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Cabin1 restrains p53 activity on chromatin

Hyonchol Jang[1], Soo-Youn Choi[1], Eun-Jung Cho[2] & Hong-Duk Youn[1]

The tumor suppressor p53 has been proposed to bind target promoters upon genotoxic stress. However, recent evidence shows that p53 occupies some target promoters without such stress, suggesting that a negative regulator might render p53 transcriptionally inactive on these promoters. Here we show that calcineurin binding protein 1 (Cabin1) is a negative regulator of p53. Downregulation of Cabin1 induces activation of a subset of p53 target genes. Cabin1 physically interacts with p53 on these target promoters and represses p53 transcriptional activity in the absence of genotoxic stress, by regulating histone modification and p53 acetylation marks. Knockdown of Cabin1 retards cell growth and promotes cell death after DNA damage in a p53-dependent manner. Thus, Cabin1 inhibits p53 function on chromatin in the quiescent state; the presence of inactive p53 on some promoters might allow a prompt response upon DNA damage.

p53 regulates many cellular functions, including cell growth, cell-cycle progression, DNA repair, senescence and death[1–5]. In unstressed cells low p53 protein levels are sustained by proteolytic degradation; upon stress p53 accumulates in the cell and becomes activated[1–5]. Although the function of p53 is largely dictated by its abundance, tight regulation is required for p53 to select targets and effect different outcomes. Because sequence-specific DNA binding of p53 is a prerequisite for the transactivation of target genes, many factors modulating the interaction between p53 and its DNA target sequences have been proposed Post-translational modifications such as phosphorylation on Ser46 and acetylation on Lys320 favor transactivation of proapoptotic genes[3]. ASPP family proteins, the Brn family of POU domain transcription factors, YB1, NF-κB, Pin1 and Hzf all guide p53 to a specific subset of targets[3,4].

However, the level of chromatin-bound p53 increases to a similar extent on pro-arrest or proapoptotic genes during apoptosis, indicating that the decision to undergo apoptosis or cell-cycle arrest is not obviously governed by selective binding of p53 (refs. 6,7). p53 recruits chromatin-modifying factors to open local chromatin structure for transcription[3]. The p300/CBP histone acetyltransferases (HATs), JMY, SNF5 and BRG1, and CAS (also known as CSE1L) affect p53 transactivation by regulating chromatin structure[3,4]. Thus, in addition to selective binding of p53 on its targets, selective recruitment of chromatin modifiers can govern selective p53 activation. Moreover, emerging evidence demonstrating that p53 occupies some of its target promoters, such as those of *Gadd45*, *Cdkn1a* (encoding p21) and *Bbc3* (also knwn as *Puma*) in the absence of genotoxic stress[6–8] suggests that a negative regulator is required to maintain p53 in a transcriptionally inactive state on these target promoters.

Cabin1 (also known as Cain) is a ubiquitously expressed 2,220-amino-acid protein that regulates the protein phosphatase activity of calcineurin and the transcriptional activity of myocyte enhancer factor 2 (Mef2)[9,10]. Regulation of both calcineurin and Mef2 by Cabin1 has been implicated in T cell apoptosis[11]. Regulation of calcineurin by Cabin1 has also been implicated in both skeletal and cardiac muscle development[12,13]. Furthermore, Cabin1 (Cain) has been shown to regulate synaptic vesicle endocytosis[14].

The mechanism of Mef2 repression by Cabin1 has been extensively studied. Cabin1 recruits chromatin-modifying enzymes, such as histone deacetylases (HDACs)[15] and a histone methyltransferase (HMT), Suv39h1, to compact chromatin in Mef2 target promoters, repressing Mef2 transcriptional activity[16].

Cabin1-null mice are embryonic lethal. Yet, mice expressing a truncated Cabin1 that lacks the C-terminal calcineurin- and Mef2-binding region, are born at the expected Mendelian ratio[17], suggesting that Cabin1 has functions besides regulating calcineurin and Mef2.

In this study, we found that Cabin1 regulates expression of a subset of p53 target genes in both human and mouse cells in the absence of genotoxic stress. To investigate whether Cabin1 could be a negative regulator of p53 on chromatin, we performed imunoprecipitation assays and quantitative chromatin immunoprecipitation (ChIP) assays. We found that Cabin1 physically interacts with p53 and negatively regulates p53 on specific p53 target promoters by regulating chromatin structure. As a consequence of negative regulation of p53 by Cabin1, knockdown of Cabin1 retards cell growth and promotes cell death upon DNA damage in a p53-dependent manner.

RESULTS

Cabin1 regulates the expression of p53 target genes

To determine other physiological functions of Cabin1, we first investigated the genes that are regulated by Cabin1 through mRNA microarray analysis of normal mouse embryonic stem cells (E14) and heterozygously deleted Cabin1 embryonic stem cells (RRP258). Downregulation of Cabin1 was confirmed at both the mRNA and

[1]National Research Laboratory for Metabolic Checkpoint, Departments of Biomedical Sciences & Biochemistry and Molecular Biology, Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea. [2]National Research Laboratory for Chromatin Dynamics, College of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea. Correspondence should be addressed to H.-D.Y. (hdyoun@snu.ac.kr).

