Published online 23 December 2015

Nucleic Acids Research, 2016, Vol. 44, No. 8 ***3629–3642***
doi: 10.1093/nar/gkv1504

Dicer interacts with SIRT7 and regulates H3K18 deacetylation in response to DNA damaging agents

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Received June 2, 2015; Revised December 9, 2015; Accepted December 10, 2015

ABSTRACT

Dicer participates in heterochromatin formation in fission yeast and plants. However, whether it has a similar role in mammals remains controversial. Here we showed that the human Dicer protein interacts with SIRT7, an NAD$^+$-dependent H3K18Ac (acetylated lysine 18 of histone H3) deacetylase, and holds a proportion of SIRT7 in the cytoplasm. Dicer knockdown led to an increase of chromatin-associated SIRT7 and simultaneously a decrease of cytoplasmic SIRT7, while its overexpression induced SIRT7 reduction in the chromatin-associated fraction and increment in the cytoplasm. Furthermore, DNA damaging agents promoted Dicer expression, leading to decreased level of chromatin-associated SIRT7 and increased level of H3K18Ac, which can be alleviated by Dicer knockdown. Taken together with that H3K18Ac was exclusively associated with the chromatin, our findings suggest that Dicer induction by DNA damaging treatments prevents H3K18Ac deacetylation, probably by trapping more SIRT7 in the cytoplasm.

INTRODUCTION

As a ribonuclease III enzyme, Dicer is essential for the biogenesis of microRNAs (miRNAs) and small interfering RNAs (siRNAs) (1–3). It is also known that Dicer is required for heterochromatin formation in fission yeast, plants and flies (4,5). Depletion of Dicer in these species leads to DNA hypomethylation and histone hyperacetylation (4,5). However, whether Dicer has a similar role in mammals remains controversial (6–11). It was first reported by Kanellopoulou *et al.* that Dicer knockout in mouse embryonic stem (ES) cells led to reduction of di/trimethyl-H3K9 and DNA methylation at centromeric repeat sequences. They proposed that small RNAs generated by Dicer processing are involved in the formation of centromeric heterochromatin (6). And two research groups demonstrated that downregulation of the miR-290 family miRNAs caused DNA hypomethylation in Dicer-deficient ES cells (7,8). Specifically, miR-290 repressed the expression of retinoblastoma-like 2 (Rbl2), a transcriptional suppressor of DNA methyltransferases (Dnmts). Reduced miR-290 expression in Dicer-deficient ES cells resulted in Rbl2 upregulation, which in turn suppressed Dnmt gene transcription and led to DNA hypomethylation (7,8). However, three other groups reported that Dicer is not required for heterochromatin formation in mouse ES cells (9–11).

Sirtuins are a family of NAD$^+$-dependent protein deacetylases that regulate genome stability, metabolism and life span (12). SIRT7 is a member of the mammalian sirtuin family. It associates with active rRNA gene (rDNA), and promotes rDNA transcription by deacetylating PAF53, a subunit of RNA polymerase I (13,14). Interestingly, SIRT7

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also represses ribosomal protein gene transcription (15). Upon ER stress, SIRT7 is induced and recruited to the promoters of ribosomal protein genes to deacetylate H3K18Ac and silence gene expression, and eventually relieves ER stress (15). SIRT7 ablation sensitizes cells to DNA damage, while its overexpression confers resistance to DNA damaging treatments (16,17). Recently, Barber *et al.* reported that SIRT7 functions as an NAD+-dependent H3K18Ac (acetylated lysine 18 of histone H3) deacetylase and is essential for maintaining the transformed state of cancer cells (18).

Although most studies show that mammalian Dicer protein is predominantly located in the cytoplasm (19,20), it is also reported that a small pool of Dicer protein interacts with RNA polymerase II and is associated with the chromatin in human somatic cells (21). Therefore, it is interesting to address whether Dicer is involved in chromatin regulation via interacting with other chromatin modifiers. In this study, we revealed that Dicer is associated with SIRT7, and is involved in regulating H3K18Ac deacetylation in human cells upon DNA damaging treatments.

MATERIALS AND METHODS

Plasmids

Cloning of Dicer knockdown and the control shRNA plasmids. The target sequences in shDicer2 and shCon plasmids were as described previously (22), and the target sequence in shDicer1 is AAGAGTTTACTAAGCACCAGG. Short hairpin oligos containing these target sequences were inserted into the Bgl III/BamH1 sites of the pSUPER.neo.GFP plasmid (Oligoengine, Seattle, WA, USA). The sequences of short hairpin oligos are as follows: shDicer1 (5′-GATCCCCGAGTTTACTAAGCACCAGGTTCAAGAGACCTGGTGCTTAGTAAACTCTTTTTA-3′ and 5′-AGCTTAAAAAGAGTTTACTAAGCACCAGGTCTCTTGAACCTGGTGCTTAGTAAACTCGGG-3′); shDicer2 (5′-GATCCCCGGCTTACCTTCTCCAGGCTTTCAAGAGAAGCCTGGAGAAGGTAAGCCTTTTTA-3′ and 5′-AGCTTAAAAAGGCTTACCTTCTCCAGGCTTCTCTTGAAAGCCTGGAGAAGGTAAGCCGGG-3′); shCon (5′-GATCCCCATTCTCCGAACGTGTCACGTTCAAGAGACGTGACACGTTCGGAGAATTTTTTA-3′ and 5′-AGCTTAAAAAATTCTCCGAACGTGTCACGTCTCTTGAACGTGACACGTTCGGAGAATGGG-3′).

Cloning of the pFlag-SIRT7(WT) plasmid. The coding sequence of SIRT7 was polymerase chain reaction (PCR)-amplified using 5′-AATTGCTAGCGGAGCGATGGCAGCCGGGGGTCTGA-3′ and 5′-AGGTCTCGAGTTATTTATCGTCGTCGTCCTTGTAATCCGTCACTTTCTTCCTTTT-3′ as primers, and cloned into the NheI/XhoI sites of pcDNA3.1-Hygro(+)(Life Technologies, Grand Island, NY, USA).

Cloning of the pFlag-SIRT7(S111A) plasmid. Serine 111 codon of SIRT7 in the pFlag-SIRT7(WT) plasmid was mutated to alanine using the TaKaRa MutanBEST Kit (Takara, Dalian, China) with the following primers (mutated nucleotides are underlined and bolded): 5′-GGCGCGGGAATC**GCT**ACGGCAGCGTCTATCCC-3′ and 5′-TGTGTAGACGACCAAGTATTTGGCGTTCCGG-3′.

The pFlag-SIRT7(dE2), pCAGGS-Flag-hsDicer (D1320A/D1709A) and pDESTmycDICER (myc-tagged Dicer) plasmids were obtained from Addgene (Cambridge, MA, USA) (23–26).

Cell culture, generation of stable cell lines and DNA damaging treatment

Cell culture. Human HEK293T, HCT116 and U2OS cell lines were acquired from ATCC (Manassas, VA, USA), and mouse embryonic fibroblast (MEF) cells were a gift from Quan Wang (Third Military Medical University, Chongqing, China). HEK293T, U2OS and MEF cells were grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA), HCT116 cells were grown in McCoy's 5A medium (Life Technologies), all supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified incubator with 5% CO_2.

Generation of stable Dicer knockdown cells. HCT116 cells were transfected with shDicer1, shDicer2 or shCon plasmids, and grown under 1000 μg/ml Geneticin (G418) selection for 2–3 weeks. Knockdown of Dicer in G418-resistant monoclones was verified by western blot using anti-Dicer antibody (ab14601, Abcam, Cambridge, MA, USA).

Generation of stable Dicer overexpressing cells. HCT116 cells were transfected with pDESTmycDICER or an empty vector pcDNA3.1, and grown under 1000 μg/ml G418 selection for 2–3 weeks. Overexpression of Dicer in G418-resistant monoclones was verified by western blot.

