

Chfr is linked to tumour metastasis through the downregulation of HDAC1

Young Mi Oh¹, Young Eun Kwon¹, Joo Mi Kim¹, Sung Jun Bae¹, Bo Keun Lee¹, Soon Ji Yoo², Chin Ha Chung¹, Raymond J. Deshaies³ and Jae Hong Seol^{1,4}

Chfr is a ubiquitin ligase that functions in the mitotic checkpoint by delaying entry into metaphase in response to mitotic stress^{1,2}. It has been suggested that Chfr is a tumour suppressor as *Chfr* is frequently silenced in human cancers³. To better understand how Chfr activity relates to cell-cycle progression and tumorigenesis, we sought to identify Chfr-interacting proteins using affinity purification combined with mass spectrometry. Histone deacetylase 1 (HDAC1), which represses transcription by deacetylating histones, was newly isolated as a Chfr-interacting protein. Chfr binds and downregulates HDAC1 by inducing its polyubiquitylation, both *in vitro* and *in vivo*. Ectopic expression of Chfr in cancer cells that normally do not express it results in downregulation of HDAC1, leading to upregulation of the Cdk inhibitor p21^{CIP1/WAF1} and the metastasis suppressors KAI1 and E-cadherin. Coincident with these changes, cells arrest in the G1 phase of the cell cycle and become less invasive. Collectively, our data suggest that Chfr functions as a tumour suppressor by regulating HDAC1.

We set out to identify new Chfr-interacting proteins using affinity purification combined with mass spectrometry. As Chfr was shown to be a ubiquitin ligase involved in protein degradation^{1,2,4,5}, we expressed a mutant version of Chfr, (Flag–Chfr^{I306A}), that lacks ubiquitin ligase activity owing to the replacement of Ile 306 with Ala. Liquid chromatography-mass spectrometry (LC-MS/MS) analysis has identified histone deacetylase 1 (HDAC1) as a Chfr-interacting protein⁶ (Fig. 1a; Supplementary Information, Fig. S1). To confirm the interaction between Chfr and HDAC1 *in vivo*, HeLa cells, which do not express endogenous Chfr⁷, were transfected with Myc–Chfr and Flag–HDAC1 expression vectors. Flag–HDAC1 was readily recovered from Chfr immunoprecipitates (Fig. 1b). It was also found that endogenous Chfr can specifically immunoprecipitate endogenous HDAC1 in HEK293T cells (which, unlike HeLa, express Chfr; Fig. 1c). We performed a pulldown assay using GST–HDAC1 expressed in *Escherichia coli* and immunopurified

Flag–Chfr. The Chfr protein was detected in fractions eluted from the GST–HDAC1 affinity column but not from the GST column (Fig. 1d). These results suggest that Chfr interacts directly with HDAC1 *in vivo*. As HDAC1 and HDAC2 form a stable heterodimer⁸, we investigated whether HDAC2 can bind Chfr. HDAC2 was co-immunoprecipitated with Chfr, whereas other HDACs, such as HDAC3 or HDAC4, were not detected in Chfr immunoprecipitates. However, other components of HDAC1-containing complexes, such as SIN3A, were detected in Chfr immunoprecipitates when large amounts of Chfr immunoprecipitates were subjected to immunoblot analysis (Supplementary Information, Fig. S2). Thus, this result indicates that Chfr–HDAC1 may form a subset of HDAC1-containing complexes.

We mapped the region of Chfr required for its interaction with HDAC1. Flag-tagged deletions of Chfr were generated (Fig. 2a) and co-expressed with Myc–HDAC1. Western blot analysis of Flag–Chfr immunoprecipitates revealed that the carboxy-terminal cysteine-rich (CR) region of Chfr is required for its interaction with HDAC1 (Fig. 2b). It has been reported that the kinase Aurora A interacts with the CR region of Chfr, whereas the kinase Plk1 interacts with the forkhead-associated (FHA) domain^{1,2,9,10}. Competition binding assays showed that the kinase Aurora A, but not Plk1, competed with HDAC1 for binding to Chfr (Fig. 2c). Next, we mapped the region of HDAC1 that is responsible for its interaction with Chfr. The C-terminal region of the HDAC1 deacetylase domain seems to be involved in its interaction with Chfr (Supplementary Information, Fig. S3). As, out of the HDAC proteins, only HDAC1 and HDAC2 can bind to Chfr directly, we aligned the amino acid sequences of the putative Chfr-binding region in the class I HDACs. The amino acids Phe 287 and Met 297, which are conserved in HDAC1 and HDAC2, were replaced with Tyr and Ile respectively, to generate the mutant HDAC1^{FM}. Chfr was immunoprecipitated with wild-type HDAC1 (HDAC1^{WT}) but not HDAC1^{FM} (Fig. 2d). Although HDAC1^{FM} did not interact with Chfr, it was fully functional as a deacetylase (Supplementary Information, Fig. S6). These results indicate that Phe 287 and Met 297 of HDAC1 are not important for its deacetylase activity, but are crucial for its interaction with Chfr.

¹School of Biological Sciences, Research Center for Functional Cellulomics, Seoul National University, Seoul 151-742, Korea. ²Department of Life and Nanopharmaceutical Sciences and Department of Biology, Kyung Hee University, Seoul 130-701, Korea. ³Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA.