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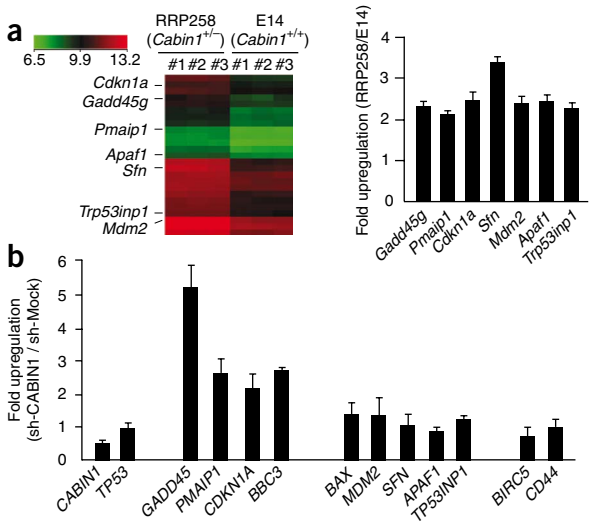

Figure 1 Cabin1 regulates the mRNA level of a subset of p53 target genes. (**a**) Microarray data show that a subset of p53 target genes is upregulated in Cabin1 haploinsufficient mouse embryonic stem cells. (**b**) Transient knockdown of CABIN1 in human cancer cells induces a subset of p53 target genes. HCT116 cells were treated with either lentiviral sh-CABIN1 or control (sh-Mock). After selecting infected cells, mRNA levels of the indicated genes were analyzed by quantitative real-time PCR. The values represent mean relative change ± s.d. ($n \geq 3$).

protein levels (**Supplementary Fig. 1a–c**). In microarray experiments that compared mRNA levels in E14 and RRP258 cells, 1,083 probes among 18,974 valid probes showed changes of more than two-fold (**Supplementary Fig. 1d,e**). A subset of p53 target genes, including *Gadd45*, *Pmaip1* (also known as *Noxa*) and *Cdkn1a*, were upregulated in *Cabin1* haploinsufficient cells (**Fig. 1a** and **Supplementary Table 1a**). Analysis of all known p53 target genes showed that a subset was upregulated in RRP258 cells (**Supplementary Table 1**). Results for some well-known targets of p53, such as *Bbc3* and *Bax*, were absent because the probes for these genes were not valid. Collectively, these results imply that Cabin1 negatively regulates p53 for selected targets.

To eliminate the possibility that these results are attributed to an adaptive response in RRP258 cells, we tested whether the increase in select p53 target genes also occurs in human cells that are transiently depleted of CABIN1. We infected HCT116 cells with lentivirus-containing short hairpin RNA (shRNA) against *CABIN1* mRNA (sh-CABIN1). After selecting infected cells, we assessed the knockdown of CABIN1 protein by immunoblotting (**Supplementary Fig. 2a**). There was no apparent change in the *TP53* mRNA level or the p53 protein level in sh-CABIN1–treated cells compared with nontargeting control shRNA (sh-Mock)-treated cells (**Fig. 1b** and **Supplementary Fig. 2b**).

Among genes that are activated by p53, *GADD45*, *PMAIP1*, *CDKN1A* and *BBC3* were upregulated in sh-CABIN1–treated cells, whereas *BAX*, *MDM2*, *SFN* (also known as *14-3-3σ*), *APAF1* and *TP53INP1* were not apparently changed (**Fig. 1b**). Expression of *BIRC5* (also called survivin) and *CD44*, genes that are repressed by p53 (refs. 18,19), was not significantly affected by CABIN1 downregulation (**Fig. 1b**).

GADD45, CDKN1A and SFN are cell-cycle arrest–related proteins, and PMAIP1, BBC3, BAX, APAF1 and TP53INP1 are proapoptotic proteins. Therefore, the responses to CABIN1 downregulation were not exclusive to cell-cycle arrest or apoptosis.

To clarify the relationship between CABIN1 and p53, we assessed whether CABIN1 directly affects p53 transcriptional activity.

