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著者	Hayashi Naoyuki, Kobayashi Masahiko, Shamma Awad, Morimura Yoko, Takahashi Chiaki, Yamamoto Ken-ichi
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Regulatory interaction between NBS1 and DNMT1 responding to DNA damage.

Naoyuki Hayashi^{1,2,3}, Masahiko Kobayashi¹, Awad Shamma², Yoko Morimura¹, Chiaki Takahashi², Ken-ichi Yamamoto¹

¹Division of Molecular Pathology, and ²Division of Oncology and Molecular Biology, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa 920-1192, Japan

³To whom correspondence should be addressed.

Division of Oncology and Molecular Biology, Cancer Research Institute,

Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan

Phone: 81-76-264-6751 Fax: 81-76-234-4521

E-mail: naohaya@staff.kanazawa-u.ac.jp

Running Title: Regulatory interaction between NBS1 and DNMT1

SUMMARY

NBS1 is the causative gene product of Nijmegen breakage syndrome (NBS), a recessive genetic disorder resulting in chromosomal instability and immunodeficiency. We isolated *DNMT1* cDNA by 2-hybrid screening by using NBS1 as bait to study its function in DNA replication and damage checkpoint. *DNMT1* encodes DNA methyltransferase 1, which maintains the genomic methylation pattern and also regulates the checkpoint pathway via interactions with various factors such as CHK1, p53, Rb, and ATM. The interaction between NBS1 and DNMT1 was observed under conditions of hydroxyl urea treatment, resulting in replication stall, and mitomycin C treatment, resulting in DNA damage. Additionally, we mapped their binding regions to the N-terminus of NBS1 (including the FHA domain) and amino acids 1401–1503 in the target recognition domain in the C-terminus of DNMT1. Under DNA replication stall conditions, DNMT1 was recruited to the *survivin* promoter by p53, and it repressed *survivin* expression via heterochromatin formation; this regulation was dependent on the *NBS1* genotype. These results suggest that DNMT1 function in the regulatory response is controlled by NBS1.

Key words: DNA methylation, DNMT1, heterochromatin, NBS1, *survivin*

NBS1, the causative gene of Nijmegen breakage syndrome (NBS), plays a crucial role in DNA damage repair and checkpoint signal transduction of checkpoint via interactions with various factors, including the Mre11/Rad50 complex, and ataxia telangiectasia-mutated (ATM) (1, 2, 3). The protein contains a forkhead-associated (FHA) domain and 2 breast cancer carboxy-terminal (BRCT) domains in its N-terminus an ATM checkpoint kinase phosphorylation site in its middle region, and regions for ATM and Mre11 binding in its C-terminus (2, 4). Thus, NBS1 contains domains for interactions with various factors, which function in checkpoint control or DNA damage repair, such as the MRN complex, consisting of Mre11, Rad50, and NBS1, which plays a major role in repair of genomic DNA double-stranded breaks. The MRN complex localizes to the sites of DNA damage, and recruits ATM kinase, a member of the PI3 kinase family, which phosphorylates p53, CHK1, CHK2, BRCA1, histone H2AX, and NBS1. The FHA and BRCT domains in the N-terminus are often found in proteins that regulate the checkpoint control system and are required for localization of γ -H2AX to DNA damage foci (3, 5). Thus, NBS1 functions as a trigger factor signaling for DNA damage repair, checkpoint control, and/or apoptosis. Furthermore, the MRN complex

colocalizes with proliferating cell nuclear antigen (PCNA) at replication forks throughout the S phase (6). Therefore, NBS1 plays an important role in genome stability via its regulatory interactions (4).

PCNA binds with DNA methyltransferase 1 (DNMT1), which primarily maintains heritable DNA methylation following DNA replication to preserve genomic methylation patterns (7, 8). In the replication machinery, the PCNA/DNMT1 complex is the target of p21 for the checkpoint response (7). DNMT1 interacts with factors responding to DNA damage, such as p53 (9) and CHK1 (10), and DNMT1 itself is recruited to the DNA damage sites (11). Furthermore, it interacts with G9a, which methylates the ninth lysine of histone H3 (K9 histone H3), and binds to HP1 β , which constitutes part of the heterochromatin (12, 13). Methylation of DNA and modification of histones are critical for heterochromatin formation. Thus, DNMT1 not only methylates DNA but also mediates heterochromatin formation to silence genes.

Under DNA stress conditions, such as replication stall, base-pair mismatch and strand breakage, DNMT1, p53, and HDAC are recruited to the promoter region of the *survivin* gene, and they increased methylation levels of both DNA and K9 histone H3 on

the *survivin* promoter to repress its expression (9). Thus, DNMT1 also functions in regulatory formation of heterochromatin to repress specific genes. Furthermore, DNMT1 is indispensable to the checkpoint system for replication stall and DNA damage because truncation of genomic *DNMT1* results in loss of the checkpoint response, while depletion of *DNMT1* by RNA interference cause activation of the checkpoint signaling (14). Therefore, DNMT1 is not only involved in maintenance of methylation patterns and heterochromatin formation responding in response to the DNA damage but is also indispensable for replication checkpoint to maintain the genome integrity.

In the present study, we demonstrated specific binding of NBS1 and DNMT1, and further, we found that this interaction is essential for DNA methylation and subsequent heterochromatin formation mediated by DNMT1.

MATERIALS AND METHODS

Plasmids. Construction of the plasmids and the PCR primers used for deletion derivatives of DNMT1 and NBS1 are shown in Table S1 and S2, respectively.

Cell culture and reagents. 293T cells were cultured in Dulbecco's modified Eagle

medium (Nissui, Japan) supplemented with 15% fetal calf serum, 2mM L-glutamine, 50 $\mu\text{g}/\text{mL}$ penicillin and 50 units/mL streptomycin. NBS patient cell line GM07166 (NBS1^{-/-}) and its NBS1-recovered line (NBS1⁺) were cultured in Dulbecco's modified Eagle medium (high glucose; Wako Japan) supplemented with 10% fetal calf serum, 2mM L-glutamine, 50 $\mu\text{g}/\text{mL}$ penicillin and 50 units/mL streptomycin (15). Treatment with hydroxyl urea (HU; Wako, Japan), mitomycin C (Kyowa Hakko Kogyo, Japan), and aphidicolin (Sigma-Aldrich, USA) were performed with final concentrations of 20 mM, 2 $\mu\text{g}/\text{mL}$, and 40 μM in each medium, respectively, and cells were harvested after incubation for 2 h.