⁴Correspondence should be addressed to J.H.S. (e-mail: jhseol@snu.ac.kr)

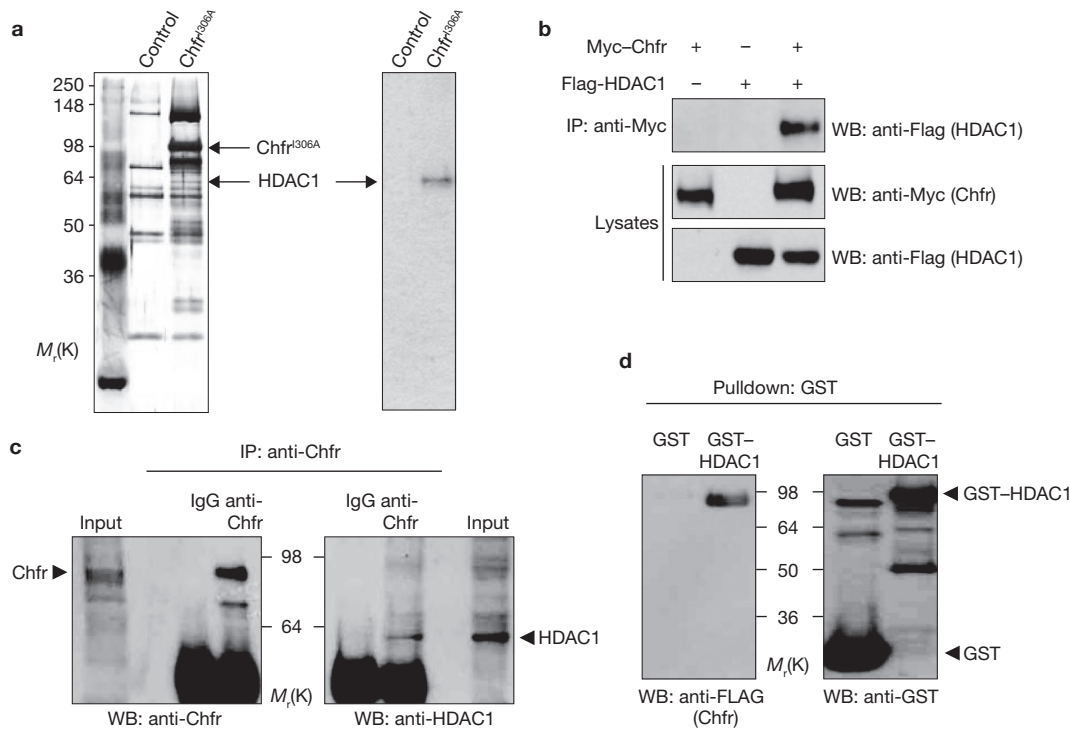


Figure 1 Chfr interacts with HDAC1 directly. **(a)** HEK293T cells were transiently transfected with pCMV-3×Flag (Control) or pCMV-3×Flag-Chfr^{1306A} (Chfr^{1306A}) and cell lysates were immunoprecipitated with anti-M2 resin. Proteins co-immunoprecipitating with Flag-Chfr were separated by SDS-PAGE, visualized with silver staining and subjected to nano-electrospray LC-MS/MS analysis. Extracts from untagged control or Flag-Chfr^{1306A} cells were evaluated in parallel by immunoblotting with anti-HDAC1 antibody. **(b)** HeLa cells were co-transfected with Myc-Chfr and Flag-HDAC1. Cell

lysates were immunoprecipitated and immunoblotted with indicated antibodies. **(c)** HEK293T cells were treated with MG132 (2 μM) for 12 h. Cell lysates were then immunoprecipitated with anti-Chfr antibody and immunoblotted with anti-Chfr or anti-HDAC1 antibodies. **(d)** GST-HDAC1 was purified from *E. coli* cells and Flag-Chfr proteins were purified from HEK293T cells with anti-M2 resin. Purified Flag-Chfr and GST-HDAC1 or GST was incubated with glutathione Sepharose for 1 h at 4 °C. Precipitates were subjected to SDS-PAGE followed by immunoblotting with anti-Flag or anti-GST antibodies.

To determine the functional consequence of the Chfr-HDAC1 interaction, we tested whether Chfr affects cellular levels of HDAC1. We found that HDAC1 accumulation varied inversely with levels of co-transfected Chfr (Fig. 3a). Co-expression of Chfr^{1306A} and Chfr lacking a CR region (Chfr^{ΔCR}) did not antagonize accumulation of HDAC1, indicating that both the substrate- and E2 (ubiquitin conjugating enzyme)-binding domains of Chfr are required for this effect (Fig. 3b). Moreover, Chfr-dependent loss of HDAC1 was blocked by the proteasome inhibitor MG132. Most importantly, expression of Chfr down-regulated endogenous HDAC1 (Supplementary Information, Fig. S5) by increasing its rate of degradation, as measured in a cycloheximide-chase assay (Fig. 3c). Together, these results suggest that the ubiquitin ligase activity of Chfr targets HDAC1 for degradation. Chfr-dependent turnover was specific, in that HDAC2 was also antagonized by co-expressed Chfr, but the levels of HDAC proteins that did not bind Chfr (HDAC3 and HDAC1^{FM}) were unchanged. During the course of these studies, we noted that the Chfr^{1306A} mutant accumulated to a higher level than HDAC1^{WT} and Chfr^{ΔCR}, suggesting that the cellular level of Chfr may be controlled by auto-ubiquitylation.

To investigate whether endogenous Chfr contributes to the regulation of HDAC1, we depleted HEK293T cells, of endogenous Chfr using RNA interference (RNAi). Analysis by reverse transcription-PCR (RT-PCR) confirmed both the specificity and efficacy of our short hairpin Chfr construct (shChfr). Importantly, the level of HDAC1 was increased when

Chfr was silenced (Fig. 3d). These results indicate that Chfr regulates the levels of endogenous HDAC1 by controlling its degradation.

Chfr possesses ubiquitin ligase activity, which suggests that Chfr mediates proteasome-dependent turnover of HDAC1 by directly ubiquitylating it. To determine whether HDAC1 can be ubiquitylated by Chfr *in vitro*, Flag-HDAC1 expressed in HeLa cells and His-Chfr or His-Chfr^{1306A} expressed in insect cells were purified. HDAC1 was ubiquitylated in a Chfr-dependent manner (Fig. 3e). To examine whether Chfr promoted ubiquitylation of HDAC1 *in vivo*, Flag-HDAC1 was co-expressed in HeLa cells with Chfr (wild-type Chfr, Chfr^{1306A} or Chfr^{ΔCR}). HDAC1 retrieved from cells that co-expressed wild-type Chfr (Chfr^{WT}) was extensively ubiquitylated (Fig. 3f). By contrast, little or no ubiquitylation of HDAC1 was observed in the absence of Chfr or when it was co-expressed with Chfr^{1306A} or Chfr^{ΔCR}. Notably, ubiquitylation of endogenous HDAC1 was decreased in HEK293T cells depleted of endogenous Chfr by shChfr (Supplementary Information, Fig. S5). Despite the major effect of Chfr on the ubiquitylation status of HDAC1, total cellular ubiquitin conjugates were not affected by either over-expression or depletion of Chfr. Taken together, these results indicate that Chfr ubiquitylates HDAC1 that is bound to its CR domain, resulting in proteasomal degradation of HDAC1.

