumably has no effect on USP22 deubiquitinase catalytic activity, completely abolished its interaction with Sirt1 (Figure 2F) and its deubiquitinase activity on Sirt1 ubiquitination (Figure 2E). Therefore, USP22 interacts with Sirt1 via its N terminus to suppress Sirt1 ubiquitination.

To study the functional roles of Sirt1 ubiquitination, we mapped the ubiquitination sites in Sirt1 protein using a proteomic approach. Sirt1 proteins in the lysates of cells transfected with FLAG-Sirt1 were purified and subjected to SDS-PAGE analysis. Coomassie brilliant blue (CBB) staining detected a strong Sirt1 protein band and faint smears above its size, suggesting the pull-down of ubiquitinated Sirt1 protein (Figure 2G). When a small fraction of eluted proteins were analyzed by western blotting, Sirt1 ubiquitination and its protein expression were confirmed (Figure 2G). Proteomic analysis identified five Sirt1 peptides that carry ubiquitin-modified lysine residues including 254, 335, 377, 499, and 523, three (254, 335, and 377) out of them locate within the HDAC domain of Sirt1 (Figures 2H and S2E). To further determine whether these five lysine residues are the predominant sites for Sirt1 ubiquitination, we generated a Sirt1 mutant by replacing all these five lysine residues with arginines (Sirt1/5KR). A dramatic reduction of ubiquitination levels to Sirt1/5KR was confirmed (Figure 2I). The low level of Sirt1/5KR ubiquitination suggested either experimental background or few remaining lysines may still exist for Sirt1 ubiquitination.

**USP22 Stabilizes Sirt1**

Since we demonstrated that USP22 deubiquitinates Sirt1, it is possible that USP22 can stabilize Sirt1. In fact, expression of USP22 significantly prolonged the half-life of Sirt1 (Figures 3A and 3B). Mutation of the zinc finger of USP22, which disrupts...
its interaction with Sirt1, abolished its ability to stabilize Sirt1. Likewise, the ubiquitin peptidase inactive form of USP22 (USP22/C185A), while it still interacts with Sirt1 (Figure 2F), failed to protect Sirt1 from degradation (Figure 3C). Conversely, knockdown of endogenous USP22 expression facilitated Sirt1 protein degradation in HCT116 cells (Figures 3D and 3E), suggesting that USP22 stabilizes Sirt1. The proteasome-specific inhibitor MG132 rescued Sirt1 protein from degradation in USP22 knockdown cells (Figure 3F), indicating that polyubiquitination induces Sirt1 degradation through a proteasomal pathway. As lysine residues 254, 335, 377, 499, and 523 were identified as the predominant ubiquitination sites in Sirt1 (Figure 2H), we then asked whether mutation of these lysine residues, which inhibited Sirt1 ubiquitination, results in Sirt1 stabilization even without USP22 overexpression. As expected, Sirt1/5KR has a significantly more prolonged half-life than its wild-type control (Figure 3G). Addition of USP22, which protects Sirt1 protein from degradation (Figure 3A), did not further alter Sirt1/5KR protein stability (Figure 3G). Collectively, our data indicate that USP22 deubiquitinates and stabilizes Sirt1 protein.

**USP22 Inhibits p53 Acetylation and Transcriptional Activity to Suppress Cell Apoptosis**

Sirt1 has been reported to deacetylate p53 and suppress p53 transcriptional activation (Vaziri et al., 2001; Yuan et al., 2011). Because USP22 inhibits the ubiquitination-mediated Sirt1 degradation, we hypothesized that USP22 inhibits p53 functions through Sirt1. In fact, USP22 dose-dependently inhibited the levels of acetylation but not protein expression of p53 (Figures 4A and 4B). In contrast to wild-type USP22, expression of the USP22/C61,63A mutant that does not interact with Sirt1 had little effect on p53 acetylation. Furthermore, the catalytically inactive form of USP22 abolished its suppressive activity on p53 acetylation (Figures 4C and 4D). Therefore, USP22 is a suppressor of p53 acetylation, a post-translational modification that is required for p53 functions. As a consequence, USP22 inhibited p53-driven puma-luciferase activity in HCT116 cells that carry a wild-type p53 gene. As a control, only basal levels of puma-luciferase activity were detected in p53 null cells, and USP22 expression had no effect on this basal luciferase activity (Figure 4E). We then tested whether USP22 expression inhibits cell apoptosis upon DNA damage. HCT116 cells were transfected with either control or USP22 expression plasmid and then exposed to etoposide. Flow cytometry analysis indicated that USP22 expression suppressed etoposide-induced apoptosis (Figure 4F). Further, knockdown of Sirt1 abolished the antiapoptotic functions of USP22, indicating that USP22 suppresses etoposide-induced apoptosis through Sirt1 (Figure S3C). Therefore, USP22 suppresses p53 transcriptional activity and proapoptotic functions.
USP22 Antagonizes p53 Functions by Stabilizing Sirt1