Generation of stable HEK293T cells that overexpress Flag-SIRT7 proteins. HEK293T cells were transfected with the pFlag-SIRT7(WT), pFlag-SIRT7 (S111A), pFlag-SIRT7(dE2) or an empty vector pcDNA3.1, and grown under the selection of 1000 μg/ml G418 [for pFlag-SIRT7(dE2) and pcDNA3.1] or 100 μg/ml hygromycin B [for pFlag-SIRT7(WT) and pFlag-SIRT7(S111A)] for 2–3 weeks. Stable SIRT7 overexpressing monoclones were then identified by western blot using anti-SIRT7 (5360, Cell Signaling Technology, Danvers, MA, USA) and anti-FLAG antibodies (0912–1, HuaAn Biotechnology, Hangzhou, China).

DNA damaging treatments. Cells were exposed to cisplatin (DDP) or doxorubicin (doxo) for 24 h, or ionizing radiation (IR) 4 h before subsequent analysis. To investigate the effect of DNA damaging agents on protein degradation, cells were treated with DNA damaging agents along with 2 μM MG132 (Sigma, Saint Louis, MO, USA).

siRNAs and transfection

siRNAs were obtained from Life Technologies (Shanghai, China). The siRNA sequences are as follows: siTAp63, CAGAAGAUGGUGCGACAAAUU and UUUGUCGCACCAUCUUCUGUU; siDicer1 AAGAGUUUACUAAGCACCAGGdTdT and CCUGGUGCUUAGUAAACU

CUUdTdT; siDicer2 AAGGCUUACCUUCUCCAGGC UdTdT and AGCCUGGAGAAGGUAAGCCUUdTdT; siSIRT7, GCCUGAAGGUUCUAAAGAAUU and UU CUUUAGAACCUUCAGGCUU; siCon, AAUUCUCC GAACGUGUCACGUdTdT and ACGUGACACGUU CGGAGAAUUdTdT. siRNA transfection was performed as previously described (22).

Protein–protein interaction assays

Immunoprecipitation (IP) for in vivo protein interaction. Cells were lysed with immunoprecipitation (IP) buffer [20 mM Hepes, pH7.4, 0.1 M KAc, 2 mM $MgCl_2$, 0.1% Tween-20, 0.5% Triton X-100, 150 mM NaCl with protease inhibitors including PMSF, Pepstain A, Aprotinin and Bestatin hydrochloride (Sigma)] at 4°C for 30 min with continuous rotation, followed by centrifugation at 13 000 *g* for 10 min. The cellular extract was precleared with Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Piscataway, NJ, USA) at 4°C for 1 h before overnight incubation with appropriate antibodies or IgG control, and then precipitated with Protein G Sepharose beads. The beads were washed three times with 1.5 ml IP buffer and eluted with protein loading buffer at 100°C for 10 min. The precipitated immune complexes were subjected to western blot. The antibodies used for IP included: anti-Dicer (ab14601, Abcam), anti-SIRT7 (H00051547-D01, Abnova, Taiwan and 5360, Cell Signaling Technology). To test the salt-sensitivity of Dicer–SIRT7 interaction, co-IP was also performed in buffer with increasing NaCl concentration. To address whether RNA is involved in Dicer–SIRT7 interaction, the cellular extract was treated with RNase A (1 mg/ml), RNase T1 (20 U/ml) and RNase V1 (20 U/ml) for 15 min at 37°C before IP.

Co-IP assays using purified recombinant Dicer and SIRT7 proteins. The recombinant human Dicer (OriGene, Rockville, MD) and His-tagged SIRT7 (Abcam) proteins were incubated together in IP buffer at 4°C. Bovine serum albumin (BSA) was used to compensate the missing protein when only one protein (Dicer or SIRT7) was included in the assay. Three hours later, the reaction mixture was added with anti-SIRT7 antibody (H00051547-B01, Abnova), anti-Dicer antibody, or IgG control, and continued to incubate at 4°C overnight before precipitation with Protein G Sepharose beads. The beads were washed three times with 1. 5 ml IP buffer, eluted and the immune complexes were subjected to western blot.

In vitro binding assay. Purified recombinant human Dicer was incubated with His-tagged recombinant SIRT7 in binding buffer (50 mM NaH_2PO_4, pH8.0, 300 mM NaCl) for 3 h. BSA was used to compensate the missing protein when only one protein (Dicer or SIRT7) was included in the assay. The mixture was applied to a Complete His-Tag Purification Column (Roche, Mannheim, Germany) and incubated for 10 min. The column was then washed with 10 column volumes of binding buffer to remove the unbound proteins, and the bound proteins were eluted with a buffer containing 50 mM NaH_2PO_4 (pH8.0), 300 mM NaCl and 250 mM imidazole. Representative unbound and bound fractions were subjected to western blot.

Co-IP assays for the Flag-tagged proteins. HEK293T cells that stably tranfected with pFlag-SIRT7(WT), pFlag-SIRT7(S111A) or pFlag-SIRT7(dE2), or transiently transfected with pCAGGS-Flag-hsDicer (D1320A/D1709A) were lysed with IP buffer at 4°C for 30 min with continuous rotation and then centrifuged at 13 000 *g* for 10 min. Equal amount of lysate was immunoprecipitated with anti-Flag M2 affinity gel (Sigma) at 4°C overnight. The gel was then washed three times with 1.5 ml IP buffer and eluted with 0.1M glycine (pH3.5) following the manufacturer's instructions. The eluates were immediately neutralized with 1M Tris (pH8.0), and subjected to western blot. The empty vector pcDNA3.1 transfected cells were used as a control.

Mass spectrometry analysis

The Dicer immunoprecipitates in HEK293T cells were extracted using SDT-lysis buffer (4% sodium dodecyl sulphate (SDS), 100 mM Tris/HCl pH 7.6 and 0.1M Dithiothreitol (DTT)), followed by LysC and trypsin-digestion using the filter aided sample preparation method as described previously (27). The ionized peptides were applied to a LTQ Orbitrap Elite mass spectrometer (Thermo Scientific, Grand Island, NY, USA). Proteins were identified from the raw mass spectrometry data by Protein Discoverer (version 1.4, Thermo Scientific), and the false discovery rate was set to 0.01.

Biochemical fractionation

Biochemical fractionation was performed as previously described with modifications (28). Briefly, HEK293T or HCT116 cells were resuspended (4×10^7 cells/ml) in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT) supplemented with protease inhibitors. Triton X-100 was added to a final concentration of 0.1%, and cells were incubated for 5 min on ice, followed by low-speed centrifugation at 1300 *g* for 5min (4°C). The supernatant (S1) was centrifuged at 14 000 *g* for 10 min (4°C) to remove cell debris and insoluble aggregates. The pelleted nuclei (P1) were then washed once in buffer A, lysed in nuclei lysis buffer (10 mM Tris–HCl, pH 7.6, 420 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT and 2 mM $MgCl_2$, protease inhibitors) and centrifuged (5 min, 1700 *g*, 4°C) to collect the insoluble chromatin (P3). The supernatant (S3), which is enriched for nucleoplasmic proteins, was adjusted to final 150 mM NaCl with low salt buffer (10 mM Tris–HCl, pH 7.6, 1 mM DTT and 2 mM $MgCl_2$). P3 was incubated in 0.25 M HCl at 4°C overnight and then centrifuged at 16 000 *g* for 15min (4°C). The collected supernatant (S4), which is enriched for chromatin-associated proteins, was neutralized with 0.25 M NaOH.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde at room temperature (RT) for 10 min, followed by blocking with 1% BSA in phosphate buffered saline containing 0.1% triton at RT for 30 min. The fixed cells were incubated with the primary antibodies at 4°C overnight, followed by incubation with Cy3- or FITC-conjugated secondary antibodies at RT for 1

h. Images were acquired via a PMT system under a Nikon A1R confocal microscope using a 60× TRIF NA1.53 objective lens at RT, and analyzed with the NIS-Elements AR4.0 software. The antibodies used for immunofluorescence included: anti-Dicer (ab14601, Abcam) and anti-SIRT7 (NB110–81762, Novus, Littleton, CO, USA and 5360, Cell Signaling Technology).

Western blot analysis

The total cell lysate, the biochemical fractionation samples or the IP eluates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to the polyvinylidene fluoride membrane (Millipore, Danvers, MA, USA). The blots were incubated with the primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and detection with ECL plus reagents (GE healthcare). The primary antibodies used included anti-Dicer (Abcam), anti-SIRT7 (5360), anti-H3K18Ac (9675S), anti-TAp63 (4892) and anti-GAPDH (2118S) (Cell Signaling Technology), anti-lamin A/C (10298-1-AP) and anti-histone H3 (17168-1-AP) (ProteinTech, Wuhan, China) and anti-Flag (0912-1, HuaAn). For co-IP experiments, about 2–5% of the cellular extract was used as input for subsequent western blot analysis.