Antibodies. Antibodies against HA and Flag tags, 3F10 and M5, respectively, were purchased from Roche. Antibodies against NBS1, DNMT1, and dimethylated K9 histon H3 were purchased from BD Biosciences Pharmingen, New England Bio Labs, and Monoclonal Antibody Institute, respectively. Immunoblotting was performed as previously described (16).

Two-hybrid screening. The plasmid pAS-NBS1, with a 2.4-kb *NcoI* fragment contained the *NBS1* coding region, was derived from pEFBOS3HA-NBS1 (15), an NBS1

expression plasmid in mammalian cell line. The screening was carried out with yeast strain PJ69-4A (17) as described previously (18)

Glutathione S-transferase (GST) pull-down and Immunoprecipitation (IP). GST-fused NBS1 protein produced in *Escherichia coli* strain BL21 (DE3) was prepared with glutathione Sepharose beads as previously described (19). HA-tagged DNMT1, derived from cDNA isolated in the 2-hybrid screening, was expressed in 293T cells. Crude extract from 293T cells containing HA-tagged DNMT1 was prepared in lysis solution (50 mM Tris HCl, pH7.5; 100 mM NaCl; 0.2% NP40; 0.5% Tween20) and then was mixed with beads bound to either GST-NBS1 fused protein or GST alone. These were incubated for 1 h on ice and then washed 4 times with the wash solution (2.5 mM Tris HCl, pH7.5; 100 mM NaCl; 10% glycerol; 1 mM EDTA; 0.01% NP40).

IP assay for endogenous proteins was performed with the antibodies described above. IgG2 (Immunotech, Czech) was used for negative control of IP experiments using the aliquot of crude extract prepared from the intact human 293T cells. Binding experiments for mapping of the binding regions were performed with anti-Flag antibody M5 and GammaBind Plus Sepharose beads (GE Healthcare, USA) in human 293T cells,

which were co-transfected with plasmids to produce HA-tagged DNMT1 and Flag-tagged NBS1 deletion derivatives. The proteins produced, preparation of crude extracts, and washing of the precipitated beads are described above.

Methylation-sensitive PCR (MSP) assay. Two micrograms of genomic DNA, which was prepared from each cell line cultured in a 150-mm dish by using the AquaPure Genomic DNA Isolation kit (Bio-Rad, USA), was treated to modify unmethylated cytosines by bisulfite treatment using the EZ DNA Methylation Kit (Zymo Research, USA). MSP assay was performed with primers specific to the bisulfite modified sequence or the unmodified sequence and Takara Taq Hot Start Version (Takara Bio, Japan). The sequences used in the MSP assay are shown in Table S3.

RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, USA). The isolated RNA was treated with RNase-free DNase. For RT-PCR, cDNA was prepared from 2 µg total RNA by using oligo dT primers and PrimeScript RT-PCR kit (Takara Bio, Japan). For PCR amplification of the *survivin* cDNA, forward and reverse primers used were 5'-TCAATCCATGGCAGCCAG-3' and 5'-TTTCTCAAGGACCACCGC-3', respectively.

Chromatin IP (ChIP) Assay. The ChIP experiment was performed as described previously (9). Soluble chromatin samples prepared with lysis buffer (50 mM Tris HCl, pH 8.0; 1% SDS; 10 mM EDTA; 1 mM PMSF) from the GM07166 (NBS1^{-/-}) cell line derived from an NBS patient and its NBS1-restored cell line (NBS1⁺), which were treated with or without HU and cross-linked by using 1% formaldehyde for 10 min at 37°C, were sonicated for 15 s twice to shear DNA to lengths between 200 and 1,000 bp. Half of each supernatant was diluted 10-fold by using the ChIP dilution buffer (15 mM Tris HCl, pH 8.0; 167 mM NaCl; 0.01% SDS; 1% Tween20; 1 mM EDTA; 1 mM PMSF) and then immunoprecipitated, and the other half was used for detection of the PCR “input” control. DNA/protein complexes were eluted from the beads with a solution containing 1% SDS and 0.1 M NaHCO₃. Then NaCl was added to final concentration of 0.2 M, and they were incubated for 6 h at 65°C to reverse the cross-links. Proteinase K (Qiagen, Germany) was added for 1 h at 45°C, and the DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Immunoprecipitated DNA was analyzed for the presence of the *survivin* promoter sequence by PCR using the specific primer pairs, Survivin-pro1 and Survivin-pro 3 for the proximal regulatory region (Table S3), and PCR

products were then visualized on 2% agarose/TAE gels. Each experiment was repeated 3 times, and band intensities were measured with NIH Image 1.63. Intensities were calculated to compare with the ChIP result for each antibody of the intact NBS1-restored cell line.

RESULTS

DNMT1 interacts with NBS1. To study the functions of NBS1 in DNA replication, we isolated its binding factors by 2-hybrid screening with approximately 3×10^5 transformants. We chose *DNMT1* cDNA to investigate the DNA replication checkpoint control from the following candidates: DNMT1, INT4, TPP1, KPNA2, KF-1, c-NAP1 and MRPL13. The isolated *DNMT1* cDNA encoded the C-terminal portion (from 1082 amino acids [aa] to the stop codon), including the catalytic domain. To examine the binding of NBS1 and DNMT1 in vitro, glutathione beads bound with GST-NBS1 proteins produced in *E. coli* strain BL21 (DE3) were prepared and were mixed with the 293T cellular extract, in which the HA-DNMT1 C-terminus isolated in the 2-hybrid screening was expressed. In precipitates of the glutathione beads from this

mixture, we detected HA-DNMT1 (Fig. 1A), confirming the results of the yeast 2-hybrid assay. We then examined the *in vivo* binding of endogenous DNMT1 to NBS1 proteins under DNA replication stall conditions with HU treatment to investigate functional aspects of the interaction *in vivo*. HU reduces the deoxynucleotide pool in the cells due to its inhibition of ribonucleotide reductase. In this assay, NBS1 was co-precipitated with an anti-DNMT1 antibody under HU treatment (Fig. 1B), and DNMT1 was also precipitated using an anti-NBS1 antibody (Fig. S1). Thus, NBS1 was bound with DNMT1 under replication stall conditions *in vivo*. To examine the DNMT1/NBS1 interaction under other DNA stresses, treatment with mitomycin C treatment a potent DNA crosslinker resulting in DNA double strand breaks, and aphidicolin which inhibits DNA polymerase directly, were also investigated (Fig. 1C). Upon mitomycin C treatment, the DNMT1/NBS1 interaction was induced in human 293T cells. This interaction, however, was not observed upon aphidicolin treatment. Direct inhibition of DNA polymerase by aphidicolin would affect the functional status of DNMT1, which binds with PCNA in the replication complex.