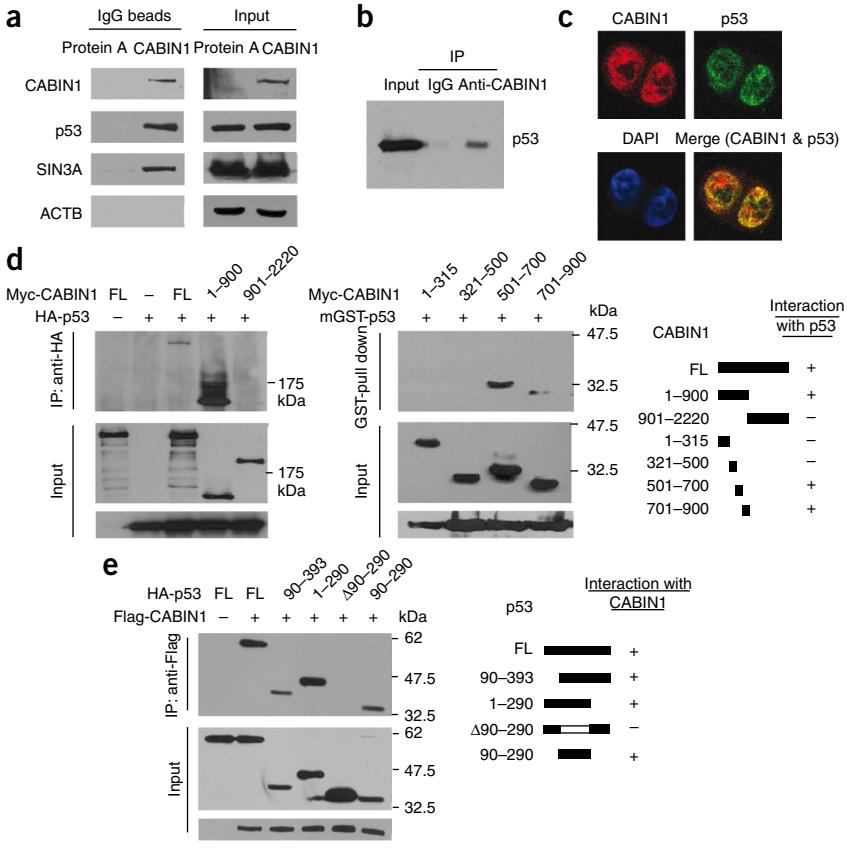

Figure 2 CABIN1 physically interacts with p53. (**a**) Protein A or protein A–tagged CABIN1 was stably overexpressed in HEK293 cells. Cell lysates were precipitated with IgG Sepharose. Precipitates were analyzed by immunoblotting with the indicated antibodies. SIN3A was used as a positive control and ACTB as a negative control. (**b**) Endogenous CABIN1 and p53 interact. Nuclear extracts from HCT116 cells were immunoprecipitated (IP) with either normal rabbit IgG or anti-CABIN1 antibody. Immunoprecipitates were then analyzed by immunoblotting with anti-p53 antibody. (**c**) CABIN1 and p53 colocalize in the nucleus. The localization of endogenous CABIN1 and p53 in HCT116 cells was analyzed by immunofluorescent staining and laser confocal microscopy. DAPI was used for nucleus staining. (**d**) Amino acids 500–900 of CABIN1 are responsible for interaction with p53. Myc-tagged CABIN1 and its fragments were overexpressed with HA-tagged p53 (left) or mGST-tagged p53 (right). Cell lysates were immunoprecipitated with anti-HA antibody and protein-A/G beads (left) or affinity-precipitated with glutathione-Sepharose beads (right). (**e**) CABIN1 binds the DNA-binding domain of p53. HA-tagged p53 and its fragments were overexpressed with Flag-tagged CABIN1. Cell lysates were immunoprecipitated with Anti-Flag M2-Agarose beads (Sigma). Immunoprecipitates were eluted from beads by competition with Flag peptide. The presence of bound p53 deletion mutants was analyzed by immunoblotting with anti-HA antibody.

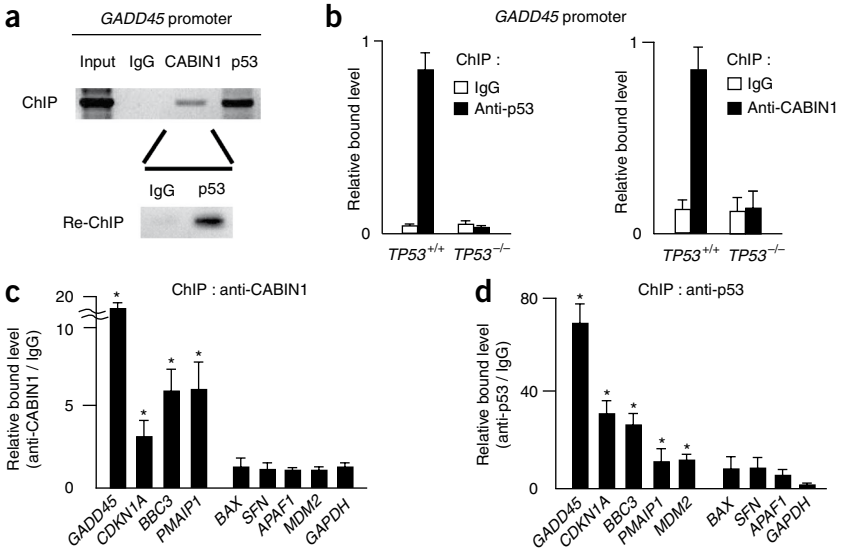

Figure 3 CABIN1 occupies a subset of p53 target promoters in the absence of genotoxic stress. (**a**) ChIP and re-ChIP show that CABIN1 and p53 coexist on the GADD45 promoter. (**b**) Recruitment of CABIN1 to the *GADD45* promoter is dependent on p53 occupancy. HCT116 (*TP53*$^{+/+}$) and HCT116 (*TP53*$^{-/-}$) cells were ChIPed with anti-p53 or anti-CABIN1 antibodies. ChIPed DNA was analyzed by real-time qPCR. (**c**,**d**) CABIN1 occupies a subset of p53 target promoters. CABIN1 (**c**) and p53 (**d**) occupancy on various p53 target promoters in HCT116 cells was determined by ChIP assays. $^{*}P < 0.05$.

We transfected H1299 cells, which express no p53, with a luciferase gene driven by a p53-response element or by a natural *PMAIP1* promoter. Co-transfection of p53 enhanced the reporter gene activity, which was repressed by the additional co-transfection of CABIN1 in a dose-dependent manner (**Supplementary Fig. 3**). The 900-amino-acid N terminus of CABIN1 ($CABIN1_{1–900}$) also repressed reporter gene activity, whereas its 1390-amino-acid C terminus ($CABIN1_{901–2220}$) did not (**Supplementary Fig. 3**), confirming the specificity of the repression. These data indicate that Cabin1 regulates the expression of a specific subset of p53 target genes.

CABIN1 physically interacts with p53

Although CABIN1 directly controls the transcriptional activity of p53 (**Supplementary Fig. 3**), knockdown of CABIN1 did not affect *TP53* mRNA levels (**Fig. 1b**). Thus, we examined the physical interaction between CABIN1 and p53. Protein A–tagged CABIN1 specifically immunoprecipitated p53 and a known binding partner, SIN3A[15], but not ACTB (**Fig. 2a**). p53 also was immunoprecipitated by Flag-tagged CABIN1 along with other CABIN1-binding partners, such as SIRT1, KDM1 (also known as LSD1) and EHMT2 (G9a) (**Supplementary Fig. 4a**,**b**).

The interaction between endogenous CABIN1 and p53 was confirmed. We incubated nuclear lysates of HCT116 cells that harbored wild-type p53 with anti-CABIN1 antibody, resulting in co-immunoprecipitation of p53 (**Fig. 2b**). Both CABIN1 and p53 resided primarily in the nuclei of HCT116 cells; a substantial portion of them merged in the same focal plane in the nucleus (**Fig. 2c**).

CABIN1 and p53 physically interact with SIN3A independently[15,20]. Thus, we asked whether the interaction between CABIN1 and p53 was mediated by SIN3A. $CABIN1_{1–900}$, which binds SIN3A and SUV39H1 (refs. 15,16), was specifically recruited by p53, in contrast to $CABIN1_{901–2220}$, which binds MEF2 (ref. 10; **Fig. 2d**). This result explains why p53 reporter gene activity was affected by $CABIN1_{1–900}$ but not $CABIN1_{901–2220}$ (**Supplementary Fig. 3**).