Quantitative real-time RT-PCR

Total RNA was prepared using Trizol reagent (Life Technologies) and incubated with RNase-free DNase I (Promega, Madison, WI, USA) for 30 min, and reversely transcribed using the M-MLV reverse transcription kit (Promega). SYBR Green real-time PCR was then performed using an ABI PRISM 7300 Sequence Detection system (Life Technologies). The primer sequences are as follows: TAp63 (29), 5′-GGTGCGACAAACAAGATTGAG-3′ and 5′-GAAGGACACGTCGAAACTGTG-3′; Dicer (22), 5′-TCCACGAGTCACAATCAACACGG-3′ and 5′-GGGTTCTGCATTTAGGAGCTAGATGAG-3′; SIRT7, 5′-AGTCTGTACCTCCTGCGTTC-3′ and 5′-GGACCCTAGACACAGGATGG-3′; GAPDH (22), 5′-ATGACATCAAGAAGGTGGTG-3′ and 5′-CATACCAGGAAATGAGCTTG-3′. The ΔΔCt method was used to measure the relative expression levels of subject genes. ΔCt was obtained by subtracting the Ct (threshold cycle) value of GAPDH from that of subject genes. And ΔΔCt was calculated by subtracting the ΔCt of control sample from that of subject sample. The fold change was calculated as $2^{-\Delta\Delta Ct}$, and the relative expression level in control sample was defined as 1.

Cell proliferation and clonogenic assays

Cell proliferation was assessed with 2×10^3 cells in 96-well plate using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) kit (Promega) according to the manufacturer's instruction. For clonogenic assay, cells were grown in medium containing 5% fetal bovine serum (FBS) for 7 days in 6-well plate and the survived colonies were stained with 0.1% crystal violet.

Statistical analysis

All statistical analyses were performed using the Student's *t*-test function of Microsoft Excel. All results represented as mean ± S.E.M. from at least three independent experiments. The difference was considered significant when the *P*-value is smaller than 0.05.

RESULTS

Dicer directly interacts with SIRT7

Our mass spectrometry analysis of Dicer immunoprecipitates revealed that SIRT7 was co-purified with Dicer (Supplementary Table S1). To validate the association between Dicer and SIRT7, we performed co-IP experiments using extracts from HEK293T and HCT116 cells. IP with an anti-Dicer antibody showed that Dicer co-precipitated with SIRT7 (Figure 1A). Reciprocally, IP with antibodies against SIRT7 revealed that SIRT7 co-precipitated with Dicer (Figure 1B). The association between Dicer and SIRT7 was attenuated by increasing salt concentration (Figure 1C), suggesting that the Dicer–SIRT7 complex is probably electrostatic in nature. In addition, treatment with ribonucleases (RNases) did not disrupt Dicer–SIRT7 interaction (Figure 1D), indicating that this interaction is not mediated by RNA. To address whether Dicer is directly associated with SIRT7, we performed co-IP experiments using purified recombinant proteins. Our results showed that recombinant Dicer effectively pulled down SIRT7, and *vice versa* (Figure 1E). The direct interaction was further validated by *in vitro* binding assay. Briefly, recombinant Dicer and His-tagged SIRT7 proteins were incubated together and subjected to His-tag column purification. The subsequent western blot revealed that His-tagged SIRT7 pulled down Dicer (Figure 1F). To map the region in SIRT7 that interacts with Dicer, we developed cell lines that stably express wild type Flag-SIRT7(WT), Flag-SIRT7(S111A) (a mutant unable to stimulate Pol I transcription) (14) or Flag-SIRT7(dE2) (a mutant lacking exon 2, which encodes a coiled-coil domain that contributes to a subset of SIRT7 interactions) (23). Cellular extracts from these stable cell lines were subjected to anti-Flag gel purification and analyzed by western blot. We found that Dicer co-precipitated with Flag-SIRT7(WT) and Flag-SIRT7(S111A), but not with Flag-SIRT7(dE2) (Figure 1G). This result indicates that the coiled-coil domain of SIRT7 is essential for Dicer–SIRT7 interaction.

Dicer and SIRT7 colocalize in the cytoplasm

To date, most studies show that mammalian Dicer protein is predominantly located in the cytoplasm (19,20), while SIRT7 is mainly a nucleolar protein (14,30,31). These observations seem against that Dicer is physically associated with SIRT7. However, it is reported that Dicer is located inside the ER lumen (32), and that a small pool of Dicer protein is also associated with the chromatin (21). In addition, Kiran *et al.* recently demonstrated that SIRT7 localizes both in the cytoplasm and in the nucleus (33). To resolve the discrepancy concerning the subcellular localization of SIRT7, we performed biochemical fractionation assay using a widely used protocol (28). Our results showed that SIRT7