To map the NBS1 binding region in DNMT1, we constructed deletion

derivatives of Flag-tagged NBS1 and HA-tagged DNMT1 (Fig. 2A), and these were expressed in human 293T cells. The binding abilities of these deletion constructs expressed in 293T cells were examined by an IP assay. We found that the N-terminal half of NBS1 (NBS1-N) binds to HA-tagged DNMT1 (Fig. 2B). NBS1-N was then used for the deletion mapping experiment owing to its more stable expression. In the mapping experiments, the deletion derivatives D3 (1397–1632 aa) and D4 (1397–1536 aa) bound to NBS1, but the deletion derivatives D2 (1082–1400 aa) and D5 (1504–1632 aa) did not; this observation indicates that the essential region of DNMT1 required for the interaction with NBS1 is in the region 1401–1503 aa (Fig. 2C). This region was included in the target recognition domain (TRD) of the DNMT1 catalytic region, which regulates binding specificity (19, 20). Additionally, the binding region in NBS1 to DNMT1 was mapped. The shortest region of NBS1 (1-115 and 178-222 aa) containing an intact FHA domain bound to DNMT1, but the deletion derivative BR1, which contained partial FHA and complete BRCT1 (46–222 aa), did not bind to DNMT1 (Fig. 3). Thus, our deletion analyses suggested that the FHA domain in NBS1 is required for the interaction with TRD in DNMT1.

NBS1 is required for regulation of heterochromatin formation. To investigate the functional relationship between NBS1 and DNMT1, we examined DNA methylation, heterochromatin formation, and binding of DNMT1 at the regulatory region of the *survivin* promoter in NBS patient cells compared to NBS1-recovered cells. The methylation status of DNA in this regulatory region was compared after inhibition of DNA replication by HU treatment. The prepared samples of genomic DNA were first cleaved with the restriction enzyme *HpaII* (methylation sensitive) or *MspI* (methylation insensitive), and then the regulatory region then amplified by PCR technique (Fig. 4A). A strong signal was detected in the sample amplified from genomic DNA digested with *HpaII* prepared from cells expressing NBS1 after HU treatment, but no signal was detected from NBS1-deficient cells. We then examined DNA methylation at the regulatory region by bisulfite method. Genomic DNA samples were subjected to bisulfite modification to change unmethylated cytosines to uracil, and a methylation sensitive PCR assay was performed with methylated- or unmethylated- specific primer sets (Fig. 4B). In the NBS patient cell line GM07166 (*NBS1*^{-/-}), *survivin* cDNA derived by RT-PCR was constitutively observed, whereas it was not observed in the

NBS1-recovered cell line (NBS1⁺) treated with HU (Fig. 4C). These observations of transcriptional expression of the *survivin* gene were consistent with the results of DNA methylation at the *survivin* promoter. Thus, regulatory methylation at the *survivin* promoter to repress its transcription was dependent on NBS1 function.

We also performed the ChIP assay by using antibodies to DNMT1 and dimethylated K9 histone H3 to investigate the relationship of DNMT1 recruitment and heterochromatin formation at the *survivin* promoter on NBS1 status, as DNMT1 is recruited by p53 to the *survivin* promoter in response to DNA stress (9). With HU treatment, levels of dimethylated K9 histone H3 on the *survivin* promoter were not increased in NBS patient cells, but the control cells, which had recovered NBS1, showed a significant increase in methylation levels of K9 histone H3 after HU treatment (Fig. 5A and B). This finding indicates that heterochromatin formation at the *survivin* promoter responding to replication stall requires NBS1 function. We also found that binding of DNMT1 to the *survivin* promoter in NBS patient cell lines was significantly reduced, although this binding ability was increased under replication inhibition conditions with HU in the NBS1-recovered cell line (Fig. 5A and C). These observations indicate that

heterochromatin formation responding to the replication checkpoint at the *survivin* promoter was consistent with the pattern of DNMT1 binding. However, DNMT1 was not recruited to the *survivin* promoter under conditions of replication stall in the presence of the Mre11 inhibitor, mirin (Fig. S2). Mirin blocked the exonuclease activity of Mre11, which was required for restoration of the stalled replication fork (22), as well as MRN-dependent ATM activation (3, 23). This result indicated that DNMT1 would function under the ATM pathway, together with our observation of DNMT1/NBS1 binding under mitomycin C treatment, which could give rise to double strand breaks (Fig. 1C). These observations thus suggested that the MRN complex function is necessary for recruiting DNMT1 to the regulatory region of the *survivin* promoter and subsequent heterochromatin formation to repress its transcription responding to replication and DNA damage checkpoint. However, constitutive binding by p53 to the *survivin* promoter was observed in NBS patient cell (Fig. 5A and D). This suggested that p53 was under DNA stress conditions, but it could not recruit DNMT1 without complete NBS1 function to regulate *survivin* gene expression. Thus p53 plays a primary role to recruit DNMT1 depending on NBS1.

DISCUSSION

The multifunctional checkpoint regulator, NBS1, has diverse functions such as double strand break repair, activation of ATM family kinases, and telomere replication (4, 24).

However, these diverse functions are co-operatively function in response to various DNA stressors such as replication stall, DNA strand lesion, and nucleotide mismatch. The observation interaction between DNMT1 and NBS1 is also likely to have an important role in the DNA stress response.