HDAC1 is a histone deacetylase that has a crucial role in controlling the expression of genes required for cell-cycle progression and tumour metastasis^{11,12}. To determine the relationship between Chfr and HDAC1

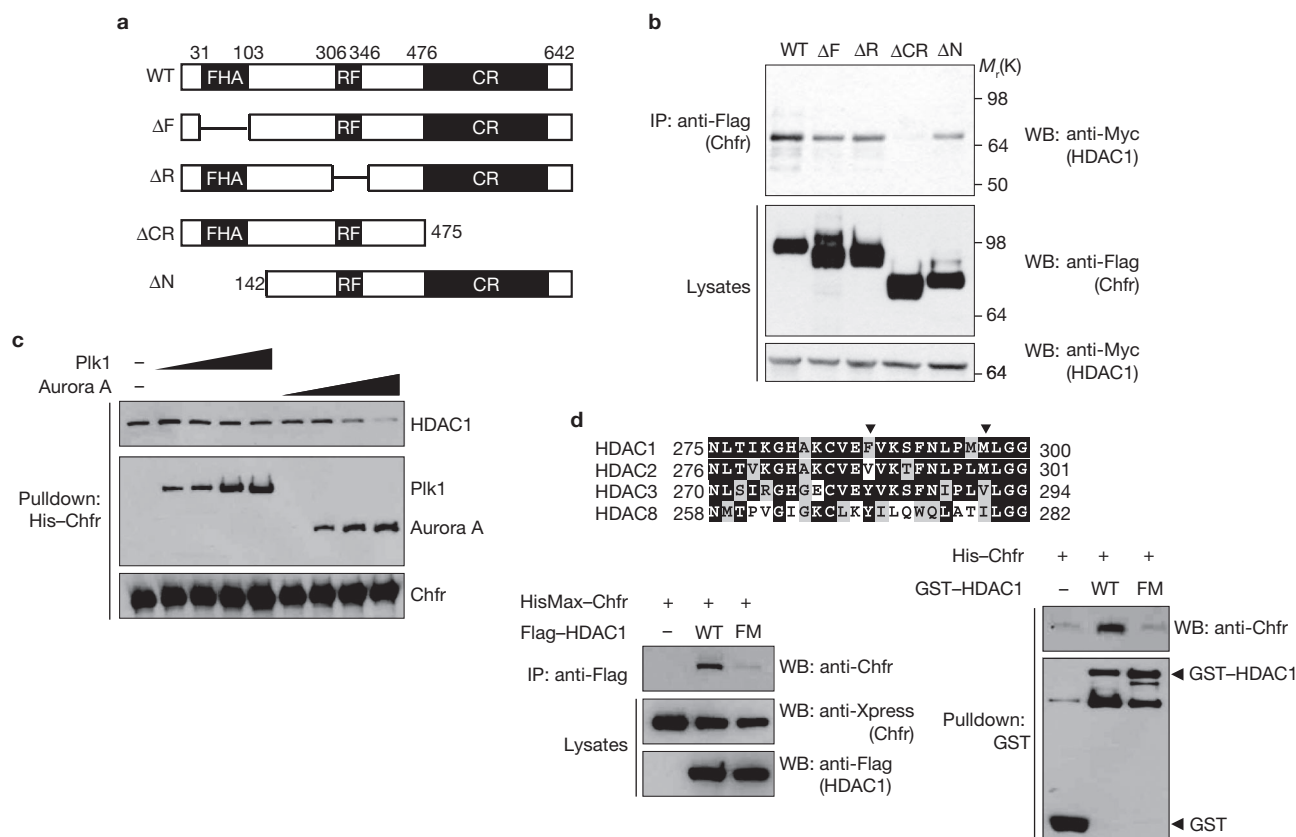


Figure 2 The C-terminal CR region of Chfr is required for its interaction with HDAC1. **(a)** Chfr deletion mutants: WT, wild-type; ΔF , FHA domain deletion; ΔR , ring finger domain deletion; ΔCR , CR domain deletion; ΔN , N-terminal deletion. **(b)** HEK293T cells were co-transfected with plasmids encoding Flag-Chfr and Myc-HDAC1. After transfection for 24 h, the cells were treated with MG132 (20 μ M) for 4 h. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. **(c)** HDAC1 and Aurora A compete with each other for binding to Chfr. GST-HDAC1 (purified from *E. coli*) and His-Chfr (purified from insect cells) were incubated with increasing amounts of Flag-Plk1 or Flag-Aurora A for 1 h at 4 $^{\circ}$ C, and pulled down by NTA resin. Precipitates were subjected to SDS-PAGE and examined by

immunoblotting with anti-Flag (Plk1 or Aurora A), anti-GST (HDAC1) or anti-His (Chfr) antibodies. **(d)** The Phe 287 and Met 297 residues in HDAC1 are crucial for its interaction with Chfr. The putative Chfr-binding sequences of Class I HDACs (275–300 amino acids of HDAC1) were aligned (upper panel). HeLa cells were co-transfected with plasmids encoding HisMax-Chfr and Flag-HDAC1 or Flag-HDAC1^{FM}. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies (left panel). His-Chfr was purified from insect cells and the GST-HDAC1 proteins were purified from *E. coli* cells. Purified GST-HDAC1 and His-Chfr were incubated with glutathione Sepharose for 1 h at 4 $^{\circ}$ C. Precipitates were subjected to SDS-PAGE followed by immunoblotting with anti-GST or anti-Chfr antibodies (right panel).

function, we first evaluated whether the deacetylase activity of HDAC1 contributes to its turnover by Chfr. HDAC1^{H141A}, a mutant that lacks deacetylase activity¹³, was transiently expressed in HeLa cells along with Chfr. Immunoprecipitation and western blot analysis revealed that HDAC1^{H141A} bound to (Fig. 3g) and was destabilized by, Chfr (Fig. 3h), suggesting that Chfr can promote turnover of HDAC1 regardless of HDAC1's activity.