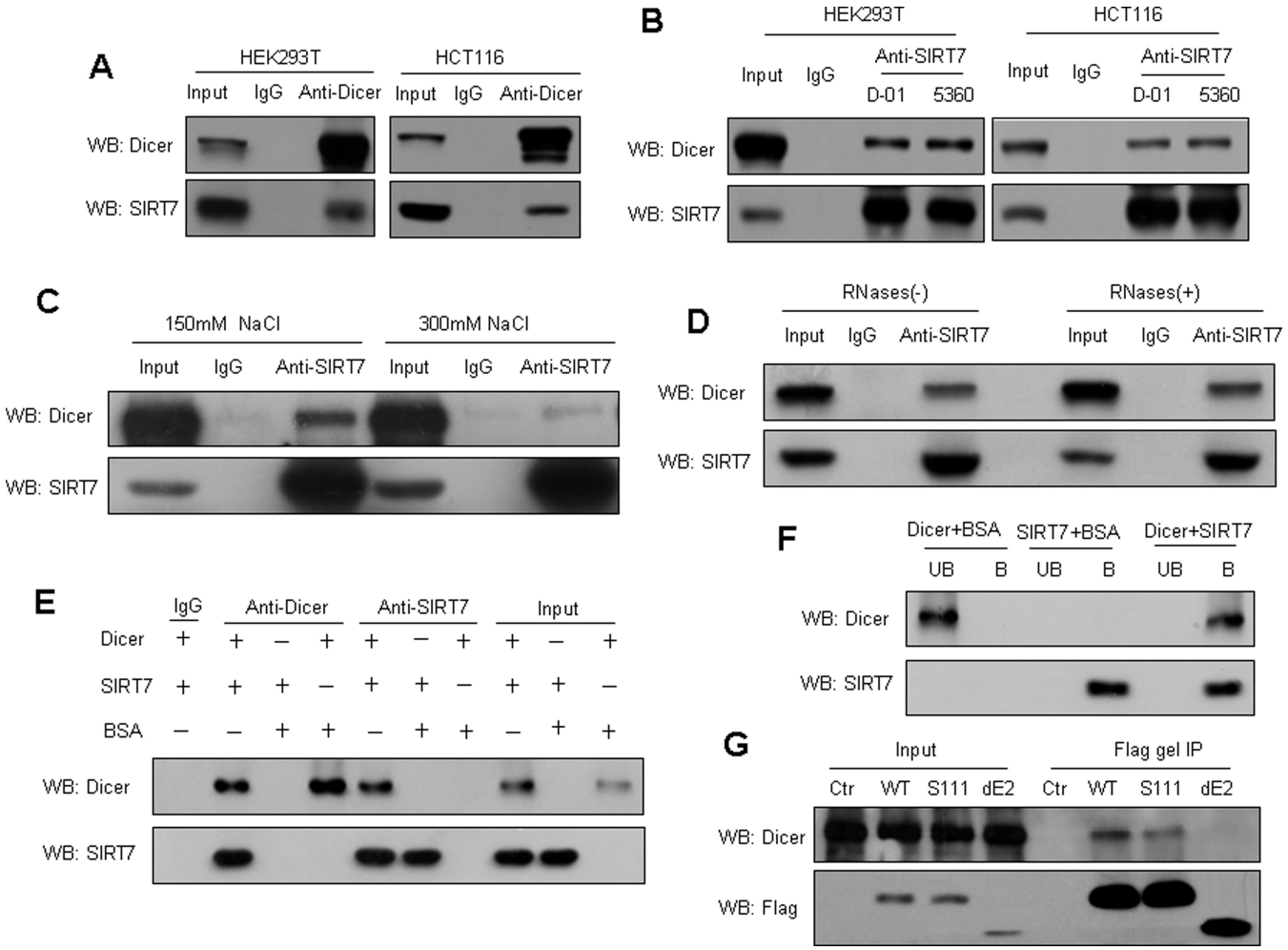

Figure 1. Direct interaction between Dicer and SIRT7. (**A**) Co-IP of endogenous Dicer and SIRT7 in HEK293T and HCT116 cells using anti-Dicer antibody. (**B**) Co-IP of endogenous Dicer and SIRT7 using two different anti-SIRT7 antibodies (H00051547-D01 and 5360). (**C**) Anti-SIRT7 (5360) co-IP using HCT116 cell lysate in the presence of increasing NaCl concentrations. (**D**) Anti-SIRT7 (5360) co-IP using HCT116 cell lysate in the presence or absence of RNases. (**E**) Direct interaction of Dicer and SIRT7 revealed by co-IP using purified recombinant Dicer and SIRT7 proteins. The recombinant proteins and IP antibodies added in each reaction are indicated on the top. BSA was used to compensate the missing protein when only one protein (Dicer or SIRT7) was included in the assay. (**F**) Interaction between recombinant human Dicer protein and purified His-tagged SIRT7 protein revealed by *in vitro* binding assay. The proteins loaded to the His-tag purification column are indicated on the top. B, representative bound fraction, UB, representative unbound fraction. (**G**) Anti-FLAG M2 gel pulled down endogenous Dicer in extracts of stable Flag-SIRT7(WT)- or Flag-SIRT7 (S111A)-expressing HEK293T cells, but not in extracts of Flag-SIRT7(dE2)-expressing HEK293T cells. Ctr: HEK293T cells transfected with the empty vector pcDNA3.1.

was mainly detected in the chromatin-associated fraction, while a small proportion was also detected in the cytoplasmic and the nucleoplasmic fractions. Unexpectedly, lamin A/C was detected exclusively in the chromatin-associated fraction (Supplementary Figure S1). Lamin A/C is a nuclear lamina protein, it is also present in the nucleoplasm and associates with chromatin (34,35). Thus, the nuclei lysis buffer (buffer B) used in this protocol is not efficient to separate nucleoplasmic proteins from chromatin-associated proteins. We hence modified this protocol by replacing buffer B with a new nuclei lysis buffer (Figure 2A). Using this modified protocol, we revealed that lamin A/C was presented in both the nucleoplasmic and the chromatin-associated fractions (Figure 2A). Dicer was mainly present in the cytoplasm, and SIRT7 was detected not only in the nucleoplasmic and the chromatin-associated fractions, but also in the cytoplasmic fraction (Figure 2A). The subcellular distribution of Dicer and SIRT7 was further validated by immunofluorescence, as colocalization of Dicer and SIRT7 was observed in the cytoplasm (Figure 2B and C; Supplementary Figures S2 and S3).

Dicer expression level affects the subcellular distribution of SIRT7

Based on three observations, including (i) SIRT7 resides not only in the nucleus but also in the cytoplasm, (ii) Dicer is mainly localized in the cytoplasm and (iii) Dicer colocalizes with SIRT7 in the cytoplasm, we proposed the following model to interpret the biological significance of physical association between Dicer and SIRT7: Dicer may trap a proportion of SIRT7 in the cytoplasm. Decreased Dicer expression would lead to a reduction, while increased Dicer expression would cause an increase of SIRT7 in the cy-

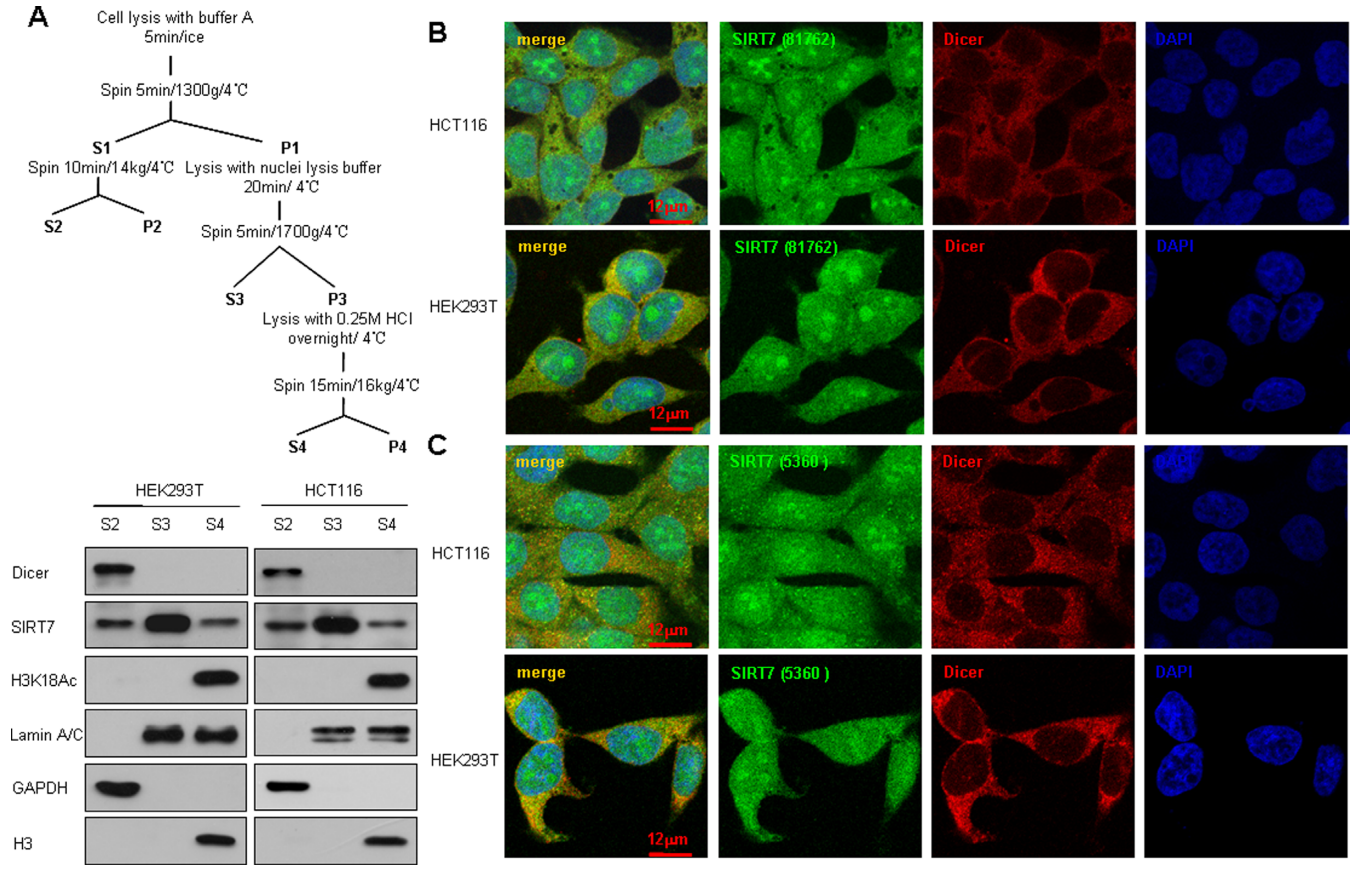

Figure 2. Colocalization of Dicer and SIRT7 in the cytoplasm. (**A**) Subcellular localization of Dicer, SIRT7, H3K18Ac, histone H3 and lamin A/C, revealed by biochemical fractionation. Upper panel, the schematic diagram of biochemical fractionation assay; bottom panel, the representative western blot images. S2, S3 and S4 represent the cytoplasmic, the nucleoplasmic and the chromatin-associated fractions, respectively. (**B** and **C**) Colocalization of Dicer and SIRT7 in the cytoplasm revealed by immunofluorescence using anti-Dicer and two different anti-SIRT7 antibodies. (B) NB110–81762; (C) 5360.