Interaction domains for NBS1/DNMT1 binding. The binding region for NBS1 in DNMT1 was mapped to the TRD in the C-terminus, which recognizes hemimethylated cytosine (25). This mapping result suggests that NBS1 directly alters DNMT1 specificity to target sites of methylation. NBS1, which bound to the TRD of DNMT1, was involved in p53-specific function of heterochromatin formation at the *survivin* promoter, as it was not observed in NBS1-deficient cells (Fig. 5). The observation that the p53-binding region in DNMT1 partially overlaps with TRD (9) might also support this notion. Another possibility is that NBS1 alters the substrate specificity of DNMT1 to form

heterochromatin in the vicinity of DNA damage sites. DNMT1 localizes to the damaged region and interacts with the DNA repair machinery (10, 26). It may be consistent with the mapping result for DNMT1 binding in NBS1, suggesting their functional role in the checkpoint system because the FHA domain in NBS1 is essential for the checkpoint response (27).

Function of DNMT1 in checkpoint system. DNMT1 is the key enzyme required for the completion of the DNA replication and also for the checkpoint response to DNA damage or DNA replication stall (10, 14, 28, 29). Our findings of the interaction between the TRD in DNMT1 and FHA domain of NBS1 suggest a functional relationship between DNA methylation and the checkpoint response because their interaction was induced in replication stall conditions in vivo (Fig. 1B). To investigate the functional role of their interaction in DNA methylation and gene silencing under checkpoint conditions, we focused on the *survivin* promoter, which has typical CpG sites and a p53 binding region for repression (9, 30). DNMT1 binds at the region containing the CpG sites and the p53 binding region under conditions of DNA damage, and it methylates these CpG sites to repress the expression of the *survivin* gene (9). DNA methylation and DNMT1 binding to

the *survivin* promoter were comparable, and methylation of K9 histone H3, which results in heterochromatin formation, was observed with the same pattern. Moreover, these observations were dependent on the *NBS1* genotype. The DNMT1/*NBS1* interaction responded to the DNA damage agent, mitomycin C (Fig. 1C); moreover, their binding and translocation to the *survivin* promoter of DNMT1 dependent on *NBS1* were inhibited by treatment with mirin, which would block the ATM pathway (Fig. S2). Recently, we found an interaction between ATM and DNMT1 (31), which was consistent with our observations. Thus, DNMT1 likely functions in general DNA stress mediated by the MRN complex and ATM (Fig. S3).

Our findings suggest that *NBS1* function is not only essential in the checkpoint system but also plays essential role in regulation of heterochromatin status via DNMT1 function.

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REFERENCES

1. Kobayashi, J., Antocchia, A., Tauchi, H., Matsuura, S., Komatsu, K. (2004) NBS1 and its functional role in the DNA damage response. *DNA Repair* **3**, 855-861
2. Zhang, Y., Zhou, J., Lim, C. (2006) The role of NBS1 in DNA double strand break repair, telomere stability, and cell cycle checkpoint control. *Cell Res.* **16**, 45-54
3. Lee, J.-H., Mand, M.R., Deshpande, R.A., Kinoshita, E., Yang, S.-H., Wyman, C., Paull, T.T. (2013) Ataxia telangiectasia-mutated (ATM) kinase activity is regulated by ATP-driven conformational changes in the Mre11/Rad50/Nbs1 (MRN) complex. *J. Biol. Chem.* **288**, 12840-12851
4. Stracker, T.H., Petrini, J.H. (2011) The MRE11 complex: starting from the ends. *Nat. Rev. Mol. Cell Biol.* **12**, 90-103
5. Kobayashi, J. (2004) Molecular mechanism of the recruitment of NBS1/hMRE11/hRAD50 complex to DNA double-strand breaks: NBS1 binds to gamma-H2AX through FHA/BRCT domain. *J. Radiat. Res.* **45**, 473-478

6. Maser, R.S., Mirzoeav, O.K., Well, J., Olivares, H., Williams, B.R., Zinkel, R.A., Famham, P.J., Petrini, J.H. (2001) Mre11 complex and DNA replication: linkage to E2F and sites of DNA synthesis. *Mol. Cell. Biol.* **21**, 6006-6016

7. Chuang, L.S.-H., Ian, H.-I., Koh, T.-W., Ng, H.-H., Xu, G., Li, B.F.L. (1997) Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* **277**, 1996-2000

8. Iida, T., Suetake, I., Tajima, S., Morioka, H., Ohta, S., Obuse, C., Tsurimoto, T. (2002) PCNA clamp facilitates action of DNA cytosine methyltransferase 1 on hemimethylated DNA. *Genes Cells* **7**, 997-1007

9. Esteve, P.-O., Chin, H.G., Pradhan, S. (2005) Human maintenance DNA (cytosine-5)-methyltransferase and p53 modulate expression of p53-repressed promoters. *Proc. Natl. Acad. Sci. USA* **102**, 1000-1005

10. Palii, S.S., Van Emburgh, B.O., Sankpal, U.T., Brown, K.D., Robertson, K.D. (2008) DNA methylation inhibitor 5-aza-2'-deoxycytidine induces reversible genome-wide

- DNA damage that is distinctly influenced by DNA methyltransferase 1 and 3B. *Mol. Cell. Biol.* **28**, 752-771
11. Mortusewicz, O., Schermelleh, L., Walter, J., Cardoso, M.C., Leonhardt, H. (2005) Recruitment of DNA methyltransferase I to DNA repair sites. *Proc. Natl. Acad. Sci. USA* **102**, 8905-8909
12. Esteve, P.-O., Chin, H.G., Smallwood, A., Feehery, G.R., Gangiselt, O., Karpf, A.R., Carey, M.F., Pradhan, S. (2006) Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. *Genes Dev* **20**, 3089-3103
13. Smallwood, A., Esteve, P.-O., Pradhan, S., Carey, M. (2007) Functional cooperation between HP1 and DNMT1 mediates gene silencing. *Genes Dev.* **21**, 1169-1178
14. Brown, K.D., Robertson, K.D. (2007) DNMT1 knockout delivers a strong blow to genome stability and cell viability. *Nat. Genet.* **39**, 289-290
15. Kobayashi, M., Hayashi, N., Takata, M., Yamamoto, K. (2013) NBS1 directly activates ATR independently of MRE11 and TOPBP1. *Genes Cells* **18**, 238-246