We mapped the p53-interacting domain of CABIN1 to its N-terminal region (amino acids 501–700 and 701–900) (**Fig. 2d**), which is distinct from the SIN3A-interacting domain, amino acids 1–315 (ref. 15). The DNA-binding domain of p53, but not the N-terminal SIN3A-binding domain[20], interacts with CABIN1 (**Fig. 2e**). Furthermore, bacterially purified His_6-tagged p53 pulled down *in vitro*–translated $CABIN1_{701–900}$ (**Supplementary Fig. 4c**). These results demonstrate that CABIN1 interacts with p53 without mediation by mSIN3.

Both $CABIN1_{501–700}$ and $CABIN1_{701–900}$ bound the DNA-binding domain of p53, and their interaction with p53 was not competitive, suggesting that these two domains bind distinct region of p53 within the DNA-binding domain (**Supplementary Fig. 4d**,**e**).

CABIN1 occupies a subset of p53 target promoters

CABIN1 represses MEF2 transcriptional activity by modulating the chromatin structure of MEF2 target promoters[16]. We investigated whether similar mechanisms exist for p53 target promoters. Because *GADD45* expression was most affected by CABIN1 downregulation (**Fig. 1b**), we studied the *GADD45* promoter. We carried out ChIP and

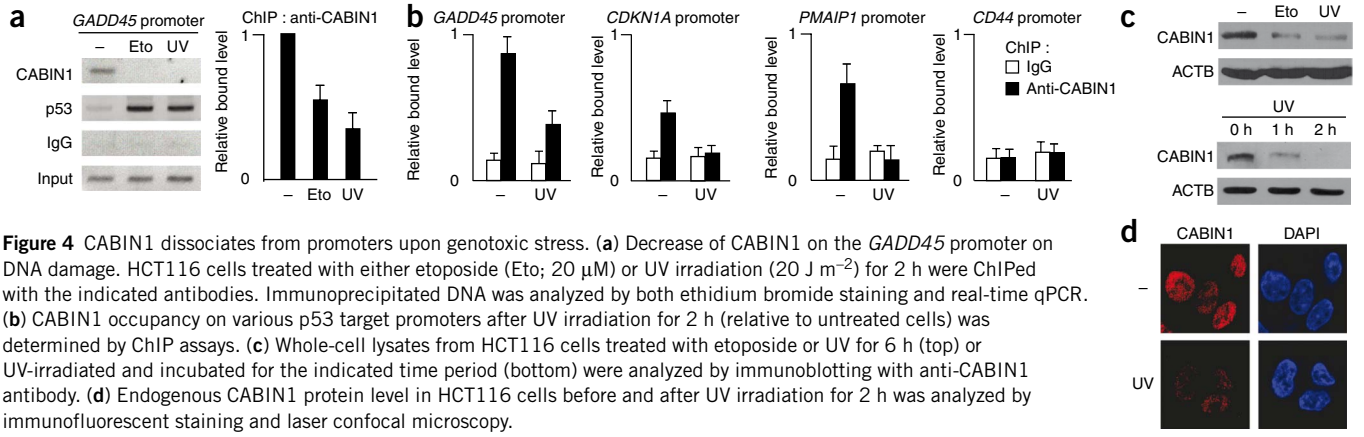

Figure 4 CABIN1 dissociates from promoters upon genotoxic stress. (**a**) Decrease of CABIN1 on the *GADD45* promoter on DNA damage. HCT116 cells treated with either etoposide (Eto; 20 μM) or UV irradiation (20 J m^{-2}) for 2 h were ChIPed with the indicated antibodies. Immunoprecipitated DNA was analyzed by both ethidium bromide staining and real-time qPCR. (**b**) CABIN1 occupancy on various p53 target promoters after UV irradiation for 2 h (relative to untreated cells) was determined by ChIP assays. (**c**) Whole-cell lysates from HCT116 cells treated with etoposide or UV for 6 h (top) or UV-irradiated and incubated for the indicated time period (bottom) were analyzed by immunoblotting with anti-CABIN1 antibody. (**d**) Endogenous CABIN1 protein level in HCT116 cells before and after UV irradiation for 2 h was analyzed by immunofluorescent staining and laser confocal microscopy.

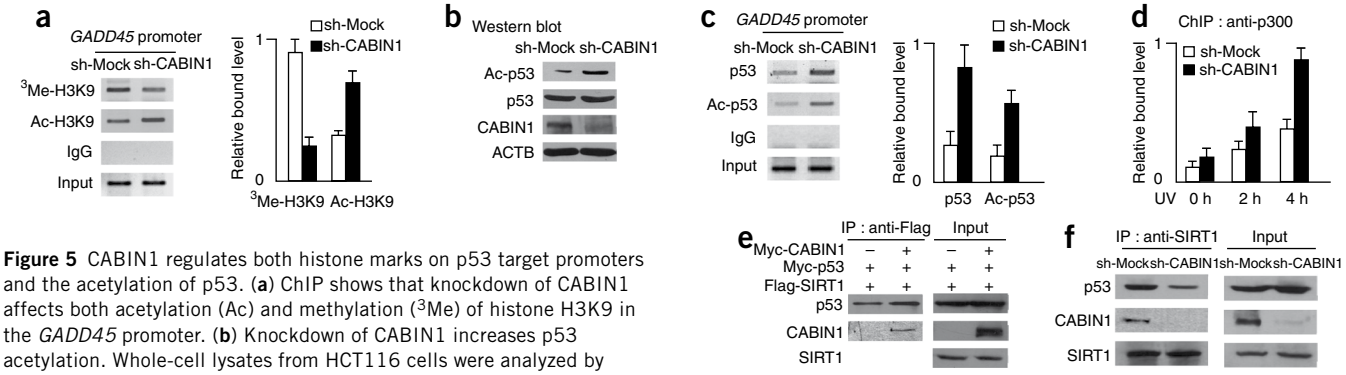

Figure 5 CABIN1 regulates both histone marks on p53 target promoters and the acetylation of p53. (**a**) ChIP shows that knockdown of CABIN1 affects both acetylation (Ac) and methylation (^{3}Me) of histone H3K9 in the *GADD45* promoter. (**b**) Knockdown of CABIN1 increases p53 acetylation. Whole-cell lysates from HCT116 cells were analyzed by immunoblotting with the indicated antibodies. (**c**) ChIP shows that knockdown of CABIN1 increases binding of both p53 and acetylated p53 to the *GADD45* promoter. (**d**) ChIP shows that p300 binds the *GADD45* promoter faster in CABIN1 knocked down cells on DNA damage, in UV-treated cells relative to untreated cells. (**e**) CABIN1 increases the physical interaction between p53 and SIRT1. IP, immunoprecipitate. (**f**) Knockdown of CABIN1 decreases the physical interaction between p53 and SIRT1. Whole-cell lysates from HCT116 cells were immunoprecipitated with anti-SIRT1 antibody and then analyzed by immunoblotting.

