A member of the ETS family, EHF, and the ATPase RUVBL1 inhibit p53-mediated apoptosis

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Tumour cells are known to be dependent on, or 'addicted to', not only oncogenes, but also some non-oncogenes. However, the mechanisms by which tumour cells are addicted to these genes have not been fully explained. Here, we show that overexpression of a member of the ETS family, EHF, is required for the survival of colon tumour cells that contain wild-type p53. We found that EHF directly activates the transcription of *RUVBL1*, an ATPase associated with chromatin-remodelling complexes. *RUVBL1* blocks p53-mediated apoptosis by repressing the expression of *p53* and its target genes. Moreover, we found that *RUVBL1* represses *p53* transcription by binding to the *p53* promoter, interfering with RNF20/hBRE1-mediated histone H2B monoubiquitination and promoting PAF1-mediated histone H3K9 trimethylation. These results indicate that EHF-mediated *RUVBL1* expression allows colon tumour cells to avoid p53-mediated apoptosis. Thus, EHF and *RUVBL1* might be promising molecular targets for the treatment of colon tumours.

Keywords: apoptosis; ETS; histone modification; p53; *RUVBL1*

EMBO reports (2011) 12, 682–689. doi:10.1038/embor.2011.81

INTRODUCTION

It has been shown that sustained activation of oncogenes is required for the survival and proliferation of tumour cells (Felsher, 2008; Weinstein & Joe, 2008). In particular, studies of conditional mouse models have shown that the inactivation of a single oncogene such as c-myc or K-ras can inhibit the survival and proliferation of tumour cells, despite the presence of other mutated genes (Fisher *et al*, 2001; Jackson *et al*, 2001). Several studies using human cancer cell lines also support the notion that tumour cells are dependent on, or 'addicted to', a single oncogene (Felsher, 2008; Weinstein & Joe, 2008). Furthermore, drugs that target specific oncogene products, such as ErbB2/Her2/Neu, Bcr–Abl and the epidermal growth factor, have been shown

to be effective in the treatment of cancer (Cobleigh *et al*, 1999; Herbst *et al*, 2004).

Sustained inactivation of tumour suppressor genes is also known to be required for the survival and proliferation of tumour cells (Xue *et al*, 2007; Meek, 2009). Inactivation of p53 is required for the survival and proliferation of tumour cells, and reactivation of p53 in p53-deficient tumours has been reported to lead to tumour regression. In response to a variety of cellular stresses—including oncogene activation, DNA damage, hypoxia and oxidative stress—p53 induces cell-cycle arrest, senescence and/or apoptosis by regulating transcription of target genes, including *p21*, *PUMA* and *BAX*. Thus, inactivation of p53 is required for the survival and proliferation of tumour cells, and reactivation of p53 in p53-deficient tumours has been reported to lead to tumour regressions (Xue *et al*, 2007). Furthermore, tumour cells can be addicted to a non-oncogene that is not mutated but has essential roles in regulating an aberrant and malignancy-specific gene expression programme (Shaffer *et al*, 2008; Kawasaki *et al*, 2009).

In this study, we show that a member of the ETS family, EHF, is required for the survival of colon tumour cells that contain wild-type p53. The ETS family of proteins function as transcriptional activators or repressors and regulate the expression of several genes involved in diverse biological processes, including cell proliferation, apoptosis and transformation (Wasylyk *et al*, 1998; Sharrocks, 2001; Seth & Watson, 2005). We demonstrate that EHF transactivates *RUVBL1* and thereby suppresses p53-mediated apoptosis by interfering with RNF20-mediated histone H2B monoubiquitination and promoting PAF1-mediated histone H3K9 trimethylation.

RESULTS AND DISCUSSION

EHF knockdown induces p53-dependent apoptosis

To identify transcription factors that are involved in aberrant gene expression in colon tumours, we searched for transcription-factor-binding motifs that are present at high frequencies in the promoter regions of genes that are upregulated in colon cancer cells. We found that putative ETS-binding sites (EBs), as well as Myc- and SP1-binding sites, are frequently detected in the promoter regions of genes that are expressed at higher levels in colon tumours than in non-cancerous tissues (supplementary Table S1 online).