16. Kobayashi, M., Ono, H., Mihara, K., Tauchi, H., Komatsu, K., Shibata, T., Shimizu, H., Uchida, K., Yamamoto, K. (2006) ATM activation by a sulfhydryl-reactive inflammatory cyclopentenone prostaglandin. *Genes Cells* **11**, 779-789.
17. James, P., Halladay, J., Craig, E.A. (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**, 1425-1436
18. Yokoyama, N., Hayashi, N., Seki, T., Pante, N., Ohba, T., Nishii, K., Kuma, K., Hayashida, T., Miyata, T., Aebi, U., Fukui, M., Nishimoto, T. (1995) A giant nucleopore protein that binds Ran/TC4. *Nature* **376**, 184-188
19. Van Hoy, M., Leuther, K.K., Kodadek, T., Johnston, S.A. (1993) The acidic activation domains of the GCN4 and GAL4 proteins are not α helical but form β sheets. *Cell* **72**, 587-594
20. Godley, L.A., Mondragon, A. (2011) Preference by exclusion. *Science* **331**, 1017-1018

21. Song, J., Rechkoblit, O., Bester, T.H., Patel, D.J. (2011) Structure of DNMT1-DNA complex reveals a role for autoinhibition in maintenance DNA methylation. *Science* **331**, 1036-1040
22. Ying, S., Hamdy F.C., Helleday, T. (2012) Mre11-dependent degradation of stalled DNA replication forks is prevented by BRCA2 and PARP1. *Cancer Res.* **72**, 2814-2821
23. Dupré A., Boyer-Chatenet L., Sattler R.M., Modi A.P., Lee J.H., Nicolette M.L., Kopelovich L., Jasin M., Baer R., Paull T.T., Gautier J. (2008) A forward chemical genetic screen reveals an inhibitor of the Mre11-Rad50-Nbs1 complex. *Nat. Chem. Biol.* **4**, 119–125
24. Lamarche, B.J., Orazio, N.I., Weizman, M.D. (2010) The MRN complex in double-strand break repair and telomere maintenance. *FEBS lett.* **584**, 3682-3695
25. Qin, W., Leonhardt, H., Pichler, G. (2011) Regulation of DNA methyltransferase 1 by interactions and modifications. *Nucleus* **2**, 392-402

26. Ha, K., Lee, G.E., Palii, S.S., Brown, K.D., Takeda, Y., Liu, K., Bhalla, K.N., Robertson, K.D. (2011) Rapid and transient recruitment of DNMT1 to DNA double-strand breaks is mediated by its interaction with multiple components of the DNA damage response machinery. *Hum. Mol. Genet.* **20**, 126-140
27. Difilippantonio, S., Celeste, A., Kruhlak, M.J., Lee, Y., Difilippantonio, M.J., Feigenbaum, L., Jackson, S.P., McKinnon, P.J., Nussenzweig, A. (2007) Distinct domains in Nbs1 regulate irradiation-induced checkpoints and apoptosis. *J. Exp. Med.* **204**, 1003-1011
28. Chen, T., Hevi, S., Gay, F., Tsujimoto, N., He, T., Zhang, B., Ueda, Y., Li, E. (2007) Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. *Nat. Genet.* **39**, 391-396
29. Unterberger, A., Andrews, S.D., Weaver, I.C., Szyf, M. (2006) DNA methyltransferase 1 knockdown activates a replication stress checkpoint. *Mol. Cell. Biol.* **26**, 7575-7586

30. Hoffman, W.H., Biade, S., Zilfou, J.T., Chen, J., Murphy, M. (2002) Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J. Biol. Chem.* **277**, 3247-3257
31. Shamma, A., Suzuki, M., Hayashi, N., Kobayashi, M., Sasaki, N., Nishiuchi, T., Doki, Y., Okamoto, T., Kohno, S., Muranaka, H., Kitajima, S., Yamamoto, K., Takahashi, C. (2013) ATM mediates pRB function to control DNMT1 protein stability and DNA methylation. *Mol. Cell. Biol.* in press.

FIGURE LEGEND

Figure 1. **Binding assays with NBS1 and DNMT1.** (A) In vitro binding of GST-NBS1 and Flag-DNMT1. Crude extract was prepared from human 293T cells transfected with the plasmid, which expressed Flag-tagged DNMT1 derived from cDNA isolated in the 2-hybrid screening, and it was used for precipitation assays with GST alone or GST-NBS1 produced in bacterial cells. (B) In vivo binding of endogenous NBS1 and DNMT1 in human 293T cells. An inhibitor of ribonucleotide reductase, HU, was added to generate replication stall. Immunoprecipitation assay was performed using an anti-DNMT1 antibody or mouse IgG2 as negative control, and immunoblot analysis for the precipitated fractions was performed with an anti-NBS1 antibody. Input samples were analyzed with aliquots of 5% and 10% of each amount used for IP to detect NBS1 and DNMT1, respectively. (C) Binding of NBS1 and DNMT1 under other DNA stressors. A cross-linker, mitomycin C, and an inhibitor of DNA polymerase, aphidicolin, were added to generate double-stranded breaks in genomic DNA, and direct alteration to the replication complex, respectively. MMC and Aphi on the lanes represent mitomycin C and aphidicolin treatments, respectively. Immunoprecipitation and immunoblot

analysis of NBS1 and DNMT1 were performed as described above.

Figure 2. Mapping the binding region of NBS1 in DNMT1. (A) Deletion derivatives of DNMT1 used for mapping experiments. Each DNA fragment was tagged with HA in the pEFBOS vector (15). The construct D1 contains the original cDNA, which was isolated via 2-hybrid screening. (B) Binding assay of N-terminal and C-terminal halves of NBS1 and HA-DNMT1 (D1) *in vivo*. N-terminal half of NBS1 that is shown as “N”, contains the start codon to 363 aa, and the C-terminal half of NBS1 that is shown as “C” contains from 364 aa to the stop codon. (C) Mapping experiments of the binding region in DNMT1 to NBS1. Deletion derivatives of HA-tagged DNMT1 are shown in Panel A. Immunoprecipitation with the anti-Flag antibody, M5, was performed as described above. Input samples were analyzed with aliquots of 5%.