toplasm. To test this hypothesis, we performed IP experiments to pull down the Dicer–SIRT7 complex using excessive amount of anti-Dicer antibody. Our results indicated that more SIRT7 proteins were co-precipitated with Dicer in Dicer overexpressing cells as compared to the control cells (Figure 3A), while fewer SIRT7 proteins were pulled down in Dicer knockdown cells (Figure 3B). In addition, Dicer knockdown led to an increase of SIRT7 in the chromatin-associated fraction with a simultaneous decrease in the cytoplasmic fraction, and the level of SIRT7 in the nucleoplasm was not significantly changed (Figure 3C; Supplementary Figure S4A). Consistently, Dicer overexpression caused a decrease of SIRT7 in the chromatin-associated fraction, and an increase in the cytoplasmic fraction (Figure 3D; Supplementary Figure S4B). Neither overexpression nor knockdown of Dicer obviously affected the level of SIRT7 protein in total cell lysate (Figure 3E and F; Supplementary Figure S4C and D). Moreover, treatment with the proteasome inhibitor MG132 did not block the decrease of chromatin-associated SIRT7 in Dicer overexpressing cells (Supplementary Figure S5A). These findings ruled out the possibility that Dicer overexpression induces SIRT7 degradation in the chromatin-associated fraction.

Dicer regulates H3K18Ac deacetylation independent of its pre-miRNA processing activity

SIRT7 is an NAD^+-dependent H3K18Ac deacetylase (18), and H3K18Ac was exclusively present in the chromatin-associated fraction (Figure 2A). We therefore investigated whether Dicer regulates H3K18Ac deacetylation. Western blot results revealed that the level of H3K18Ac was increased in Dicer-overexpressing cells (Figure 3E; Supplementary Figure S4C), and slightly decreased in Dicer knockdown cells (Figure 3F; Supplementary Figure S4D). To address whether the pre-miRNA processing activity of Dicer is required for regulating H3K18Ac deacetylation, we transfected HEK293T cells with pCAGGS-Flag-hsDicer (D1320A/D1709A), a plasmid that encodes a mutant Dicer protein devoid of pre-miRNA processing activity (26). Our results revealed that overexpression of this mutant Dicer protein also induced a decrease of chromatin-associated SIRT7 (Supplementary Figure S6A), hence leading to an increase of H3K18Ac (Supplementary Figure S6B). Moreover, this mutant Dicer protein was able to interact with the endogenous SIRT7 protein (Supplementary Figure S6C).

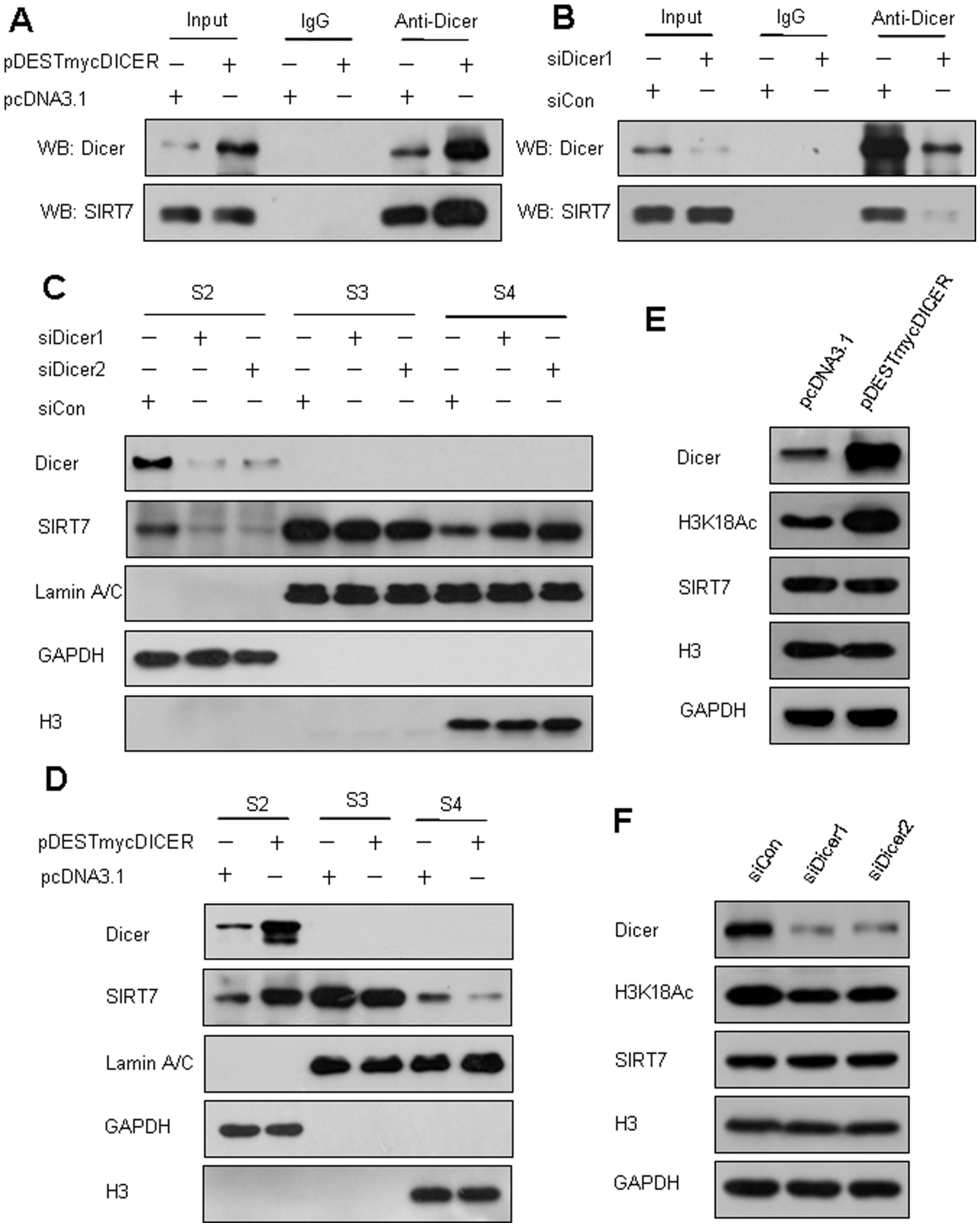

Figure 3. Dicer expression level affects SIRT7 subcellular distribution and H3K18Ac level in HEK293T cells. (**A** and **B**) Co-IP of Dicer and SIRT7 using excessive amount of anti-Dicer antibody in pDESTmycDICER (A) or siDicer1 (B) transiently transfected cells. (**C**) Increased level of chromatin-associated SIRT7 and decreased level of cytoplasmic SIRT7 in siDicer-transfected cells, revealed by biochemical fractionation. (**D**) Decreased level of chromatin-associated SIRT7 and increased level of cytoplasmic SIRT7 in pDESTmycDICER transiently transfected cells, revealed by biochemical fractionation. (**E** and **F**) Representative western blot images of Dicer, H3K18Ac, SIRT7 and histone H3 in pDESTmycDICER (E) or siDicer (F) transiently transfected cells. S2, S3 and S4 represent the cytoplasmic, the nucleoplasmic and the chromatin-associated fractions, respectively.

DNA damaging agents induce Dicer expression

It has been demonstrated that TAp63 binds to Dicer promoter and induces its expression (36), and DNA damage upregulates TAp63 and increases its DNA binding activity (29,37). These observations promoted us to investigate the effect of DNA damage on Dicer expression. Treatment with DNA damaging agents, including cisplatin (DDP), doxorubicin (doxo) and ionizing radiation (IR), led to upregulation of Dicer and TAp63 in HEK293T and HCT116 cells (Figures 4A and 5; Supplementary Figure S7A). To address whether TAp63 plays a role in regulating Dicer expression upon DNA damage, we knocked down TAp63 in DNA damaging treated cells. Our results revealed that TAp63 knockdown partially suppressed DNA damaging agent-induced Dicer upregulation (Figure 5).