Our finding that USP22 inhibits the p53 transcriptional activity and stabilizes Sirt1 suggests that USP22 inhibits p53 acetylation and antiapoptotic functions in a Sirt1-dependent manner. To test this possibility, we analyzed the effects of USP22 and Sirt1 coexpression on p53 acetylation. As shown in Figure 5A, USP22 and Sirt1 synergistically inhibited p53 acetylation. The deacetylase activity of Sirt1 is required for its synergy with USP22 in suppressing p53 acetylation, as expression of the HDAC inactive mutant of Sirt1, Sirt1/HY, failed to inhibit p53 acetylation (Figure 5B). Notably, expression of USP22 failed to suppress p53 acetylation in sirt1 null MEF cells (Figure S3A) or in sirt1 null MEF cells (Figure 5C). Reintroduction of Sirt1 null MEF cells with Sirt1 alone partially inhibited p53 acetylation and its target genes expression. HCT116 cells stably expressing control or USP22-specific shRNA were treated with etoposide (20 nM) for 24 hr followed by 2 hr MG132 treatment (20 μM). The expression levels of p53 (middle panel) and USP22 (bottom panel) were confirmed by western blotting.

Figure 5. USP22 Inhibits p53 Functions through Sirt1

(A) USP22, Sirt1, p300, and p53 expression plasmids were transfected into HCT116 cells. P53 acetylation was analyzed by immunoprecipitating with anti-FLAG antibody and western blotting with anti-acetyl-lysine antibody (top panel).

(B) USP22, p300, Sirt1, or its deacetylase inactive mutant plasmid were transfected into HCT116 cells. Endogenous p53 acetylation was analyzed.

(C) Indicated plasmids were transfected into Sirt1 null MEF cells, and p53 acetylation was analyzed. The expression levels of p53, USP22, and Sirt1 were confirmed in (A)–(C).

(D) Puma luciferase plasmids were cotransfected with or without USP22 plasmid into wild-type or Sirt1 null MEF cells. The luciferase activities were analyzed.

(E) Myc-USP22 and Flag-p53 expression plasmids were transfected into HEK293 cells. The interaction between USP22 and p53 was analyzed as in Figure 1B.

(F) FLAG-p53 plasmids were transfected alone or together with Myc-tagged USP22 into wild-type or Sirt1 null MEF cells, and the interaction of USP22 and p53 was analyzed (top panel). The expression levels of p53 (middle panel) and USP22 (bottom panel) were confirmed by western blotting.

(G) USP22 knockdown promotes p53 acetylation and target genes expression. HCT116 cells stably expressing control or USP22-specific shRNA were treated with etoposide (20 nM) for 24 hr followed by 2 hr MG132 treatment (20 μM). The expression levels of USP22, Sirt1, acetylated p53, p53, and its target genes were determined. Tubulin was used as loading control.

(H) Total RNA was isolated from HCT116 cells stably expressing control or USP22-specific shRNA. The mRNA levels of usp22, sirt1, and p53 (top panel) or p53 target genes (bottom panel) were determined by real-time PCR.

(I) HCT116 cells were transfected with siRNA against Sirt1 or USP22 alone or together. The transfected cells were treated with etoposide as in Figure 4F, and the apoptotic cells were analyzed. Error bars represent data from three independent experiments (mean ± SD).
detected in wild-type but not in sirt1 null MEF cells (Figure 5F). Therefore, the interaction of USP22 with p53 is probably mediated by Sirt1. Collectively, our data indicates that USP22 inhibits p53 functions through Sirt1 deacetylase.