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Received 15 November 2010; revised 31 March 2011; accepted 1 April 2011; published online 27 May 2011

Fig 1 | Knockdown of EHF induces apoptosis of colon tumour cells. Annexin V assays were performed with HCT116 (upper), HCT116(p53^{-/-}; lower left) or DLD-1 (lower right) cells transfected with the indicated siRNA. siRNAs targeting two sequences of each ETS were used (except for ETV6). Data are the means $\pm$ s.d. of three independent experiments. PI, propidium iodide; siCont, cells transfected with control siRNA; siRNA, small-interfering RNA.

To explain the role of the ETS family of transcription factors in colon cell tumorigenesis, we examined the effects of small-interfering RNAs (siRNAs) targeting ETS proteins on cell viability. We found that knockdown of some members of the ETS family proteins—ETV4, ETV6, ELK1, ELF2, ERF or EHF—resulted in marked increases in apoptotic death of the colon tumour cell line HCT116, as determined by Annexin assays (Fig 1, upper panel; supplementary Fig S1 online). When similar experiments were performed with HCT116(p53^{-/-}) cells—a derivative of HCT116 cells in which p53 was disrupted by homologous recombination (Bunz *et al*, 1998)—EHF knockdown led to less apoptotic death (Fig 1, lower left). Knockdown of EHF also did not induce significant apoptosis in DLD1 cells, which contain a mutated p53 (Fig 1, lower right). Thus, EHF knockdown might induce apoptotic death, at least in part, in a p53-dependent manner. On the other hand, knockdown of either ETV4, ETV6, ELK1, ELF2 or ERF induced apoptotic death of HCT116(p53^{+/+}), HCT116(p53^{-/-}) and DLD1 cells with similar efficacies. These results indicate that some of the ETS family members are required for the survival of colon tumour cells.

RUVBL1 expression is upregulated directly by EHF

We next attempted to clarify the mechanisms underlying ETS-mediated protection of colon tumour cells from apoptotic cell death. We focused our analysis on EHF, as it is overexpressed in colon tumours (Kleinbaum *et al*, 1999) and its function is related to p53 (Fig 1). To identify the target genes of EHF, we profiled the transcriptome of HCT116 cells transfected with an siRNA targeting EHF (siEHF). Among those genes that were downregulated in cells transfected with siEHF compared with control cells, we selected

those that contain ETS-binding motifs in their promoter regions and that are overexpressed in colon tumour cells. We found that seven genes, including RUVBL1, meet these criteria (supplementary Table S1 online).

RUVBL1 is a member of the AAA + (ATPase associated with diverse cellular activities) family of proteins that is associated with several chromatin-remodelling complexes and has important roles in transcriptional regulation, the DNA damage response, telomerase activity, snoRNP assembly, cellular transformation and cancer metastasis (Gallant, 2007; Huber *et al*, 2008; Jha & Dutta, 2009). To confirm that EHF is involved in the upregulation of RUVBL1 in colon tumour cells, we transfected siEHF into HCT116 cells and examined RUVBL1 messenger RNA (mRNA) and protein expression by quantitative reverse transcription-PCR (qRT-PCR) and immunoblotting, respectively. We found that siEHF significantly repressed RUVBL1 mRNA and protein expression (Fig 2A,B). We also found that overexpression of EHF resulted in an increase in RUVBL1 mRNA and protein expression in HeLa cells, which contain low levels of endogenous EHF (Fig 2C). These results indicate that RUVBL1 expression is upregulated by EHF in colon tumour cells.

We next examined whether EHF could upregulate the transcription of a series of reporter constructs containing various fragments of the RUVBL1 promoter and 5' untranslated region (-1,940 to +75), which was fused upstream of a luciferase gene (wild type (WT)-1, -2, -3, -4 and -5 shown in Fig 2D). When transfected into HeLa cells, the activities of WT-1, -2, -3 or -5 were enhanced by co-expression of EHF (Fig 2D). By contrast, the activity of WT4 was not enhanced by overexpression of EHF. Inspection of the sequence that is present in WT5 but not WT4 revealed three possible EBSs. We found that EHF overexpression did not enhance activities of the WT5 reporters when each of the central GGAA motifs was replaced by TTAA (MT-1, -2 and -3). These results indicate that EHF upregulates RUVBL1 expression by binding to these EBSs.