observed that both CABIN1 and p53 bound the *GADD45* promoter in the absence of genotoxic stress (**Fig. 3a**). CABIN1 and p53 occupy the *GADD45* promoter simultaneously, because CABIN1-bound chromatin was clearly re-ChIPed with anti-p53 (**Fig. 3a**).

The recruitment of CABIN1 to *GADD45* promoter is dependent on p53 occupancy on the promoter. CABIN1 occupied *GADD45* promoter in HCT116 (*TP53*$^{+/+}$) cells but not in HCT116 (*TP53*$^{-/-}$) cells (**Fig. 3b**). Further analysis of CABIN1 occupancy on various p53 target promoters showed that CABIN1 occupied a specific set of p53 target gene promoters such as *GADD45*, *CDKN1A*, *BBC3* and *PMAIP1* (**Fig. 3c**). CABIN1 promoter occupancy was not proportional to the p53 promoter occupancy. p53 occupied more *CDKN1A* promoter than *PMAIP1* promoter (**Fig. 3d**), but CABIN1 occupies more *PMAIP1* promoter than *CDKN1A* promoter (**Fig. 3c**). In addition, although the *MDM2* promoter showed significant p53 occupancy[21], it showed no apparent CABIN1 occupancy (**Fig. 3c**).

Upon treatment with etoposide or UV irradiation, CABIN1 dissociated from the *GADD45* promoter, whereas more p53 bound to the promoter (**Fig. 4a**). We obtained similar results for the *CDKN1A* and *PMAIP1* promoters but not for the *CD44* promoter (**Fig. 4b**). Dissociation of CABIN1 from p53 target promoters might be due to CABIN1 protein degradation. The CABIN1 protein level was decreased by etoposide treatment or UV irradiation (**Fig. 4c**). This decrease is probably due to protein degradation, because the CABIN1 levels went down rapidly within 2 h after UV irradiation (**Fig. 4c**,**d**).

CABIN1 regulates chromatin structure on p53 target promoters

In the *GADD45* promoter, knockdown of CABIN1 decreased trimethylation of the Lys9 of histone H3 (H3K9), a marker of repressed chromatin[22], and increased H3K9 acetylation, a marker of active chromatin[23] (**Fig. 5a**). Therefore, CABIN1 renders the chromatin of select p53 target promoters unsuitable for transcription. Knockdown of CABIN1 also increased the level of acetylated p53 (Ac-p53) (**Fig. 5b**), thereby elevating levels of bound Ac-p53 on the *GADD45* promoter (**Fig. 5c**). The transcriptional coactivator p300 bound the *GADD45* promoter faster in CABIN1-downregulated cells following DNA damage (**Fig. 5d**).

To investigate how CABIN1 affects the acetylation status of p53, we examined the relationship between CABIN1 and SIRT1, a p53 deacetylase[24,25], because SIRT1 interacts with CABIN1 (**Supplementary Fig. 4a**,**b**). CABIN1$_{1–900}$ mediated the binding to SIRT1 (data not shown), and bacterially purified His$_6$-tagged SIRT1 pulled down both *in vitro*–translated CABIN1$_{321–700}$ and CABIN1$_{501–900}$ but not CABIN1$_{1–315}$ (**Supplementary Fig. 4f**). SIRT1 specifically immunoprecipitated p53, and the addition of CABIN1 increased levels of immunoprecipitated p53 (**Fig. 5e**). Endogenous

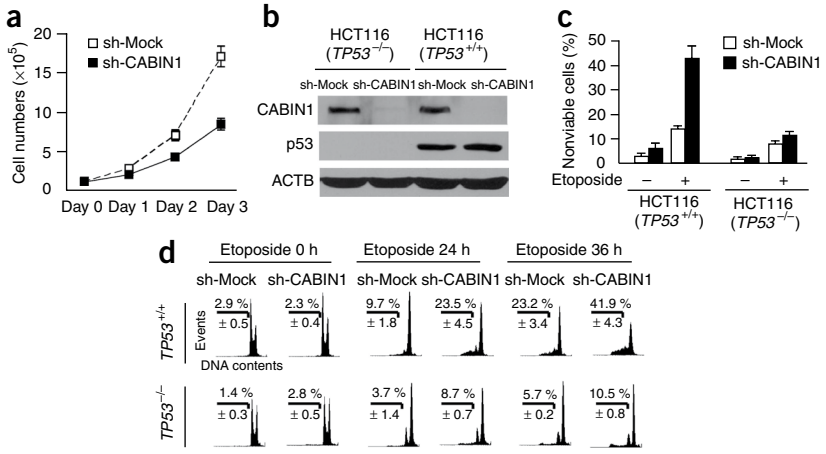

Figure 6 Knockdown of CABIN1 retards cell growth and promotes cell death on DNA damage. (**a**) Growth rates of HCT116 cells infected with either lentiviral sh-CABIN1 or control lentivirus were measured by cell counting. (**b**) Protein levels of CABIN1 and p53 in HCT116 (*TP53*$^{+/+}$) and HCT116 (*TP53*$^{-/-}$) cells infected with lentiviral sh-CABIN1 or control lentivirus were analyzed by immunoblotting. (**c**) HCT116 (*TP53*$^{+/+}$) and HCT116 (*TP53*$^{-/-}$) cells were infected with lentiviral sh-CABIN1 or control lentivirus. After treatment with etoposide for 24 h, cells were stained with FITC-conjugated Annexin V and propidium iodide (PI). The proportion of nonviable cells was measured by flow cytometry. Means ± s.d. ($n \geq 3$) are shown. (**d**) Apoptotic HCT116 cells were analyzed by measuring the population in sub-G1 phase among the PI-stained cells harvested at the indicated times. A representative figure and means ± s.d. ($n \geq 3$) are shown.