To further prove that USP22 acts on p53 by stabilizing Sirt1, we tested whether inactivation of USP22 increases endogenous p53 acetylation. Figures 5G and 5H show that knockdown of USP22 expression resulted in reduced Sirt1 protein but not its mRNA expression levels, confirming that USP22 regulates Sirt1 expression at the posttranslational level. To support this, treatment of cells with a proteasome inhibitor MG132 largely protected Sirt1 from degradation (Figure 5G). As a consequence, there was a significant increase of endogenous p53 acetylation and p53 transcriptional activity because both the protein and the mRNA expression levels of p21 and BAX, two transcriptional products of p53, were increased in USP22 knockdown cells (Figures 5G and 5H). Moreover, knockdown of either USP22 or Sirt1 promoted etoposide-induced cell apoptosis, which is further enhanced by a combined Sirt1/USP22 knockdown (Figure 5I). Therefore, our studies collectively demonstrated that USP22-mediated deubiquitination of Sirt1 inhibits p53 acetylation and transcriptional activity and p53-mediated apoptosis in response to DNA damage.

**USP22 Is Required during Early Embryogenesis**

Our studies demonstrated that USP22 suppresses transcriptional activity and apoptotic functions of p53 by stabilizing Sirt1 when cells face DNA damage. To determine the in vivo physiological functions of USP22, we generated usp22 knockout mice utilizing gene-targeted ES cells by replacing exon 1 of the usp22 gene with the neomycin-resistance gene, which leads to a deletion of the ATG start codon and a frame shift of the remaining usp22 mRNA (Figures S4A and S4B). usp22 heterozygous mice were born without phenotypic abnormalities in expected Mendelian ratios and survived well past one year of age with normal fertility. Breeding of usp22 heterozygous mice did not yield any homozygous offspring (Figure 6A). Of 55 total live born pups, 34.5% (19/55) were wild-type and 65.5% (36/55) were heterozygous. To establish at what stage of usp22 null mouse development resulted in embryonic lethality, we crossed heterozygous mice and harvested embryos at progressively earlier stages of development. We were unable to identify live usp22 null embryos at 10.5 days and after postimplantation stage (Figure 6A). Several dead embryos were confirmed to be usp22 homozygous at E10.5 (Figure 6B). In addition, we were able to identify usp22 null embryos at E9.5 or earlier close to Mendelian ratios but significantly smaller in size. Histological analysis by H&E staining revealed retardation...
in embryonic development (Figures 6B and 6C). Therefore, USP22 appears to be critical for the early stages of embryonic development.

Our findings so far support that USP22 functions as a critical antiapoptotic factor by facilitating Sirt1-mediated suppression of p53 functions. To further validate the role of USP22 in cell apoptosis, we generated usp22 null mouse embryonic fibroblasts (MEFs). The lack of both usp22 mRNA and USP22 protein in usp22 null MEFs were confirmed (Figures 6D and 6F). Of note, usp22 null MEFs exhibited dramatically increased apoptosis (Figure 6G). Consistent with our finding that USP22 knockdown promotes cell apoptosis through destabilizing Sirt1, a dramatic reduction in Sirt1 protein levels was detected in usp22 null MEFs (Figure 6F). This dramatic reduction of Sirt1 protein expression is regulated at the posttranscriptional level, because its mRNA levels are not affected by USP22 deficiency (Figure 6D), confirming our findings that USP22 is required to stabilize Sirt1 protein. This destabilization of Sirt1 protein caused by USP22 deficiency results in elevated p53 acetylation (Figures 6F and S4C) and target gene transcription because a significant increase in both protein and mRNA levels of p21 and BAX, as well as in the mRNA level of PUMA, was detected in usp22 null MEFs when compared to that in the wild-type control (Figures 6E and F). As BAX, p21, and Puma are de novo target genes of p53, our data collectively demonstrate that USP22 is required to enhance Sirt1-mediated suppression of p53 transcriptional activity and suppression of cell apoptosis during mouse embryonic development. In fact, suppression of p53 expression largely inhibited the cell apoptosis caused by usp22 deficiency (Figure 6G), further supporting our conclusion that USP22 regulates cell apoptosis through p53.