To confirm that EHF transactivates RUVBL1 directly, we investigated whether EHF binds to the RUVBL1 promoter region *in vivo*. HeLa cells were transfected with Flag-tagged EHF, and subjected to chromatin immunoprecipitation (ChIP) assays. We found that a DNA fragment (R1_350 in Fig 2E) encoding the three EBSs identified above was immunoprecipitated in significant amounts by Flag antibody (Fig 2E, left). By contrast, two control fragments (R1_5,000 and R1_3,000) were not immunoprecipitated in the same experiment. Moreover, ChIP assays using EHF antibody showed that endogenous EHF is associated with the R1_350 region in the RUVBL1 promoter (Fig 2E, right). Taken together, these results indicate that EHF transactivates RUVBL1 expression directly, by binding to the EBSs located in its promoter region.

RUVBL1 negatively regulates p53 expression

To clarify the importance of EHF-mediated RUVBL1 overexpression in colon tumour development, we examined the effects of an siRNA targeting RUVBL1 (siRUVBL1) on cell viability, as determined by Annexin V staining. We found that knockdown of RUVBL1 resulted in marked increases in the apoptotic death of HCT116 and RKO cells, which contain wild-type p53, but not of DLD1 or Caco2 cells that contain mutated p53 (Fig 3A). siRUVBL1-mediated apoptosis of RKO cells was suppressed in cells transfected with an siRNA

Fig 2 | EHF directly regulates the expression of *RUVBL1*. (A) qRT-PCR analysis of *RUVBL1* expression in HCT116 cells transfected with siRNA targeting a scrambled sequence (siCont) or EHF (siEHF1, siEHF2). Before fold-change calculation, values were normalized to the signal generated from actin mRNA. (B) HCT116 cells were transfected with siCont, siEHF1, or siEHF2 and subjected to immunoblotting analysis with the indicated antibodies. Actin was used as a loading control. (C) qRT-PCR analysis of *RUVBL1* expression in HeLa cells transfected with the Flag-tagged EHF expression plasmid (top). Cell lysates were subjected to immunoblotting analysis with the indicated antibodies (lower three panels). (D) Schematic representation of the *RUVBL1* promoter luciferase reporter constructs used (left). Derivatives of the wild-type *RUVBL1* promoter containing mutations in potential EBS (GGAA → TTAA) were constructed as shown. Luciferase assays were performed with HeLa cells transfected with a reporter plasmid, and the Flag-tagged EHF or the empty expression plasmid. Bars represent luciferase activity in cells transfected with Flag-tagged EHF divided by the activity in cells transfected with the empty plasmid. The pRL-tk Renilla luciferase reporter was co-transfected to normalize transfection efficiency. $^{**}P < 0.01$, $^{***}P < 0.001$. (E) Upper panel: schematic representation of the human *RUVBL1* promoter region. Amplified regions are indicated by black bars. The region around -350 contains EBS1, EBS2 and EBS3. The regions around -3,000 and -5,000 were amplified as controls. Lower left: ChIP assays were performed on HeLa cells transfected with an empty (white) or Flag-tagged EHF (grey) expression vector using antibodies against Flag. Lower right: ChIP assays were performed with HCT116 cells using EHF antibody. The promoter region of NKX3.1 was amplified as a positive control, $^{*}P < 0.05$. Data are the means $\pm$ s.d. of three independent experiments. ChIP, chromatin immunoprecipitation; IB, immunoblotting; IgG, immunoglobulin G; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription-PCR; siRNA, small interfering RNA; siCont, cells transfected with control siRNA; siEHF, siRNA targeting EHF; TSS, transcription start site.

targeting p53. Furthermore, HCT116(p53^{-/-}) cells showed significantly less siRUVBL1-induced apoptosis than parental HCT116 cells. Fluorescence-activated cell-sorting analysis showed similar results

(Fig 3B). These results indicate that RUVBL1 overexpression leads to the escape of colon tumour cells from p53-mediated apoptotic cell death. In addition, the fact that siRUVBL1 could induce the

Fig 3 | RUVBL1 knockdown induces p53-dependent apoptosis of colorectal cancer cell lines. (A) Annexin assays were performed with the indicated colon tumour cells that had been transfected with the indicated siRNA; *** $P < 0.001$. (B) Fluorescence-activated cell-sorting analyses were performed with the indicated colon tumour cells that had been transfected with the indicated siRNAs; ** $P < 0.01$. (C) Annexin assays were performed with RKO cells (left) and HCT116 cells (right) transfected with siEHF and/or RUVBL1-NLS; ** $P < 0.01$, *** $P < 0.001$. (D) qRT-PCR analyses of the indicated genes in RKO cells transfected with the indicated siRNA. Data are the means $\pm$ s.d. of three independent experiments. (E) HCT116 cells were transfected with siCont, siRUVBL1-1, or siRUVBL1-2 and subjected to immunoblotting analysis with the indicated antibodies. Actin was used as a loading control. IB, immunoblotting; NLS, nuclear localization signal; PI, propidium iodide; qRT-PCR, quantitative reverse transcription-PCR; siRNA, small-interfering RNA; siCont, cells transfected with control siRNA; siEHF, siRNA targeting EHF; siRUVBL1, siRNA targeting RUVBL1.