To explore the effect of turnover on HDAC1 function, we examined the effect of gain- or loss-of-function of Chfr on HDAC1-dependent transcriptional regulation. A vector encoding HAT(p300) was co-transfected with Flag-HDAC1^{WT}, Flag-HDAC1^{FM} and/or Flag-Chfr^{WT}. Histone H4 acetylation stimulated by HAT(p300) was markedly diminished by HDAC1^{WT} expression, but this decline was reversed by expression of Chfr^{WT}. However, the decrease in histone H4 acetylation caused by HDAC1^{FM} was not reversed by Chfr^{WT} (Fig. 4a). As it was reported that HDAC1 also deacetylates non-histone proteins¹⁴, we evaluated the effects of Chfr on p53 deacetylation. HAT(p300)-stimulated acetylation of p53 was diminished by HDAC1, but this decline was also reversed by

Chfr (Supplementary Information, Fig. S6). These results suggest that Chfr controls the deacetylase activity of HDAC1 towards both histone and non-histone proteins.

Given that Chfr-mediated degradation of HDAC1 modulates acetylation of HDAC1 substrates, we next examined whether Chfr derepresses genes regulated by HDAC1. Transcription of *p21* is inhibited by HDAC1 activity¹⁵. Real-time PCR (rtPCR; Fig. 4b) and RT-PCR (Fig. 4c) revealed that the amount of *p21* mRNA was increased on expression of Chfr, whereas the Chfr^{I306A} mutant had no effect. Similarly, the level of the endogenous p21 protein was increased on expression of Chfr but not Chfr^{I306A}. To evaluate in more detail the effect of Chfr on transcriptional regulation by HDAC1, we used a *p21-luciferase* reporter gene. In HeLa cells, Chfr^{WT}, HDAC1-specific shRNA (shHDAC1) or the HDAC inhibitor TSA enhanced expression of the reporter gene. By contrast, the expression of Chfr^{I306A} or Chfr Δ CR had essentially no effect. The similar effects of Chfr, shHDAC1 and TSA on the *p21* reporter suggest that Chfr may induce p21 expression by repressing HDAC1. To test this hypothesis directly, we silenced