DISCUSSION

The current study demonstrates that USP22 is a deubiquitinase of Sirt1 and leads to suppression of p53 transcriptional activity and p53-mediated cell apoptosis both during embryonic development and in response to DNA damage. This conclusion is supported by the following evidence: First, Sirt1 interacts with both USP22 and p53 to form a complex in cells. Second, USP22 removes polyubiquitin chains that conjugate to and drive Sirt1 degradation through the proteasome pathway. Third, the USP22-mediated stabilization of Sirt1 leads to decreasing levels of p53 acetylation and suppression of p53-mediated transcriptional activity and apoptotic functions. Fourth, the suppressive activity of USP22 on p53 functions depends on intact Sirt1 because UPS22 failed to inhibit p53 transcriptional activity in sirt1 null cells. Finally, genetic deletion of usp22 gene in mice leads to early embryonic lethality, and loss of USP22 functions causes decreased Sirt1 protein expression.

USP22 is critically required for embryogenesis because the targeted deletion of usp22 gene in mice leads to early embryonic lethality. While the underlying molecular mechanisms are to be determined, our finding that USP22 suppresses p53 activity through Sirt1 stabilization suggests that increased p53 functions and cell apoptosis account for, at least partially, the embryonic lethality due to usp22 deficiency. Similar to usp22 null mice, most of the sirt1 null embryos are smaller than their wild-type or heterozygous littermates, indicating that they were retarded in certain developmental processes (McBurney et al., 2003). This retardation is likely due to cell apoptosis caused by the elevated p53 transcriptional activity because Sirt1-deficient cells exhibited p53 hyperacetylation and increased p53 target gene expression and cell apoptosis (Cheng et al., 2003). Indeed, we detected a more than 90% reduction of Sirt1 protein expression in usp22 null MEFs. In addition, it is clear that the increased p53 protein expression causes early embryonic lethality in mdm2 null mice because it can be rescued by p53 deficiency (Jones et al., 1995; Léveillard et al., 1998; Montes de Oca Luna et al., 1995). As an E3 ubiquitin ligase, MDM2 critically controls p53 protein levels through ubiquitination-mediated protein degradation under both physiological and pathological conditions. P53 protein expression is increased in mdm2 null cells (Boyd et al., 2000). Therefore, increased p53 activity seems to be a common molecular mechanism underlying the defect in the embryonic development in usp22, sirt1, and mdm2 null mice. Meanwhile, it needs to be noted that, in addition to the USP22-Sirt1-p53 pathway, other molecular mechanisms are likely involved in embryonic development because most sirt1 null mice die within 12 hr after birth, in contrast to E10.5 of usp22-deficient mice.

In addition to the identification of a physiological function of USP22 in mouse embryonic development, our findings here provide a possible molecular explanation for usp22 as a putative cancer stem cell gene through its antagonizing action toward p53 tumor suppressor Sirt1. Acetylation of p53 has been found as a critical regulatory mechanism to promote its DNA-binding and transcriptional activity (Gu and Roeder, 1997; Luo et al., 2001; Luo et al., 2000; Tang et al., 2008; Tang et al., 2008). Sirt1-mediated deacetylation can directly inhibit p53 transcriptional activity for the transcription of apoptosis-related target genes including p21, BAX, PUMA, and NOXA (Langley et al., 2002b; Motta et al., 2004; Vaziri et al., 2001). While the roles and functions of Sirt1 in cancer development have become increasingly complex and are still not well understood, Sirt1 clearly has a suppressive effect on p53 tumor suppressor. Therefore, USP22 may function as an oncogene by antagonizing p53 through Sirt1 stabilization during the early stages of tumor development before p53 mutations occur.