apoptotic death of HCT116(p53^{-/-}) cells suggests that RUVBL1 could suppress apoptosis that is mediated not only by p53, but also by other molecules. It remains to be investigated whether p63 and/or p73 is involved in RUVBL1-mediated apoptotic cell death.

To assess the extent to which RUVBL1 upregulation mediates the suppression of apoptotic cell death by EHF, we overexpressed RUVBL1 in cells in which EHF expression was knocked down by

siRNA. As exogenously expressed RUVBL1 localizes mainly to the cytoplasm, we added the nuclear localization signal (NLS) from the SV40 large T-antigen to the amino-terminus of RUVBL1 (RUVBL1-NLS; supplementary Fig S2 online). When RKO and HCT116 cells were transfected with RUVBL1-NLS, it localized mainly to the nucleus similar to endogenous RUVBL1, and siEHF-mediated apoptotic cell death was partly suppressed (Fig 3C).

Fig 4 | RUVBL1 represses *p53* expression by interfering with H2B monoubiquitination and promoting H3K9 trimethylation. (A) Schematic representation of the human *p53* gene. (B–D) ChIP assays were performed with RKO cells transfected with siRUVBL1 or siCont using ubH2B (B), RUVBL1 (C) or RNF20 (D) antibodies. G-TR, P-TR and B-TR are derived from the TR of the *GAPDH*, *PUMA* and *BAX* genes, respectively. G-TSS, P-TSS and B-TSS are derived from the TSS of the *GAPDH*, *PUMA* and *BAX* genes, respectively. * $P < 0.05$, ** $P < 0.01$. (E) qRT-PCR analysis of *p53*, *RUVBL1* and *RNF20* in RKO cells transfected with siRUVBL1 and/or siRNA targeting RNF20 (siRNF20) or siCont. Data are the mean $\pm$ s.d. of three independent experiments. (F,G) ChIP assays were performed with RKO cells transfected with siRUVBL1 or siCont using PAF1 (F) or H3K9Me3 antibodies (G). G-TSS is derived from the TSS of the *GAPDH* gene, * $P < 0.05$. (H) qRT-PCR analysis of *p53*, *PAF1* and *CDC73* in RKO cells transfected with siPAF1, siCDC73 or siCont. Data are the mean $\pm$ s.d. of three independent experiments. (I) Aberrant expression of EHF and RUVBL1 allows colon tumour cells to avoid *p53*-mediated apoptosis. EHF directly activates the transcription of *RUVBL1*. RUVBL1 represses *p53* transcription by interfering with RNF20/hBRE1-mediated histone H2B monoubiquitination and promoting PAF1-mediated histone H3K9 trimethylation, thereby inhibiting apoptosis of colon tumour cells. DS, derived from the 3' UTR; qRT-PCR, quantitative reverse transcription-PCR; siCont, cells transfected with control siRNA; siRNA, small-interfering RNA; siRUVBL1, siRNA targeting RUVBL1; TR, derived from the transcribed region; TSS, the transcription start site; UP, derived from the promoter region; UTR, untranslated region.

These results indicate that RUVBL1 is an important target gene of EHF that mediates suppression of apoptotic cell death in colon tumours. As the effect of siEHF was only partly ablated by over-expression of RUVBL1-NLS, EHF might also inhibit apoptotic cell death by mechanisms that are independent of RUVBL1 upregulation.

We next examined whether RUVBL1 is involved in the regulation of the expression of *p53* and its target genes, *PUMA* and *BAX*, which have crucial roles in *p53*-mediated apoptotic cell death. We found that knockdown of RUVBL1 using siRNA resulted in marked increases in the mRNA and protein expression levels of *p53* and its target genes in RKO cells (Fig 3D,E), but not in Caco2 cells (supplementary Fig S3 online). This effect of siRUVBL1 was not observed when cells were co-transfected with siRNA against *p53*. These results indicate that RUVBL1 over-expressed in tumour cells negatively regulates *p53* expression, and thereby interferes with *p53*-mediated apoptosis.