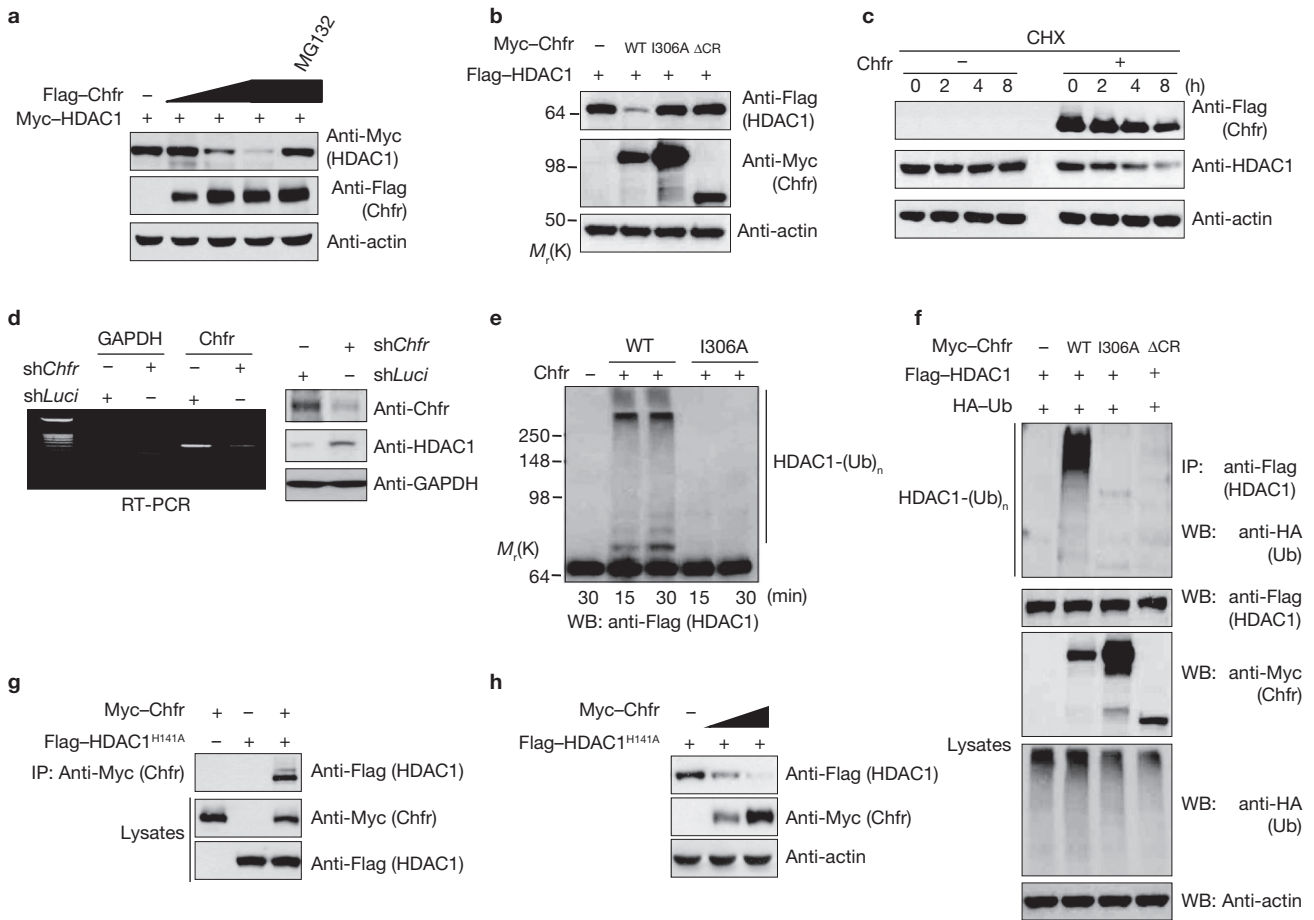


Figure 3 Chfr negatively regulates and ubiquitylates HDAC1 *in vitro* and *in vivo*. (a) HeLa cells were co-transfected with Myc-HDAC1 (0.5 μ g) and Flag-Chfr (0.5, 1 and 2 μ g) plasmids. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-Flag or anti-Myc antibodies. After transfection for 24 h, the cells were treated with MG132 (20 μ M) for 4 h. (b) Flag-HDAC1 (0.5 μ g) was co-transfected into HeLa cells with 2 μ g of Chfr^{WT}, Chfr^{I306A} or Chfr^{ΔCR} expression vectors. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with indicated antibodies. Uncropped images of the scans are shown in Supplementary Information, Fig. S8. (c) HeLa cells were transfected with Flag-Chfr. At 24 h after transfection, the cells were treated with cycloheximide (200 μ g ml⁻¹, CHX) for 8 h. Cell lysates were subjected to immunoblotting with anti-Flag or anti-HDAC1 antibodies. (d) HEK293T cells were transfected with 2 μ g of shChfr or a negative control vector (shLuci). After incubation for 72 h, total RNA was prepared and subjected to RT-PCR for Chfr and GAPDH mRNA. Cell lysates were subjected

to immunoblotting in parallel with anti-Chfr or anti-HDAC1 antibodies. (e) The purified Flag-HDAC1 (1 μ g) from HeLa cells was incubated at 37 °C for the indicated times in the presence or absence of His-Chfr or His-Chfr^{I306A} (3 μ g each) purified from insect cells. After incubation, each sample was subjected to SDS-PAGE and immunoblotting with anti-Flag antibody. (f) HeLa cells were transfected with plasmids encoding Flag-HDAC1, HA-ubiquitin and Myc-tagged Chfr (Chfr^{WT}, Chfr^{I306A}, or Chfr^{ΔCR}). After incubation for 24 h, cells were treated with MG132 (2 μ M) for 12 h and then collected for analysis. (g) HeLa cells were co-transfected with Myc-Chfr and Flag-HDAC1^{H141A}. After transfection for 24 h, the cells were treated with MG132 (20 μ M) for 4 h. Cell lysates were immunoprecipitated with anti-Myc antibody, and immunoblotted with the indicated antibodies. (h) HeLa cells were co-transfected with Flag-HDAC1^{H141A} (0.5 μ g) and Myc-Chfr (0.5 and 2 μ g) plasmids. The stability of HDAC1^{H141A} was analysed by SDS-PAGE and immunoblotting with anti-Flag or anti-Myc antibodies.

HDAC1 by RNAi in HeLa cells transfected with a Chfr expression vector. Compared with cells expressing Chfr^{WT} alone, no further induction of p21 expression was seen (Fig. 4d). Moreover, Chfr reversed the repressive effect of HDAC1^{WT}, but could not counteract HDAC1^{FM}. These results indicate that Chfr does indeed activate p21 expression by binding to and mediating the turnover of HDAC1.

The role of HDAC1 in cellular function is very complex. HDAC inhibitors arrest the cell cycle in the G1 or G2 phase^{15,16}. In the absence of HDAC1, cells arrest either in the G1 phase or at the G2-M phase transition, resulting in loss of mitotic cells, cell growth inhibition and an increase in the percentage of apoptotic cells¹⁷. As our data suggest that Chfr promotes p21 gene expression by downregulating HDAC1, we examined the effect of Chfr expression on cell-cycle progression.

HeLa cells transfected with Flag-Chfr were cultured for various times and subjected to FACS (fluorescence-activated cell sorting) analysis to determine their DNA content (Fig. 4f). The fraction of cells in G1 and the level of p21 were significantly increased by 48 h in culture. Consistent with the hypothesis that Chfr expression arrests cells in G1, immunofluorescence confirmed that cells expressing Flag-Chfr contained little or no phospho-histone H3 and cyclin B after 48 h in culture (Fig. 4f). Both the fraction of G1-phase cells and p21 levels decreased at later time-points, probably owing to an increase in the number of cells that were not transfected with Flag-Chfr, as Chfr levels also decreased at 72 h. These results indicate that Chfr over-expression leads to cell-cycle arrest in G1. Similar results were obtained with the dominant-negative HDAC1^{H141A} (Supplementary Information, Fig. S7a-c). Collectively,