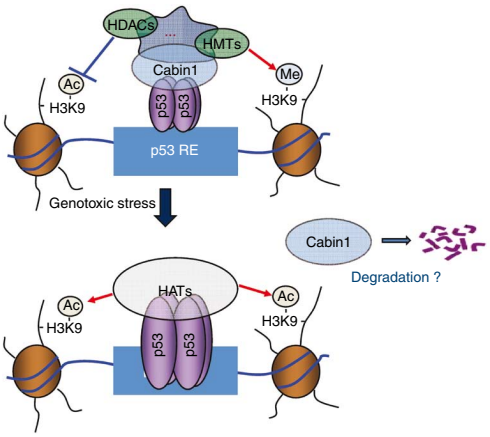

Figure 7 Mechanism of negative regulation of p53 by Cabin1. In the absence of genotoxic stress, Cabin1 renders p53 transcriptionally inactive on chromatin by recruiting various enzymes, such as HMTs and HDACs. Cabin1 also reduces p53 binding to target promoters by regulating the acetylation status of p53. On genotoxic stress, Cabin1 protein is likely degraded, and more p53 binds to the target promoters. p53 recruits histone acetyltransferases (HATs) and other transcriptional coactivators to initiate transcription.

SIRT1 immunoprecipitated both endogenous p53 and CABIN1 from HCT116 cell lysates (**Fig. 5f**). Knockdown of CABIN1 diminished the interaction between SIRT1 and p53 (**Fig. 5f**). These data suggest that CABIN1 increases the interaction between SIRT1 and p53 and thereby reduces p53 acetylation.

Physiological outcome of CABIN1 knockdown

Finally, we investigated the physiological consequence of negative regulation of p53 by Cabin1. Knockdown of CABIN1 in HCT116 cells retarded cell growth (**Fig. 6a**), as expected, owing to upregulation of the cell-cycle arrest–related genes *GADD45* and *CDKN1A* (**Fig. 1b**). Knockdown of CABIN1 also induced several proapoptotic genes, such as *PMAIP1* and *BBC3* (**Fig. 1b**). Knockdown of CABIN1 alone, however, did not cause significant cell death (**Fig. 6b**).

Because the mRNA levels of p53 target genes increased to a greater extent in CABIN1-downregulated cells subjected to genotoxic stress (**Supplementary Fig. 5a**), was applied the same treatment to cells to augment the effect of CABIN1 depletion. We knocked down CABIN1 in both HCT116 ($TP53^{+/+}$) and HCT116 ($TP53^{-/-}$) cells (**Fig. 6b**) and then subjected them to etoposide treatment. Under these conditions, CABIN1-downregulated HCT116 ($TP53^{+/+}$) cells showed a significant increase in the proportion of nonviable cells (**Fig. 6c** and **Supplementary Fig. 5b**). In contrast, the effect of CABIN1 downregulation on cell viability was relatively unapparent in HCT116 ($TP53^{-/-}$) cells (**Supplementary Fig. 5b**). We also measured the extent of apoptosis after etoposide treatment by analyzing the number of cells in sub-G1 phase. Similarly, CABIN1 knockdown enhanced cell death on etoposide treatment in HCT116 ($TP53^{+/+}$) cells (**Fig. 6d**). CABIN1 depletion in HCT116 ($TP53^{-/-}$) cells also increased cell death on etoposide treatment (**Fig. 6d**), although this effect was minor compared with that seen in HCT116 ($TP53^{+/+}$) cells. Because CABIN1 is a huge protein that interacts with various chromatin-modifying enzymes, additional pathways governing cell growth or DNA damage–induced cell death may exist.

Treatment with UV irradiation instead of etoposide also produced similar results (**Supplementary Fig. 5c**). These phenomena—growth retardation and promotion of cell death on DNA damage in CABIN1-depleted cells—were not restricted to HCT116 ($TP53^{+/+}$) cells. In mouse C2C12 cells, Cabin1 depletion upregulated select p53 target genes and caused growth arrest (**Supplementary Fig. 6**). Cabin1-downregulated C2C12 cells also showed increased apoptosis upon etoposide treatment (**Supplementary Fig. 6**).

On the basis of these results, we propose a negative-regulatory mechanism of p53. In the absence of genotoxic stress, Cabin1 and p53 co-occupy select p53 target promoters. Cabin1 renders p53 transcriptionally inactive by recruiting various enzymes, such as HMTs and HDACs. Cabin1 also reduces p53 binding to target promoters by regulating the acetylation status of p53. Upon genotoxic stress, Cabin1 protein is presumably degraded, and thus more p53 can bind to its target promoters, When activated, p53 recruits histone acetyltransferases (HATs) and other transcriptional coactivators to initiate transcription (**Fig. 7**), but the presence of transcriptionally inactive p53 on the promoter may allow it to respond promptly to DNA damage.

DISCUSSION

p53 represses the transcription of many genes, such as *AFP*, *BCL2* and *HBV*, by preventing other transactivators from binding to the promoter. In addition, p53 suppresses *MAP4*, *STMN1* and *HSP90AB1* by recruiting HDACs through SIN3A[2]. All of these genes are actively repressed by p53 under stress, and factors that are involved in this process assist p53 to achieve its goal. Cabin1 differs from these factors in that it impedes p53 activation of target genes under normal culture conditions.