DNA damaging treatments induce a decrease of chromatin-associated SIRT7 and an increase of H3K18Ac, which is partially blocked by Dicer knockdown

So far we have found that DNA damaging agents induced Dicer expression, and that Dicer may trap SIRT7 in the cytoplasm. We then investigated whether DNA damaging agents affect the subcellular distribution of SIRT7. Co-IP experiments using excessive amount of anti-Dicer antibody revealed that more SIRT7 proteins were associated with Dicer upon DNA damaging treatments (Figure 4B; Supplementary Figure S7B). Biochemical fractionation results revealed that DNA damaging treatments caused an increase of SIRT7 in the cytoplasmic fraction, and a decrease in the chromatin-associated fraction (Figure 4C and D; Supplementary Figure S7C and D). Consistently, DNA damaging treatments increased the level of H3K18Ac, without affecting the protein levels of SIRT7 and total histone H3 (Figure 4A; Supplementary Figure S7A).

Interestingly, treatment with MG132 did not block the decrease of chromatin-associated SIRT7 in DNA damaging treated cells (Supplementary Figure S5B), ruling out the possibility that DNA damaging treatments induced SIRT7 degradation in the chromatin-associated fraction. In addition, Dicer knockdown partially blocked the increase of cytoplasmic SIRT7, and the decrease of chromatin-associated SIRT7 in DNA damaging treated cells (Figure 6A and B; Supplementary Figure S8A and B). These observations suggest that DNA damaging treatments induce SIRT7 redistribution from the chromatin to the cytoplasm via upregulating Dicer expression. Consistently, the increase of H3K18Ac upon DNA damaging treatments was partially inhibited by Dicer knockdown (Figure 6C and D; Supplementary Figure S8C and D).

DISCUSSION

In summary, we revealed a direct interaction between Dicer and SIRT7, which is mediated by the coiled-coil domain of SIRT7. In addition, we found that a proportion of SIRT7 was trapped in the cytoplasm, probably through interacting with Dicer. Moreover, treatment with DNA damaging agents promoted Dicer expression, causing an increase of SIRT7 in the cytoplasm and simultaneously a decrease in the chromatin-associated fraction, as well as an elevated H3K18Ac level. Dicer knockdown prevented the decrease of chromatin-associated SIRT7, and partially blocked the increase of H3K18Ac upon DNA damaging treatments. Furthermore, neither Dicer overexpression nor DNA damaging treatments induced degradation of chromatin-associated SIRT7. Taken together, our findings indicate that more SIRT7 proteins were trapped in the cytoplasm upon DNA damaging treatments, probably by upregulating Dicer expression. Further investigations are needed to address whether Dicer upregulation blocks the nuclear transportation of newly synthesized SIRT7 or facilitates the relocation of SIRT7 from the chromatin to the cytoplasm. As SIRT7 is an H3K18Ac deacetylase, and H3K18Ac is exclusively present in the chromatin-associated fraction, our findings hence suggest that Dicer induction by DNA damaging agents prevents H3K18Ac deacetylation, possibly via trapping more SIRT7 protein in the cytoplasm. Histone acetylation associates with open and actively transcribed euchromatic domains (38). Therefore, we proposed that Dicer upregulation helps maintaining an open chromatin state upon DNA damaging treatments, while depletion of Dicer may prevent DNA damaging agent-induced chromatin relaxation by promoting H3K18Ac deacetylation (Figure 7).

It was reported that H3K18 is acetylated by CBP/p300 at DNA double-stranded breaks (39), so we proposed that, in addition to acetylation by CBP/p300, repression of H3K18Ac deacetylation also contributes to the increase of H3K18Ac upon DNA damaging treatments. We and others have reported that depletion of Dicer leads to accumulation of spontaneous DNA damage (22,40–42), therefore Dicer knockdown may affect the acetylation status of H3K18 via two different mechanisms: (i) decreased Dicer expression results in an increase of chromatin-associated SIRT7, which in turn leads to H3K18Ac deacetylation; (ii) the accumulation of spontaneous DNA damage in Dicer knockdown cells slightly induces CBP/p300-mediated H3K18 acetylation. The combined effect may explain why Dicer knockdown did not lead to an obvious decrease in the level of H3K18Ac. However, in the presence of DNA damaging agents, the effect of Dicer knockdown on CBP/p300-mediated H3K18 acetylation might be negligible. Consequently, Dicer knockdown led to an obvious decrease of H3K18Ac in cells treated with DNA damaging agents.

Although Dicer is essential for heterochromatin formation in fission yeast and plants (4,5), it remains controversial whether it plays a similar role in mammalian cells (6–11). While three groups reported that Dicer depletion leads to heterochromatin decondensation (6–8), other reports suggest that Dicer is not required for heterochromatin formation in mouse ES cells (9–11). Surprisingly, we found here that Dicer knockdown blocked the increase of H3K18Ac upon DNA damaging treatments. Our findings to some extent are consistent with the report by Benetti *et al.* (8), who showed that depletion of Dicer in mouse ES cells leads to an increase of heterochromatic histone marks, and a decrease of active chromatin histone marks at telomeric chromatin, even though the level of global DNA methylation is decreased in Dicer-null ES cells. Altogether, these observations indicate that the role of Dicer in chromatin regulation