As a protein involved in a variety of biological functions, the activity and expression of Sirt1 is tightly regulated at many levels. Our study here demonstrates that Sirt1 is polyubiquitinated and targeted for proteasomal degradation. Indeed, abolishing Sirt1 ubiquitination by replacing the ubiquitin-conjugating lysine residues with arginines prolongs Sirt1 half-life. USP22 removes polyubiquitination of Sirt1 to control its protein stability and functions. Therefore, USP22 is a positive regulator of Sirt1. The E3 ligase(s) that catalyzes Sirt1 ubiquitination remains to be identified. While our study here identified Trim28, a RING-finger-containing protein, as a Sirt1-interaction partner, we could not detect any effect of altered Trim28 expression on Sirt1 ubiquitination and protein stability, implying that Trim28 is not an E3 ligase of Sirt1.

Previous studies have demonstrated that USP22 is a component of the SAGA (yeast Spt-Ada-Gcn5-Acetyl transferase) complex, a chromatin-modifying transcription coactivator, and
the SAGA complex integrity and proper subdomain organization are important for its function (Köhler et al., 2010; Pijnappel and Timmers, 2008; Samara et al., 2010). The fact that our proteomic analysis identified GCNS, another bona fide component of the SAGA complex (Grant et al., 1997; Grant et al., 1998; Roberts and Winston, 1997; Wang et al., 1998), as one of the Sirt1-interacting proteins, suggests that USP22-mediated deubiquitination of Sirt1 is possibly regulated by the SAGA complex. It will be very interesting to further investigate whether and/or how GCNS and other SAGA complex proteins regulate USP22-mediated deubiquitination of Sirt1 and other USP22 substrates in vivo.

EXPERIMENTAL PROCEDURES

Isolation of Sirt1 Interactors by a Proteomic Approach

HEK293 cells were transfected with FLAG-tagged Sirt1 expression plasmids. The transfected cells were lysed with NP-40 lysis buffer. Sirt1 proteins were immunoprecipitated with anti-FLAG Ab-conjugated agarose, and the immune complex was eluted from the agarose with 100 mM FLAG peptide (Sigma Aldrich). The eluted proteins were digested with trypsin and characterized by mass spectrometry.

In Vivo Ubiquitination Assay

Transfected cells with various combinations of plasmids DNA were lysed with RIPA Buffer (50 mM Tris-HCl, pH 8; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate, and 0.2% SDS), followed by precleaning with protein G sepharose beads. Cell lysates were immunoprecipitated with anti-Sirt1 antibodies and analyzed by western blotting with anti-HA or anti-Ub Abs.

Apoposis Analysis

The cells were left untreated or treated with 20 nM etoposide (Sigma) for 24 hr or indicated time. Treated cells were collected and cell apoptosis was determined by annexin V (Sigma) staining, using flow cytometry as described (Chen et al., 2009; Gao et al., 2008).

Generation of USP22 Knockout Mice and Embryonic Fibroblasts

The mouse usp22 locus was cloned from strain C57BL/6 genomic library. A 1.5 kb fragment that carries usp22 exon 1 was subcloned into pENTR3C-loxp-P- MCS-LoxP-Neo-FRT vector using SaI1 and Not1 sites (Wang et al., 2008b). The pDONR vectors, pDONR P4-1PR and pDONR P2R-P3, supplied in the MultiSite Gateway Three-Fragment Vector Construction Kit (Invitrogen) were used to generate the 5′ and 3′ homology arm constructs. The ES cells, derived from an F1 embryo between C57BL/6 mice, were used for transfection, and homologous recombination was checked by Southern blot analysis. Two independent usp22+/– ES clones were injected into eight-cell embryos. Chimeric males with greater than 80% agouti coat color were bred to C57BL/6 females, and germ line transmission of the mutant allele was identified by PCR analysis of embryo- and tail-derived genomic DNAs. MEFs were generated and used as described (Gao et al., 2008).

Histological Analysis of Mouse Embryos

Mouse embryos were removed from the uterus, and the yolk sac was harvested for genotyping. Embryos were fixed in 10% formalin for 2 days and embedded with OCT and frozen in –80°C overnight. Eight micrometer tissue sections were prepared, attached to aminopropyl triethoxysilane-coated slide glasses, and subjected to H&E staining as reported (Chen et al., 2011). The detailed materials and methods of this study are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2012.03.024.
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