Nonproteolytic negative regulators of p53, such as Sirt1, Smyd2, Kdm1 (LSD1), Ppp1r13l (iASPP), Noc2l (NIR) and Bach1 have been identified[24–31]. Sirt1 negatively regulates p53 via deacetylation[24,25]. Smyd2 and Kdm1 restrict p53 binding to its target promoters via methylation and demethylation[26,27]. Ppp1r13l blocks p53 binding to cell death–related promoters, a function that is counteracted by Pin1 (refs. 28,29). Notably, CABIN1 physically interacts with SIRT1 and KDM1 (**Supplementary Fig. 4a**,**b**), with the interaction with SIRT1 regulating p53 acetylation (**Fig. 5**). In this context, it will be interesting to determine whether the interaction between Cabin1 and Kdm1 affects the methylation of p53.

NOC2L co-occupies the *CDKN1A* promoter with p53 under normal conditions and stress, inhibiting histone acetylation[30]. Bach1 is recruited to the *Perp* and *Cdkn1a* promoters under normal culture conditions and impedes p53-mediated cellular senescence[31].

In this report, we show that CABIN1 occupies a subset of p53 target promoters in the absence of genotoxic stress. CABIN1 is rapidly degraded upon DNA damage (**Fig. 4c**,**d**), suggesting that CABIN1 mainly regulates cellular response in undamaged conditions. Although CABIN1 knockdown alone induced several target genes, the increase was relatively low compared to the DNA damage–induced increase in such genes (**Supplementary Fig. 5a**). Thus, CABIN1 knockdown alone may not fully activate p53 to sufficiently cause cell death (**Fig. 6**). CABIN1 knockdown, however, partially activates p53, allowing it respond quickly to DNA damage. It has been reported that in unstressed cells p53 is already recruited to target gene-regulatory sequences, but it requires activation[6]. Various coactivators are required to fully activate p53 (refs. 3,4). From these reports and our findings, we suggest that, on some promoters, Cabin1 hinders p53 from recruiting coactivators in unstressed cells. Upon DNA damage, release of Cabin1 from p53 enables access of coactivators and full activation of p53.

Several regulators of p53 showing promoter specificity have been identified[3]. PPP1R13B (ASPP1) and TP53BP2 (ASPP2) guide p53 to the proapoptosis targets[32], whereas Hzf selectively binds pro-arrest targets[33]. CAS selectively associates with a subset of p53 target promoters such as *PIG3* and *P53AIP1*, but not *CDKN1A*, showing no global discrimination between proapoptotic and growth-inhibitory

genes[34]. The molecular basis of their promoter selectivity, however, has not been not elucidated. CABIN1 selectively occupies the *GADD45*, *PMAIP1* (*NOXA*) and *BBC3* (*PUMA*) promoters but not the *BAX*, *MDM2* and *CD44* promoters (**Fig. 3c**). CABIN1 seems to occupy promoters that are preoccupied by p53. All the targets showing CABIN1 promoter occupancy are occupied by p53 in normal culture conditions. However, not all the promoters showing p53 occupancy are associated with CABIN1 (**Fig. 3c**,**d**). Studies on additional factors governing p53 target promoter specificity of CABIN1 may be interesting.

Chromatin structure of local p53 target gene promoter regions should be 'opened' for transcription. Cabin1 increases H3K9 methylation and decreases H3K9 acetylation of selective p53 target gene promoter regions, thereby making chromatin unsuitable for transcription. Cabin1 binds both the H3K9 methyltransfereases, Suv39h1 (ref. 16) and Ehmt2, and the histone deacetylases, HDAC1, HDAC2 (ref. 15) and Sirt1. Cabin1 has also been reported to compete against p300 for binding with Mef2 (ref. 15). Thus, Cabin1 might recruit these repressive chromatin modifiers and exclude activating chromatin modifiers such as p300/CBP HATs. Consequently, binding of p53 to its target might not be sufficient for transcription. Cabin1 and repressive chromatin modifiers should be released from p53 for access of HATs to enable opening of local chromatin structure. Cabin1 not only regulates local chromatin structure near p53 response elements but also regulates DNA binding of p53. Knockdown of Cabin1 apparently increased the levels of chromatin-bound p53, partially via regulating p53 acetylation marks (**Fig. 5b**,**c**). Thus, Cabin1 might act as a reservoir of inactive p53, keeping p53 close to regulatory genomic sequences. In case of DNA damage, Cabin1 is rapidly degraded; then, p53 can be provided locally and can recruit coactivators for an immediate transcriptional response. This fine, nonproteolytic regulation may provide p53 with target specificity, leading to numerous potential outcomes.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

H.J. planned the research, performed almost all the experiments and wrote the manuscript; S.-Y.C. performed some of the immunoprecipitation experiments; E.-J.C. supervised the research; H.-D.Y. planned and supervised the research and wrote the manuscript.

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ONLINE METHODS

Cells and transfection. We purchased normal mouse embryonic stem cells (E14) and heterozygously Cabin1-deleted cells (RRP258) from the Mutant Mouse Regional Resource Centers (MMRRC). We cultured stem cells in DMEM containing 15% (v/v) FBS (Hyclone), 2 mM L-glutamine, 1% (v/v) nonessential amino acids, 55 μM β-mercaptoethanol, antibiotics (all from Gibco) and 1,000 U ml^{-1} of ESGRO (Chemicon) on gelatin-coated plates.

We obtained HEK293 and H1299 cells from ATCC and HCT116 (*TP53*$^{+/+}$) and HCT116 (*TP53*$^{-/-}$) cells from B. Vogelstein (The Johns Hopkins University Medical Institutions). We cultured these cells in DMEM containing 10% (v/v) FBS and 50 U ml^{-1} each of streptomycin and penicillin.

We generated HEK293 cells stably expressing protein A–tagged CABIN1 by G418 selection of pcDNA3.0-TAP-CABIN1–transfected cells and HEK293 cells stably expressing Flag-tagged CABIN1 by hygromycin selection of pcDNA5-FRT/Flag-CABIN1–transfected cells using the Flp-in system (Invitrogen).