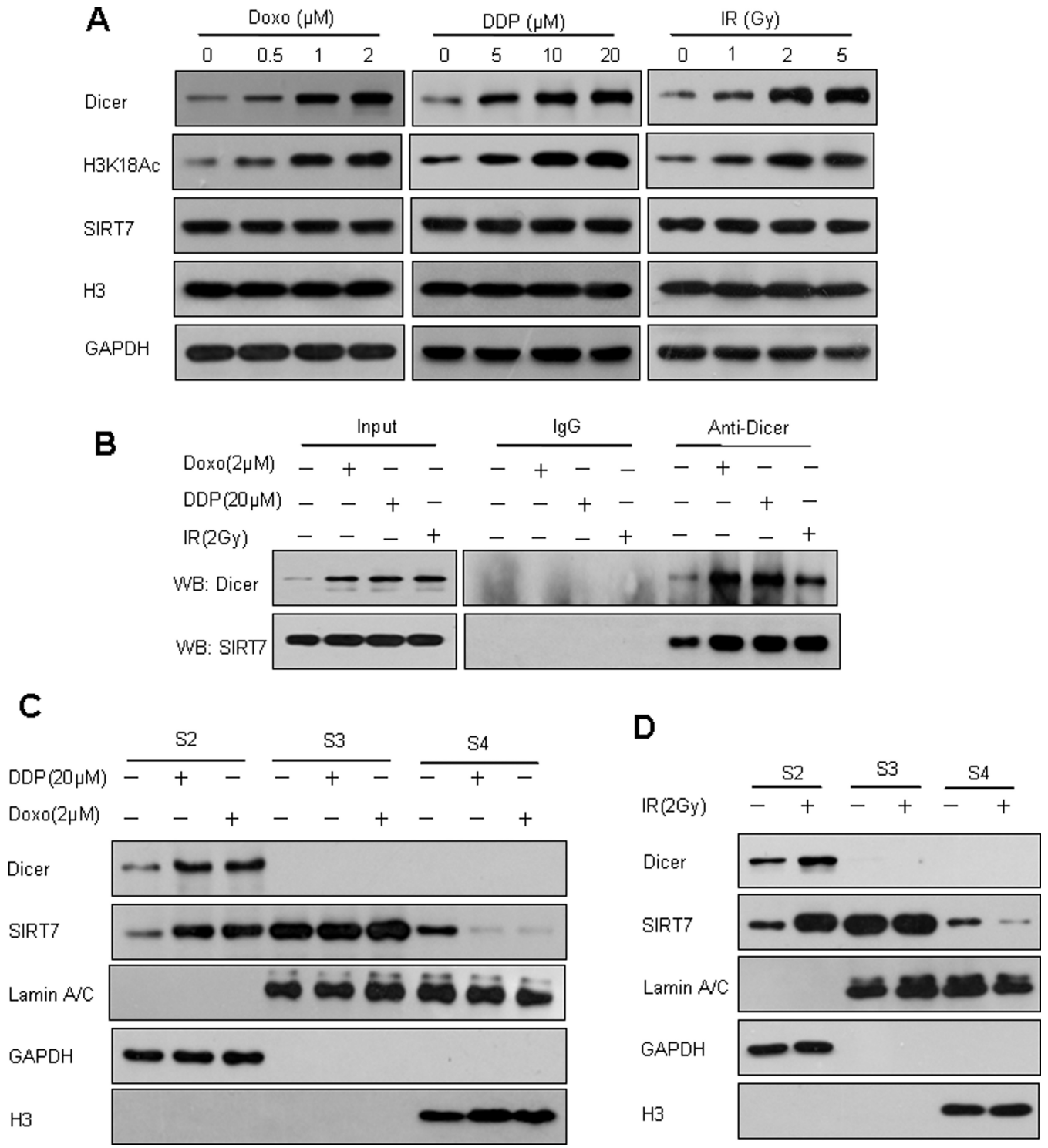

Figure 4. DNA damaging agents upregulate Dicer, causing a decrease of chromatin-associated SIRT7 and an increase of H3K18Ac in HEK293T cells. (**A**) Representative western blot images of Dicer, H3K18Ac, SIRT7 and histone H3 in cells treated with different doses of DNA damaging agents. (**B**) Co-IP of Dicer and SIRT7 using excessive amount of anti-Dicer antibody in DNA damaging treated cells. (**C** and **D**) Decreased level of chromatin-associated SIRT7 and increased level of cytoplasmic SIRT7 in DDP- or doxo-treated cells (C) or IR-treated cells (D), revealed by biochemical fractionation. S2, S3 and S4 represent the cytoplasmic, the nucleoplasmic and the chromatin-associated fractions, respectively.

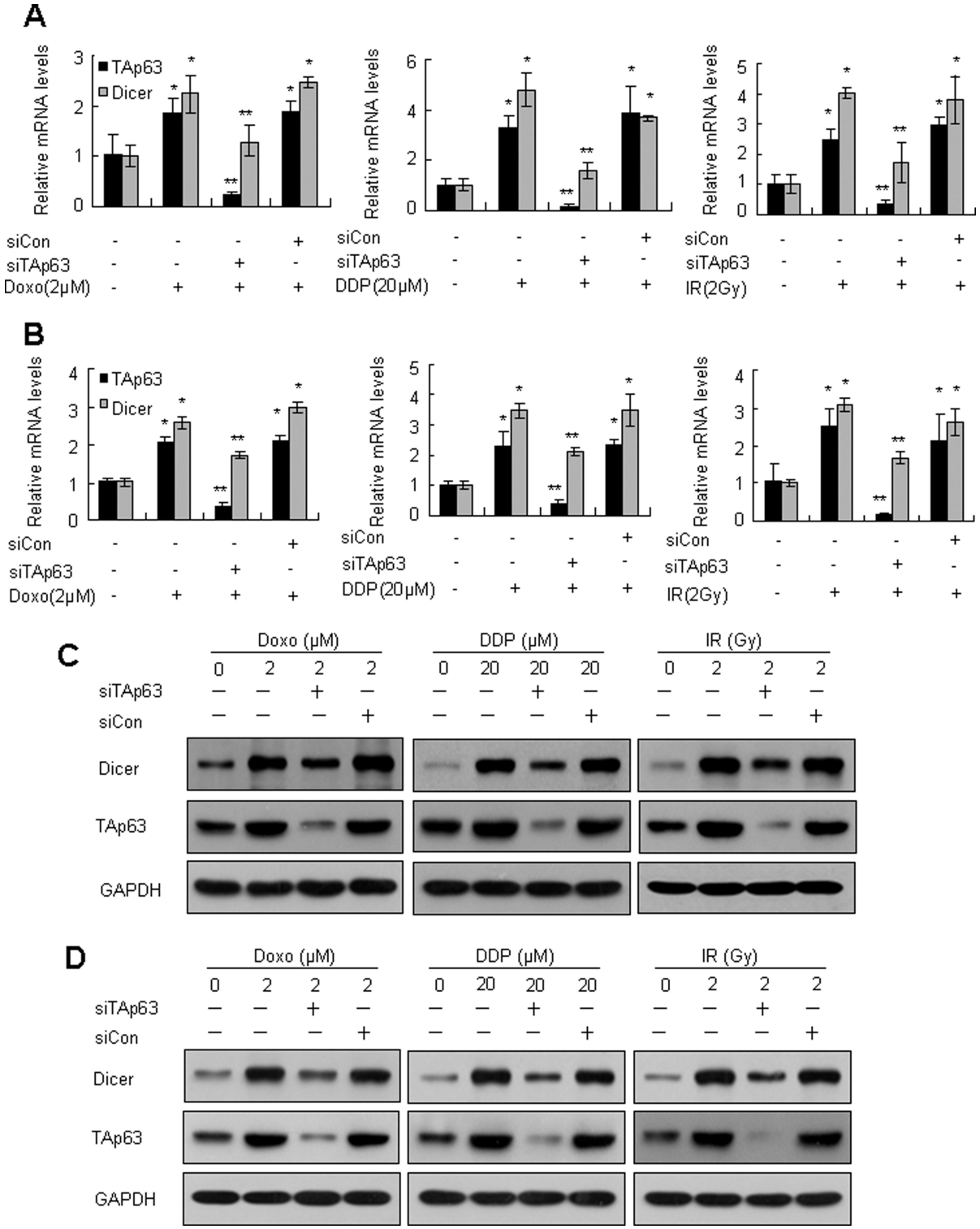

Figure 5. TAp63 knockdown prevents DNA damaging agent-induced Dicer upregulation. (**A** and **B**) Expression of TAp63 and Dicer detected by real-time RT-PCR in HEK293T (A) or HCT116 (B) cells. Data represent means ± S.E.M. from three independent experiments. * Compared to cells neither exposed to DNA damaging agents nor transfected with siRNAs, $P < 0.05$; ** Compared to cells exposed to DNA damaging agents but not transfected with siRNAs, $P < 0.05$. (**C** and **D**) Expression of Dicer and TAp63 determined by western blot in HEK293T (C) or HCT116 (D) cells.