RUVBL1 inhibits histone H2B monoubiquitination

It has been reported that RUVBL1 is present in the PAF1 complex, which regulates monoubiquitination of histone H2B (Yart et al, 2005; Ding et al, 2009). Monoubiquitinated histone H2B (ubH2B) is associated with the transcribed regions of highly expressed genes (Minsky et al, 2008). There is accumulating evidence indicating that monoubiquitination of histone H2B is a necessary step for transcriptional elongation by RNAP II (Pavri et al, 2006). Hence, we suggested that RUVBL1 might downregulate *p53* expression by regulating monoubiquitination of histone H2B. To test this hypothesis, we examined the monoubiquitination state of histone H2B associated with the *p53* gene in RKO cells transfected with siRNA against RUVBL1. ChIP analysis with ubH2B antibody showed that RUVBL1 knockdown resulted in a significant increase in ubH2B associated with the transcribed and 3'-untranslated regions of *p53*, but not *GAPDH*, *PUMA* or *BAX* (Fig 4A,B). In addition, RUVBL1 knockdown did not change the overall amount of ubH2B (supplementary Fig S4 online). On the other hand, ChIP analysis with RUVBL1 antibody showed that RUVBL1 is associated with the transcription start site (TSS) and transcribed regions of *p53*, *GAPDH*, *PUMA* and *BAX* (Fig 4C).

It has been shown that RNF20/hBRE1, the main H2B-specific E3 ubiquitin ligase, enhances *p53* expression through ubH2B (Shema et al, 2008). We therefore examined whether RNF20 is involved in siRUVBL1-mediated enhancement of *p53* expression. ChIP analysis with RNF20 antibody showed that RNF20 was associated with TSS and transcribed regions of *p53*, but not of *GAPDH*, *PUMA*, and

BAX in cells transfected with siRUVBL1 (Fig 4D). No such association was seen in cells transfected with control siRNA (siCont). Furthermore, when RKO cells were transfected with siRUVBL1 along with siRNA targeting RNF20 (siRNF20), siRUVBL1-mediated upregulation of *p53* expression was abrogated (Fig 4E). These results indicate that in RUVBL1-knockdown cells, RNF20 is recruited to the promoter of *p53*, where it induces *p53* expression by monoubiquitination of histone H2B.

RUVBL1 promotes histone H3K9 trimethylation

It has been shown that the PAF1 complex represses transcription by inducing H3K9 trimethylation (Yang et al, 2010). We therefore examined whether PAF1-mediated H3K9 trimethylation is involved in RUVBL1-mediated repression of *p53* expression. ChIP analysis with PAF1 antibody showed that knockdown of RUVBL1 led to a decrease in the amount of PAF1 and H3K9 trimethylation associated with the TSS region of *p53*, but not the *GAPDH* gene (Fig 4F,G). We also found that knockdown of PAF1 or CDC73, a component of the PAF1 complex, resulted in the upregulation of *p53* expression (Fig 4H). Under these conditions, RUVBL1 knockdown did not change the overall amount of H3K9Me3 and the mRNA expression levels of PAF1 and CDC73 (supplementary Fig S4,S5 online). Thus, our results indicate that the RUVBL1-PAF1 complex represses *p53* expression, at least in part by inducing H3K9 trimethylation.

RUVBL1 and transcription factors

RUVBL1 has been shown to function as a transcriptional repressor of Myc, β -catenin and NF- κ B (Gallant, 2007; Huber et al, 2008; Jha & Dutta, 2009). We therefore examined whether RUVBL1-dependent *p53* repression is mediated through interaction with these transcription factors. We found that knockdown of either Myc, β -catenin or NF- κ B using siRNA did not change the expression levels of *p53* in RKO cells (supplementary Fig S6 online). We also found that knockdown of RUVBL1 along with Myc, β -catenin or NF- κ B1 did not alter *p53* expression (supplementary Fig S6 online). These results indicate that Myc, β -catenin and NF- κ B are not involved in RUVBL1-mediated repression of *p53* expression.