We carried out DNA transfections using the calcium phosphate co-precipitation method or Lipofectamine reagent (Invitrogen).

DNA constructs and purification of recombinant proteins. We described the various CABIN1 expression vectors previously[16]. We described mammalian expression vectors for full-length or deletion mutants of p53, p53-driven luciferase reporter genes and the bacterial expression vector for His_6-p53 previously[35]. We generated pcDNA3.0-TAP-CABIN1 by subcloning the EcoR1-Sal1 product of pSG5-myc-CABIN1 into pcDNA3.0-TAP. TAP is a tandem-affinity tag of both protein A and CBP. We obtained the expression vector for HA-KDM1 from S.-T. Kim and the expression vector for HA-EHMT2 from K. L. Wright.

We generated the Flag-SIRT1 expression vector by cloning the PCR products from cDNA of HEK293 cells into the pECE-Flag vector and the bacterial expression construct for His_6-SIRT1 by subcloning the PCR fragment from pECE-Flag-SIRT1 into pET-21a (Novagen). We expressed His_6 fusion proteins in the *Escherichia coli* strain BL21 (DE3) and isolated the proteins using the TALON metal-affinity resin (Clontech) according to the manufacturer's instructions.

Microarray. Total RNA from E14 and RRP258 cells were extracted with Trizol (Invitrogen). Macrogen (Korea) performed Illumina mouse-6 bead arrays.

Lentiviral sshort hairpin RNA–mediated knockdown of CABIN1. We purchased lentiviral vectors containing the human CABIN1-targeting sequences pLKO.1-sh-CABIN1 #1 (RHS3979-98826932), #2 (RHS3979-98826939), #3 (RHS3979-98826925), #4 (RHS3979-98826918), #5 (RHS3979-98826911) and mouse Cabin1-targeting sequences pLKO.1-sh-Cabin1 #1 (RMM3981-98497479) and sh-Cabin1 #4 (RMM3981-98497503) from Open Biosystems. As a control, we used the pLKO.1 vector. We produced lentivirus according to the manufacturer's protocol using the BLOCK-iT Lentiviral RNAi expression system (Invitrogen). Between 36 h and 48 h after lentiviral infection, infected cells were selected with puromycin for 2 d and then used for experiments. Because pLKO.1-sh-CABIN1 #1 was most effective, we used it in most experiments, where it is not specifically noted otherwise.

Quantitative reverse trancsriptase–polymerase chain reaction analysis of relative mRNA levels. We extracted total RNA with Trizol (Invitrogen) and reverse transcribed it (AMV Reverse Transcriptase XL, Takara). We quantified the mRNA levels by real-time PCR with the SYBR Green qPCR Kit (Finnzymes, F-410L) on the iQ5 Real-time PCR Detection System (Bio-Rad). To analyze relative mRNA expression levels, we normalized the results to *ACTB* and *HPRT1* using the $2^{-\Delta\Delta^{CT}}$ calculation method. We listed sequences of the primers for real-time PCR in **Supplementary Table 2a**.

Reporter gene assay. We transfected H1299 cells with pCMV-β-gal and reporter plasmids along with the indicated expression vectors. We measured luciferase activities 30 h after transfection with a Sirius luminometer (Berthold Detection Systems). We normalized luciferase activities to β-galactosidase acitivity.

Immunoprecipitation and immunoblot. We carried out immunoprecipitation and immunoblotting as described[16]. We purchased anti-ACTB, anti-Flag (M2) and anti-EHMT2 antibodies from Sigma; anti-HA (16B12) and anti-Myc (9E10) from Covance; anti-p53 (DO-1), anti-p53 (FL393), anti-SIRT1 (sc-15404) and anti-CDKN1A (sc-6246) from Santa Cruz Biotechnology; anti–acetyl-p53 (K373/382, 06-758) and anti-SUV39H1 from Upstate; and anti-KDM1 from Abcam. Affinity-purified rabbit anti-CABIN1 polyclonal antibodies, anti-CABIN1 (N92) and anti-CABIN1 (C1) were generated against purified GST-tagged CABIN1 (amino acids 1–92) and a peptide corresponding to CABIN1 (931–946), respectively (AbFrontier, Korea).

Confocal microscopy. We fixed HCT116 cells with 4% (w/v) paraformaldehyde and permeabilized them with 0.5% (w/v) Triton X-100. Then, we stained the cells with anti-CABIN1 polyclonal antibody and Alexa Fluor 568–conjugated anti-rabbit antibody (Invitrogen) and with anti-p53 (DO-1) monoclonal antibody and Alexa Fluor 488–conjugated anti-mouse antibody (Invitrogen). We observed immunofluorescence under a Zeiss LSM 510 laser scanning microscope.

Chromatin immunoprecipitation and Re-ChIP. We carried out ChIP assays as described[16]. We used anti-p53 (FL393, Santa Cruz Biotechnology), anti-CABIN1 (C1), anti–acetyl-p53 (K373/382, 06-758, Upstate), anti–Ac-H3K9 (#9671, Cell Signaling), anti–Tri-MeH3K9 (07-442, upstate) and anti-p300 (sc-584, Santa Cruz) antibodies. As a control, we used anti-rabbit IgG (Santa Cruz). We listed the sequences of primers that were used to amplify ChIP-enriched DNA that spanned the p53 response elements in p53 target promoters in **Supplementary Table 2b**.

For re-ChIP, we eluted chromatin that was enriched in anti-CABIN1 (C1) antibody precipitates by competition with epitope peptides that were used in the generation of anti-CABIN1 (C1) antibody. Then we ChIPed the eluates with anti-p53 antibody.

Detection of apoptosis. To assess the extent of apoptosis after DNA damage, we stained cells with both Annexin V-FITC and propidium iodide according to the manufacturer's protocol using the ApoScan Annein V FITC Apoptosis Detection Kit (BioBud, Korea). Alternatively, we fixed cells in 70% (v/v) ethanol and stained them with a solution containing RNase A (50 μg ml^{-1}) and propidium iodide (50 μg ml^{-1}).

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