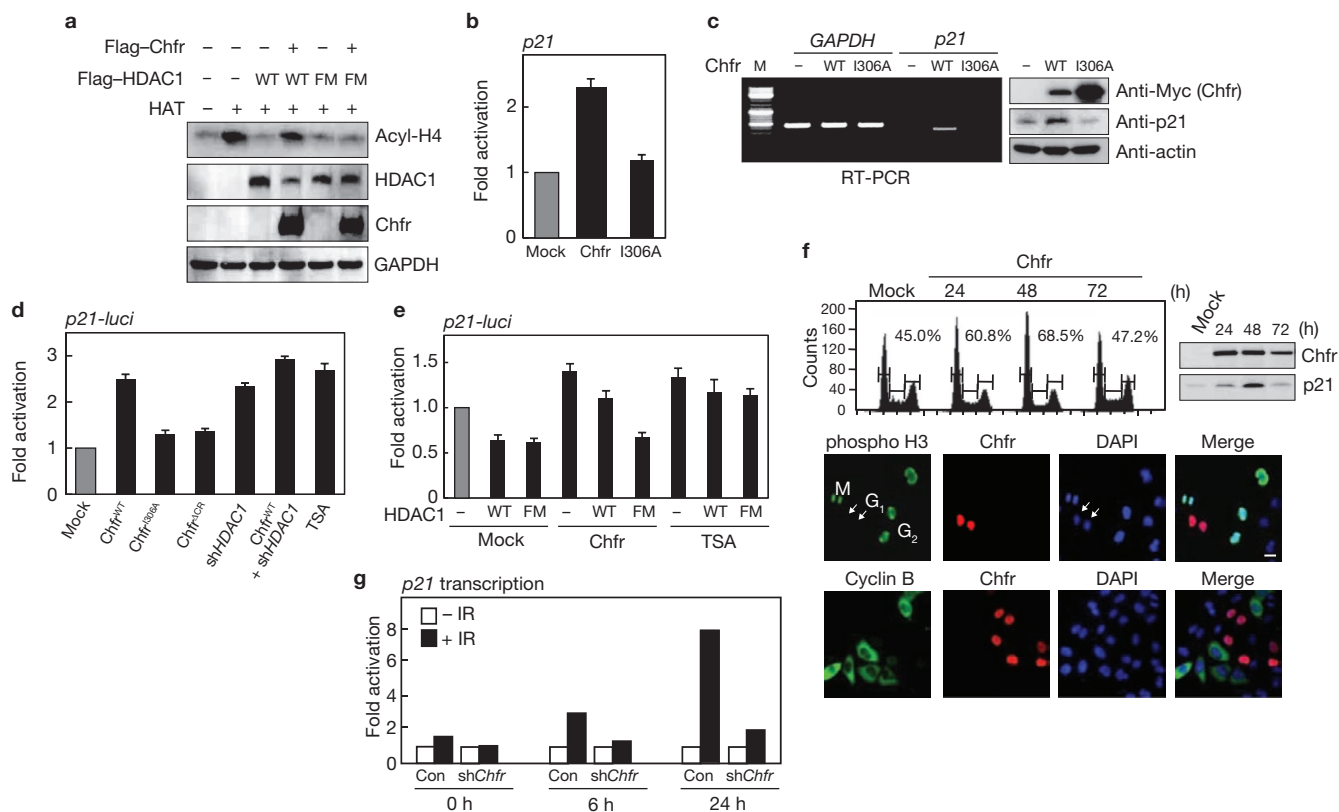


Figure 4 Chfr stimulates the *p21* transcription that was repressed by HDAC1. (a) HEK293T cells were co-transfected with HAT(p300), Flag-HDAC1^{WT}, Flag-HDAC1^{FM} and/or Flag-Chfr. After incubation for 24 h, cell lysates were analysed by immunoblotting with anti-Flag antibodies. Histones were purified as described previously²⁵, and acetylated histone was detected by an anti-Acyl-H4 antibody. Uncropped images of the scans are shown in Supplementary Information, Fig. S8. (b, c) HeLa cells were transfected with 2 μ g of empty, Chfr or Chfr^{I306A} vector. After incubation for 48 h, total RNA was prepared. Real-time PCR (b) and RT-PCR (c) were carried out using specific primers for *p21* and *GAPDH* mRNAs (mean \pm s.d., $n = 3$). The corresponding cell lysates were subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies. Uncropped images of the scans are shown in the Supplementary Information, Fig. S8. (d, e) Chfr relieves transcriptional

repression on a *p21*-luciferase reporter. HeLa cells were transfected with the indicated vectors (d). After incubation for 48 h, luciferase activity was measured and normalized by β -galactosidase assay (e). Cells were treated for 2 h with TSA (5 μ M) before assay (mean \pm s.d., $n = 3$). (f) HeLa cells transfected with Flag-Chfr or an empty vector were cultured for the indicated times and subjected to FACS analysis to determine their DNA content. They were also subjected to immunoblot analysis with anti-Flag or anti-p21 antibodies. For immunocytochemistry, HeLa cells were transiently transfected with Flag-Chfr. After incubation for 48 h, cells were stained with anti-Flag (Chfr), anti-phospho-histone H3 or cyclin B antibodies. Scale bars represent 10 μ m. (g) HEK293T cells were irradiated with IR (15 Gy) and incubated for the indicated times. Total RNA was prepared from cells and real-time PCR was then carried out using specific primers for *p21* and *GAPDH*.

these results suggest that Chfr over-expression causes p21-dependent cell-cycle arrest in G1 and that Chfr may contribute to checkpoint function during interphase of the cell cycle as well as during mitosis.

To further clarify the physiological role of Chfr in *p21* gene expression, HEK293T cells transfected with either shChfr or a control vector, were irradiated with ionizing radiation (IR), incubated for various periods and subjected to real-time PCR. After exposure to IR for 24 h, the transcription level of *p21* showed an 8-fold increase in the control cells, but not in the shChfr cells (Fig. 4g). Our results suggest that Chfr can regulate progression through different stages of the cell cycle by controlling the turnover of different proteins at each stage.

Although Chfr is ubiquitously expressed in normal tissues, it is frequently downregulated in human cancers³, mainly because of hypermethylation of its promoter region^{18–20}. To address whether Chfr participates in tumour development, we assessed the expression level of Chfr and HDAC1 in human prostate (PC-3) or breast (MCF7) cancer cells. Whereas Chfr was expressed in normal, non-metastatic prostate (RWPE1) and breast (MCF10A) cells, little or no Chfr was observed in

the metastatic PC-3 and MCF7 cancer cells. Interestingly, the levels of HDAC1, Plk1 and Aurora A, all potential substrates of Chfr, were much higher in metastatic cancer cells, compared with normal cells (Fig. 5a). In addition to its role in p21 expression, HDAC1 may also have an important role in tumour metastasis through its ability to repress expression of the metastasis suppressor gene *KAI1* (ref. 12) and the invasion suppressor gene *E-cadherin*^{21,22}. To evaluate whether Chfr could have a role in metastasis, we examined whether it influenced the expression of *KAI1* or *E-cadherin* in metastatic cancer cells. using real-time PCR in MCF7 cells (which do not express endogenous Chfr) revealed that expression of both *KAI1* and *E-cadherin* mRNA was markedly stimulated by Chfr. Consistent with increased transcription of *KAI1* and *E-cadherin*, levels of the KAI1 and E-cadherin proteins were also elevated in cells expressing Chfr (Fig. 5b). To determine the potential effect of Chfr and HDAC1 on metastatic capacity, we forced expression of Chfr or downregulated HDAC1 expression in MCF7 cells and evaluated the invasive activity of the cells in a Matrigel invasion assay²³. Over-expression of Chfr, RNAi-mediated knockdown of *HDAC1* or treatment with TSA decreased the