EHF/RUVBL1 signalling and tumour cell survival

We have shown that human colon tumour cells that contain wild-type *p53* are addicted to EHF/RUVBL1 signalling. Furthermore, we have shown that overexpression of EHF in colon tumour cells

suppresses apoptosis by upregulating RUVBL1, which in turn represses p53 expression by interfering with RNF20-mediated histone H2B monoubiquitination and promoting PAF1-mediated

histone H3K9 trimethylation (Fig 4I). Thus, aberrant expression of EHF and RUVBL1 might be essential for the survival of colon tumour cells that contain wild-type p53. In addition, as some

ETS family members are known to be activated by Ras-MAP (Mitogen-activated protein) kinase signalling (Wasylyk et al, 1998; Sharrocks, 2001; Seth & Watson, 2005), it is also possible that direct activation of EHF contributes to colon-tumour-cell survival. The reason that these proteins are also overexpressed in colon tumour cells that contain mutated p53 might be that they have other important functions, including the regulation of cell-cycle progression, senescence, metastasis, DNA damage repair and telomerase activity (Gallant, 2007; Huber et al, 2008; Jha & Dutta, 2009). We speculate that therapies targeting EHF or RUVBL1 might be useful for molecular targeted therapy of colon tumours. The detailed mechanisms by which RUVBL1 specifically suppresses p53 expression and the mechanisms underlying aberrant EHF upregulation in colon tumour cells remain to be explained.

METHODS

Antibodies. The following commercial antibodies were used: FLAG (F3165, Sigma), EHF (ETS3 Clone 5A5, Lab Vision), RUVBL1 (10210-2-AP, ProteinTech), p53 (sc-126, Santa Cruz), PUMA (Ab-1, CALBIOCHEM), BAX (6A7, BD Biosciences), Actin (A-2066, Sigma), ubH2B (NRO3, MEDIMABS), RNF20 (NB100-2242, Novus Biologicals), PAF1 (A300-172A, Bethyl Laboratories) and H3K9Me3(s8898, Abcam).

RNA interference. Stealth siRNA duplexes were purchased from Invitrogen. Cells were transfected with stealth RNA duplexes using Lipofectamine RNAiMAX (Invitrogen). The sequences of siRNAs are shown in supplementary Table S2 online. Validated Stealth negative control RNAi duplex with high GC content (Invitrogen) was used as a control.

qRT-PCR analysis. Total RNA was isolated using the TRIzol (BIOLINE), and 1 mg RNA was reverse transcribed with SuperScript III Reverse transcriptase (Invitrogen) using an oligo(dT)18 primer. qRT-PCR analysis of cDNA and genomic DNA was performed on a LightCycler 480 (Roche Applied Science) with Syber Green PCR mastermix (Applied Biosystems). Primers for mRNA expression are shown in supplementary Table S3 online.

Apoptosis. Phosphatidylserine exposure at the cell surface was detected using MEBCYTO Apoptosis Kit (MBL). For fluorescence-activated cell-sorting analysis, cells were fixed with 70% ice-cold ethanol at -20°C . After washing with Phosphate-Citrate Buffer (PCB; 192 mM Na_2HPO_4 , 4 mM citric acid), 1 ml of propidium iodide-RNase solution (phosphate-buffered saline containing 10 μg of propidium iodide and 10 μg of DNase-free RNase (Sigma)) was added per sample and incubated at 25°C for 20 min. DNA content was determined by flow cytometry (FACSCalibur; Becton Dickinson).

ChIP assay. ChIP assays were performed according to the manufacturer's instructions (Upstate). For each assay, cells from approximately one confluent 10 cm plate were used. For the RNAi-coupled ChIP analyses in Fig 4, RKO cells were treated for 72 h with siRNA duplexes and then subjected to ChIP assays as described above. Primers for quantitative PCR are shown in supplementary Table S4 online.

Immunoblotting. Cells (5×10^6) were lysed for 20 min with lysis buffer (1% Nonidet P40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 50 mM sodium fluoride) containing Halt Protease Inhibitor Cocktail (Thermo Scientific). After centrifugation at $23,100 \times g$ for 20 min at 4°C , samples were resolved by SDS-PAGE and then transferred

to polyvinylidene difluoride membranes. Blots were blocked with 3% skimmed milk in Tris-buffered saline (TBS) plus Tween 20 before probing with antibodies.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

This work was supported by Research Program of Innovative Cell Biology by Innovative Technology (Integrated Systems Analysis of Cellular Oncogenic Signaling Networks), Grants-in-Aid for Scientific Research on Innovative Areas (Integrative Research on Cancer Microenvironment Network), the Takeda Science Foundation and in part by the Global Center of Excellence Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms), MEXT, Japan. We thank A. Nishida, A. Niida, R. Koyama-nasu, A. Watanabe, A. Nonaka and H. Aburatani for discussions.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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