Figure 3. N-terminus in NBS1 (including the FHA domain) mapped as the DNMT1 binding region. (A) Deletion derivatives of Flag-tagged NBS1, shown as FB1, BR1 and F, were used for the mapping assay. Flag-tagged derivatives were expressed in the pEFBOS vector in human 293T cells. Numbers above the bars represents the position of the aa residues in the NBS1 coding region. (B) Immunoprecipitation of HA-tagged

DNMT1 with Flag-tagged NBS1 deletion derivatives. DNMT1 (D4) used in this experiment is shown in Fig. 2A. The input samples loaded were an aliquot of 5% of that used for immunoprecipitation run on an SDS-PAGE gel.

Figure 4. **Regulated DNA methylation in the *survivin* promoter is dependent upon**

NBS1 function. Methylation status at the *survivin* promoter was examined in an NBS

patient cell line GM07166 (NBS1^{-/-}) and its NBS1-recovered line (NBS1⁺). (A) PCR

assay to detect DNA methylation used the methylation sensitive restriction enzyme

HpaII. Sequences of the primers Suvivin-pro1 and Suvivin-pro3 to detect of the *survivin*

promoter are shown in Table S3. Methylation-insensitive restriction enzyme *MspI*, which

cuts the same sequence as *HpaII*, was used for the control experiment. (B) PCR assay of

bisulfite-modified genomic DNA. The primer sequences used for the MSP assay at the

survivin promoter are shown in Table S1. Methylated DNA would be amplified with the

primers Suvivin-pro1M and Suvivin-pro3M, and unmethylated DNA would be amplified

with the primers Suvivin-pro1U and Suvivin-pro3U (Table S3). Intact genomic DNA as

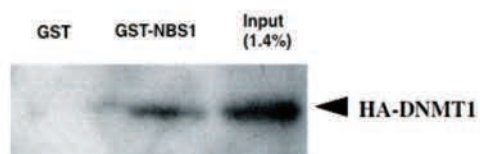
a control experiment was amplified with the primers Suvivin-pro1 and Suvivin-pro3. (C)

RT-PCR analysis of *survivin* transcripts in an NBS patient cell line GM07166 (NBS1^{-/-})

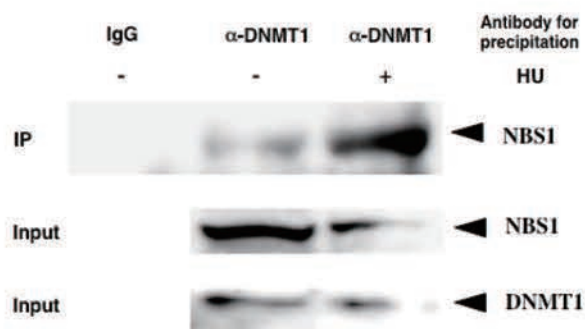
and its NBS1-recovered line (NBS1⁺). The upper part represents the RT-PCR product of the *survivin* transcripts, and the lower part represents the RT-PCR product of *GAPDH* transcripts as an internal control. In each panel, “+” represents the sample treated with 20 mM HU for 2 h to inhibit DNA replication.

Figure 5. Regulation of methylation at the ninth lysine of histone H3, and regulatory binding of DNMT1 and p53 at the *survivin* promoter are required for NBS1 function. (A) Chromatin immunoprecipitation (ChIP) analysis at the *survivin* promoter in the NBS patient cell line GM07166 (NBS1^{-/-}) and its NBS1-recovered line (NBS1⁺) was performed with anti-dimethyl K9 histone H3, anti-DNMT1, and anti-p53. Four lanes of the left half of each panel show the bands amplified with immunoprecipitated genomic DNA and the specific primers, Survivin-pro1 and Survivin-pro3 (Table S3). Results of input control reactions are shown in the 4 lanes of the right half of each panel. Panels B, C, and D represent relative band intensities of the experiment repeated in triplicate compared to each result of the intact NBS1-restored cell line.

A



B



C

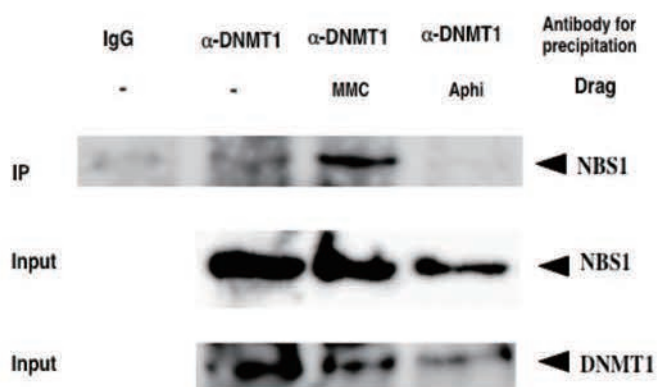
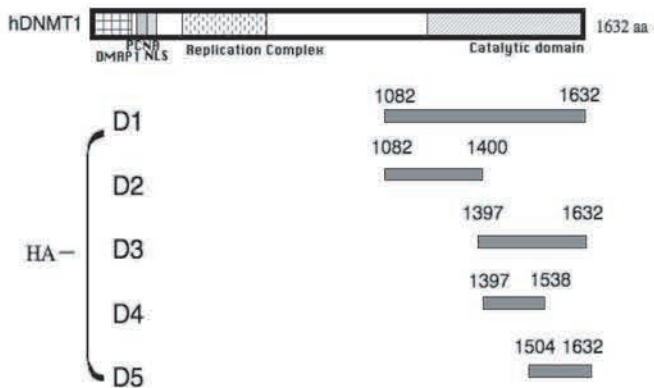
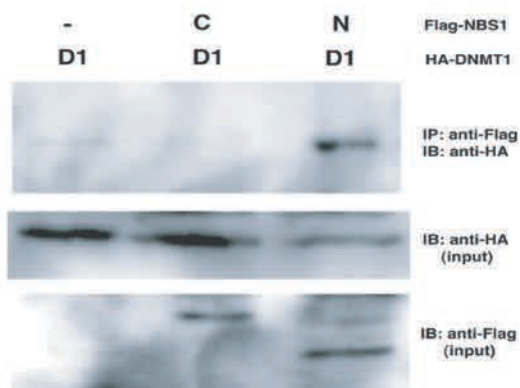