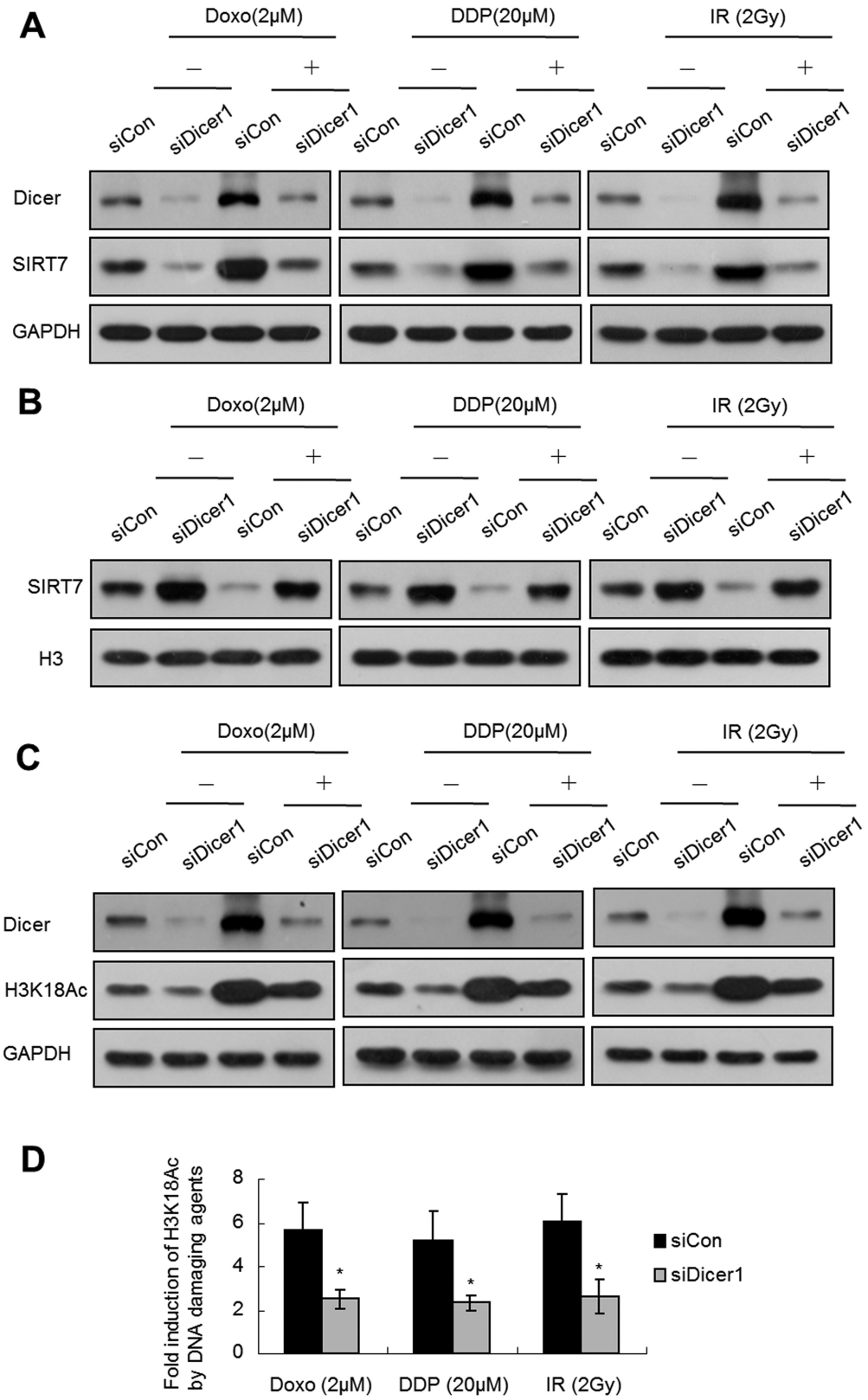

Figure 6. Dicer knockdown prevents the reduction of chromatin-associated SIRT7 and the increase of H3K18Ac in DNA damaging treated HEK293T cells. (**A**) Dicer knockdown blocked the increase of SIRT7 in the cytoplasmic fraction of DNA damaging treated cells. (**B**) Dicer knockdown prevented the reduction of SIRT7 in the chromatin-associated fraction of DNA damaging treated cells. (**C**) Dicer knockdown partially prevented the increase of H3K18Ac in DNA damaging treated HEK293T cells. (**D**) Quantification of H3K18Ac levels in (C). Fold induction of H3K18Ac by DNA damaging agents = the level of H3K18Ac in DNA damaging treated cells/the level of H3K18Ac in non-DNA damaging treated cells. * $P < 0.05$.

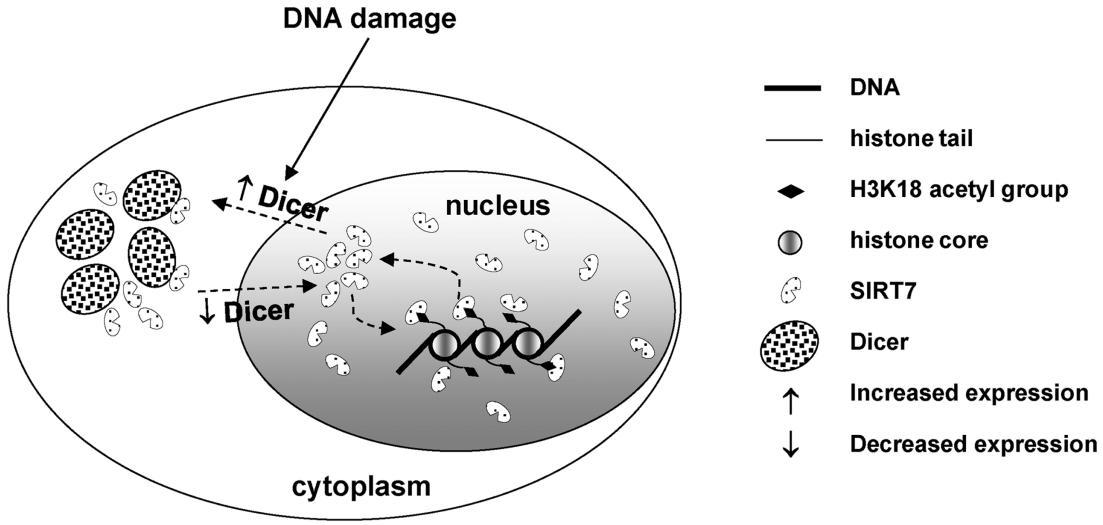

Figure 7. A schematic model depicting how DNA damaging agents prevent deacetylation of H3K18Ac via upregulating Dicer expression.

is much more complex than expected and may be context specific.

The subcellular localization of SIRT7 seems controversial. Most studies indicate that SIRT7 is predominantly distributed in the nucleolus (14,30,31). Using subcellular fractionation assay, Kiran *et al.* showed that SIRT protein is present both in the nucleus and the cytoplasm, and the cytoplasmic SIRT7 is 2.5 kDa larger than the nuclear one (33). However, their immunocytochemistry results revealed that the cytoplasmic localization of SIRT7 is present only in primary fibroblasts but not in epithelial cells (33). In this study, we revealed that SIRT7 is present not only in the nuclear fractions, but also in the cytoplasmic fraction, and that the SIRT7 protein exhibited the same molecular weight in different cellular fractions. In addition, we found that the cytoplasmic staining of SIRT7 is observed not only in fibroblasts, but also in epithelial cells (Figure 2; Supplementary Figures S2 and S3). The reason for the discrepancy is unclear. Although the molecular functions of SIRT7 in the cytoplasm remain unknown, there is sufficient evidence against that SIRT7 is exclusively localized in the nucleolus: First, SIRT7 physically associates and colocalizes with Dicer in the cytoplasm, and Dicer–SIRT7 interaction is further supported by two SIRT7 interactome data (23,43). In addition, these SIRT7 interactome data reveal that SIRT7 interacts with dozens of proteins that are exclusively localized in the cytoplasm (23,43). These findings support that SIRT7 resides in the cytoplasm. Second, ChIP-sequencing data indicate that SIRT7 binds to the promoters of protein-coding genes that mostly localize outside the nucleolus (18), suggesting that SIRT7 is not confined to the nucleolus. Third, biochemical fractionation and immunofluorescence experiments using different antibodies indicate that SIRT7 resides both in the cytoplasm and in the nucleus, and the specificity of these antibodies was validated in SIRT7 knockdown cells (Supplementary Figure S2).

Due to its pleiotropy, the role of Dicer in cellular transformation and tumorigenesis remains controversial. Dicer has been reported as a haploinsufficient tumor suppressor (44,45). However, Sekine *et al.* found that knockout of Dicer in hepatocytes promotes hepatocarcinogenesis (46). Kumar *et al.* reported that Dicer knockdown promotes tumor cell proliferation, and enhances cellular transformation and tumorigenesis (47), while other groups demonstrated that Dicer knockdown suppresses the growth and tumorigenic capacity of different cancer cell lines (48,49). We demonstrated here that Dicer knockdown in HCT116 cells impaired cell growth and colony formation (Supplementary Figure S9A and B). Surprisingly, Dicer overexpression also repressed cell proliferation and colony formation (Supplementary Figure S9C and D). SIRT7-linked H3K18 deacetylation is essential for oncogenic transformation, and SIRT7 knockdown impaired the proliferation of HT1080 and U2OS cells (18). Further investigations are needed to address whether the effect of Dicer overexpression on cell growth and colony formation would be partially attributed to the reduction of chromatin-associated SIRT7 and hence hypoacetylation of H3K18Ac. In summary, our study suggest that Dicer overexpression may in part contribute to the growth inhibitory effect of DNA damaging agents, which may have implication in cancer chemotherapy and radiotherapy.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENT

We wish to thank Bin Tan (Chongqing Medical University, Chongqing, China), Xiao Chen, Guiqiang Yang and Yixiang Han (Wenzhou Medical University, Wenzhou, China) for technical assistance, and Quan Wang (Third Military Medical University, Chongqing, China) for providing MEF cells.

FUNDING

National Sciences Foundation of China [81171967, 31271383, 81572780 to K.F.T.; 31200976 to G.L.]; Na-

tional Major Special Science and Technology Project [2013ZX10002002 to K.F.T.]; Zhejiang Provincial National Sciences Foundation [LZ16H160004 to K.F.T.]. Funding for open access charge: National Sciences Foundation of China [81171967, 31271383, 81572780 to K.F.T.; 31200976 to G.L.]; National Major Special Science and Technology Project [2013ZX10002002 to K.F.T.]; Zhejiang Provincial National Sciences Foundation [LZ16H160004 to K.F.T.].
Conflict of interest statement. None declared.

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