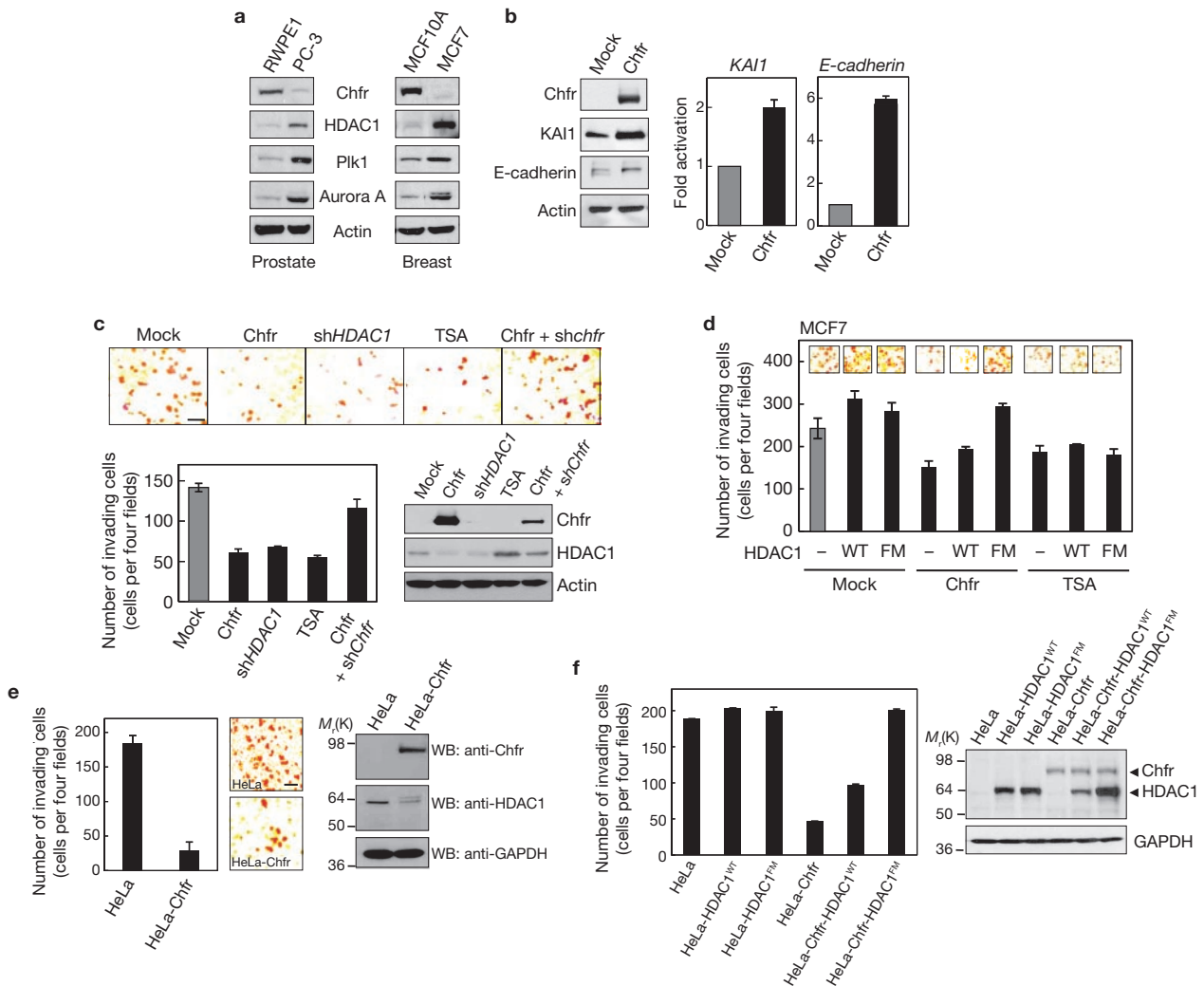


Figure 5 Chfr modulates the invasive activity of metastatic cancer cells. (a) Immunoblot analysis with anti-Chfr or anti-HDAC1 antibodies indicates that endogenous HDAC1 is highly stabilized in PC-3 or MCF7 metastatic cancer cells, in which Chfr is not expressed. RWPE1 or MCF10A cells are normal, non-metastatic, prostate or breast cells, respectively. (b) MCF7 cells transfected with Flag-Chfr were cultured for 48 h and cell lysates were then subjected to immunoblotting with anti-Flag (Chfr), anti-KAI1 or anti-E-cadherin antibodies. Total RNA was prepared from the same cells and subjected to real-time PCR using specific primers for *KAI1*, *E-cadherin* and *GAPDH* (mean \pm s.d., $n = 3$). (c) The invasive activity of MCF7 cells expressing Chfr, shHDAC1, or Chfr/shChfr was assayed in Matrigel chambers. Cells were treated for 2 h with TSA (5 μ M) before assay. The cells that had migrated to the lower chamber of the filter were stained with Giemsa (upper panel) and quantified (lower left panel; mean \pm s.d., $n = 3$). Cell lysates were evaluated by immunoblotting with anti-Flag (Chfr) or anti-HDAC1 antibodies (lower right panel). Scale bars represent 10 μ m. Uncropped images of the scans are shown in Supplementary