Fig. 1

A



B



C

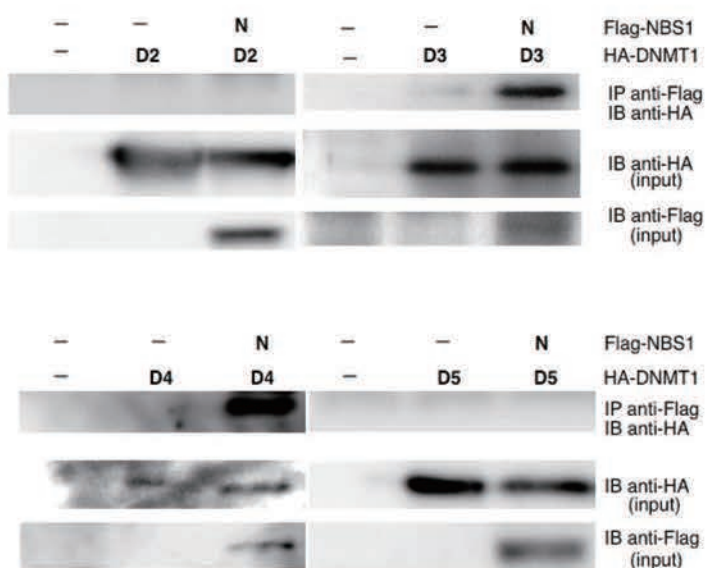
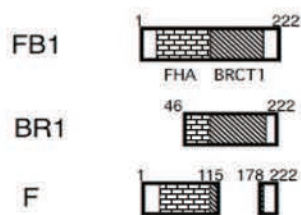
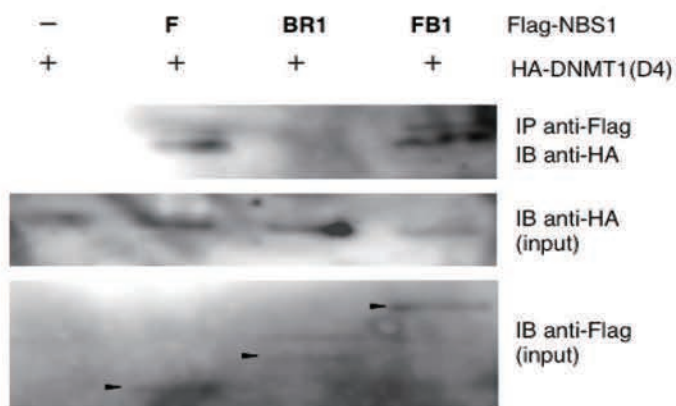


Fig. 2

A**B****Fig. 3**

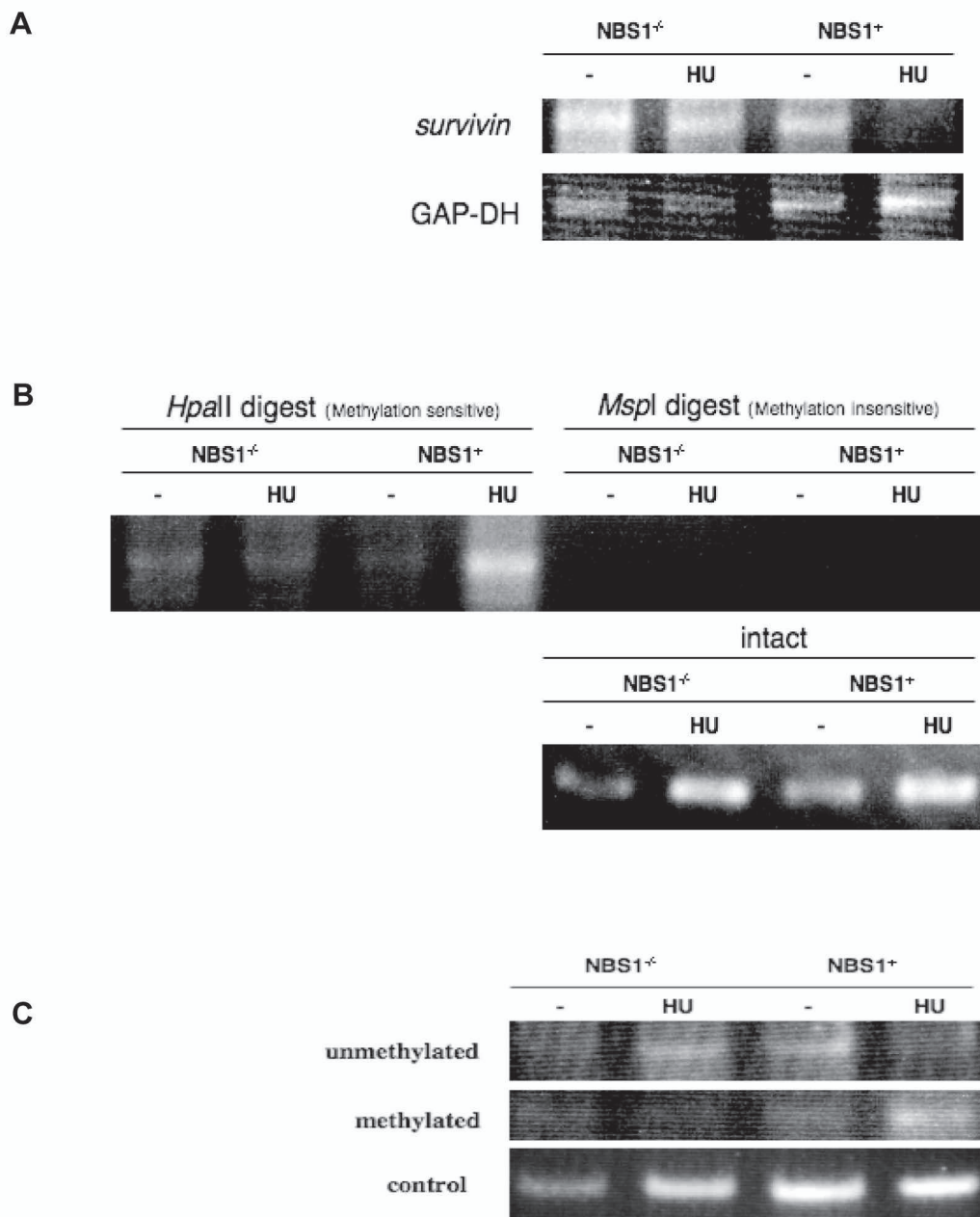
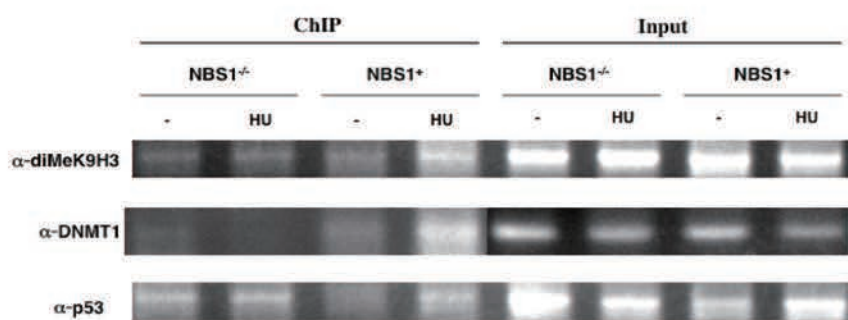
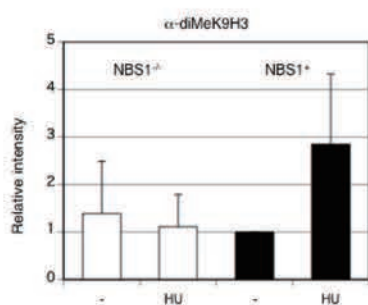
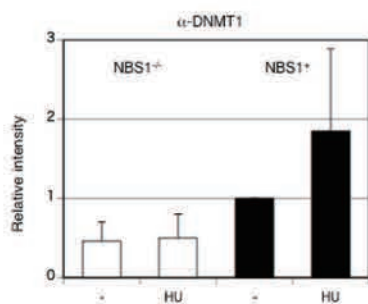
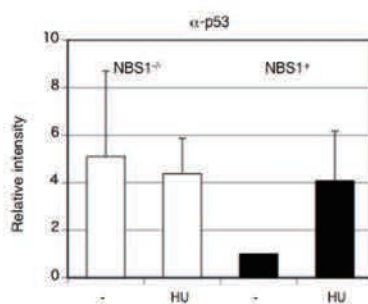


Fig. 4

A**B****C****D****Fig. 5**

Supplemental Tables

Table S1. The plasmids used in this study.

Plasmid	Gene	Reference
pASNBS1	GAL4dbd-NBS1	(15)
pACT library	GAL4act-cDNA library derived from human B cell	(18)
pEFBOS-3HA	CMVp-three copies of HA tag	(16)
pEFBOS-Flag	CMVp-Flag tag	(16)
pGEX-KG	tacp-GST (glutathione S-transferase)	(19)

Table S2. The primer sequences used for construction of deletion derivatives.

DNMT1 primers

D2	: NBB1-BamN	5'- CGGATCC G GGA CCT GCC CGA G -3'
	DNMT1-4370	5'- CAC TAG TGC CGA GGC TCC ATT CCG CAC CT -3'
D3	: DNMT1-4380	5'- GAA TGG ATC CTC GGC ACT GGA G -3'
	DNMT1-BamC	5'- GGG GAT CCT GGT GCC AGA AAC -3'
D4	: DNMT1-4380	5'- GAA TGG ATC CTC GGC ACT GGA G -3'
	DNMT1-4800	5'- GACTAGTTGTGCTGAAGAAGCC -3'
D5	: DNMT1-4700	5'- CGGATCCAGGCAGTTCAACACCCTCAT -3'
	DNMT1-BamC	5'- GGG GAT CCT GGT GCC AGA AAC -3'

NBS1 primers

NBS1-NSal	5'- CGT CGA CAT GTG GAA ACT GCT GCC C -3'
NBS1-FHABR1	5'- GAA GAT CTG TTT TCT TTC CTG CCG -3'
NBS1-SHAA200	5'- CGCCCGAAATGCTGCTGTGTTAACTGCTAAC -3'

Table S3. The primer sequences used for MSP and ChIP assay at the *survivin* promoter.

Primer Name	Sequence
Survivin-pro1	5'-GACCACGGGCAGAGCCACGCGGCG-3'
Survivin-pro3	5'-CCACCTCTGCCAACGGGTCCCGCG-3'
Survivin-pro1M	5'-AACCACGAACAAAACCACGCGACG-3'
Survivin-pro2M	5'-CCACCTCTACCAACGAATCCCGCG-3'
Survivin-pro1U	5'-AACCACAAACAAAACCACACAACA-3'
Survivin-pro2U	5'-CCACCTCTACCAACAAATCCCACA-3'

Supplemental Figures

Figure S1. **DNMT1 was also precipitated with NBS1.** Immunoprecipitation was performed with the contrary combination of the antibodies in Fig. 1B. Mouse IgG2 as negative control was used. Immunoblot analyses to the precipitated fractions and as input samples, 5% and 10% of aliquot used for immunoprecipitation was analyzed to detect NBS1 and DNMT1, respectively.

Figure S2. **Mirin, Mre11 inhibitor, inhibited DNMT1 localization to the *survivin* promoter.** ChIP analysis at the *survivin* promoter in NBS1 recovered line of GM07166 was done with anti-DNMT1 antibody. Mirin (Focus Biomolecules, USA) was added in 100 μ M of final concentration before HU treatment. Three lanes of the left half represented results of ChIP assay with the specific primers, Survivin-pro1 and Survivin-pro3 (Table S3). Results of input control reactions were shown in 3 lanes of the right half.

Figure S3. **Models for DNMT1 recruitment to the *survivin* promoter in DNA stress condition.** (A) In the control cells, DNA damage signal is transmitted via ATM and MRN complex. DNMT1 is recruited to the *survivin* promoter where p53 binds. Thus heterochromatin structure is formed there to silence the *survivin* gene. Under the DNA stress condition, NBS1, which is bound at TRD in the catalytic region of DNMT1, may affect its methylation activity. (B) In NBS patient cell line, mutated NBS1 binds with neither ATM nor Mre11. In spite of constitutive binding of p53 at the *survivin* promoter, DNMT1 is not recruited there. (C) Under mirin treatment, which inhibits nuclease activity of Mre11 and signal transmission to ATM kinase, DNMT1 is not recruited to the *survivin* promoter. In this condition, interaction between DNMT1 and NBS1 was also reduced (data not shown).