Information, Fig. S8. (d) The invasive activity of MCF7 cells expressing HDAC1^{WT}, HDAC1^{FM} and/or Chfr was assayed in Matrigel chambers. Cells were treated for 2 h with TSA (5 μ M) before assay. The cells that had migrated to the lower chamber of the filter were stained with Giemsa and quantified (mean \pm s.d., $n = 3$). (e) The invasive activity of HeLa cells that stably expressed Chfr^{WT} was assayed in Matrigel chambers. The invading HeLa or HeLa cells reconstituted with wild-type Chfr (HeLa-Chfr) were stained with Giemsa (middle panel) and quantified (left panel, mean \pm s.d., $n = 3$). Cell lysates were evaluated by immunoblotting with anti-Chfr or anti-HDAC1 antibodies (right panel). Scale bars represent 10 μ m. (f) The invasive activity of HeLa or HeLa-Chfr cells that stably expressed HDAC1^{WT} or HDAC1^{FM} was assayed in Matrigel chambers. The cells that had migrated to the lower chamber of the filter were stained with Giemsa and quantified (left panel, mean \pm s.d., $n = 3$). Cell lysates were evaluated by immunoblotting with anti-Flag (Chfr or HDAC1) or anti-GAPDH antibodies (right panel). Uncropped images of the scans are shown in Supplementary Information, Fig. S8.

ability of MCF7 cells to invade Matrigel by approximately 60%, compared with control cells (Fig. 5c). Furthermore, expression of HDAC1^{FM} or HDAC1^{WT} slightly increased the invasive activity of MCF7 cells, but this increase was reversed by treatment with TSA (Fig. 5d). Conversely, co-expression of Chfr blocked the increase in invasiveness caused by expression of HDAC1^{WT}, but had no effect on invasiveness induced by expression of HDAC1^{FM}. Taken together, our data suggest that Chfr malfunction in cancer cells can lead to HDAC1 stabilization, which

in turn blocks expression of metastasis suppressors, such as KAI1 and E-cadherin. To further determine whether endogenous Chfr could have a role in metastasis, we evaluated the invasive activity of HeLa cells that had been stably reconstituted with Chfr (HeLa-Chfr). The invasive activity of the HeLa-Chfr cells was about six times lower than that of HeLa cells (Fig. 5e). Furthermore, in MCF10A cells, which naturally express endogenous Chfr, RNAi-mediated knockdown of *Chfr* increased the ability of cells to invade Matrigel, and this increase was partially reversed

by shRNA-mediated depletion of *HDAC1* (Supplementary Information, Fig. S7d). These data suggest that endogenous Chfr affects metastasis, at least in part, by downregulating HDAC1. To strengthen the connection between Chfr-mediated HDAC1 downregulation and cell migration, we evaluated the invasive activity of HeLa or HeLa-Chfr cells, which stably expressed HDAC1^{WT} or HDAC1^{FM} (Fig. 5f). HDAC1^{FM} is resistant to Chfr-mediated downregulation, and HDAC1^{FM}-expressing cells are resistant to Chfr-mediated reduction in cell migration. These data directly demonstrate that the observed changes in cell migration are caused by Chfr-mediated HDAC1 downregulation.

Chfr was originally identified on the basis of its role in mediating cell-cycle checkpoint arrest triggered by mitotic stress²⁴. In our study we found that Chfr mediates ubiquitylation and turnover of HDAC1, which is a key transcriptional regulator. HDAC1 could promote cancer by at least two distinct mechanisms: 1) repression of p21 expression, resulting in the loss of a p21-dependent G1 checkpoint, which arrests cells in the G1 phase in response to DNA damage, and 2) repression of metastasis suppressor gene expression, such as *KAI1* and *E-cadherin*. We show here that forced expression of Chfr in cancer cells, in which its expression is normally extinguished, results in downregulation of HDAC1, upregulation of p21, *KAI1* and *E-cadherin* and reduced invasiveness. Taken together, our data uncover a mechanism by which loss of Chfr activity could contribute to the development of human cancer. □

METHODS

Cell culture and transfection. HEK293T, HeLa and MCF7 cells were grown at 37 °C in DMEM supplemented with 100 U ml⁻¹ penicillin, 1 µg ml⁻¹ streptomycin and 10% FBS. All transfections were carried out using LipofectaminePlus (Invitrogen).

Plasmids and antibodies. *Chfr* complementary DNA (cDNA) was isolated from a human brain cDNA library and cloned into pcDNA-Myc, pcDNA4-HisMax, p3×Flag-CMV10 and pFastBac vector. *Chfr*-specific (sh*chfr*) or *HDAC1*-specific (sh*HDAC1*) shRNA were synthesized and cloned into pSilencer 2.0-U6 (Ambion). Target sequences for shRNAs were as follows: sh*Chfr*, 5'-AGAUACCUCAGGUGCAGGU-3'; sh*HDAC1*, 5'-AAGCAGATGCAGAGATTCAAC-3'. Antibodies used for experiments were as follows: anti-Flag and β-actin (1:5000; Sigma); anti-acetylated histone H4, anti-cyclin B, anti-Plk1 and anti-E-cadherin (1:1000; Upstate Biotechnology); anti-HDAC1, anti-KAI1, anti-Myc and anti-HA (1:1000; Santa Cruz); anti-Xpress (1:5000; Invitrogen); anti-Aurora A and anti-Cyclin A (1:2000; Cell Signaling Technology); peroxidase-conjugated AffiniPure goat anti-rabbit and anti-mouse IgGs (1:5000; Bio-Rad); rabbit polyclonal anti-Chfr antiserum was raised against a recombinant His-Chfr (1:1000).

Assays for protein-protein interaction. Cells were lysed in buffer A consisting of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 mM EDTA and 0.2% Triton X-100 and a 1× protease inhibitor cocktail (Roche). Cell lysates were incubated with anti-M2 resin (Sigma) or 9E10 resin for 2 h at 4 °C. The resins were collected by centrifugation, and then washed three times with buffer A and twice with buffer B, which consisted of 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1 mM EDTA. Bound proteins were eluted by 0.2% SDS and separated by SDS-PAGE, followed by staining with silver or immunoblotting with the appropriate antibodies.

To analyse the interaction of Chfr and HDAC1 *in vitro*, GST-HDAC1 was purified from *E. coli* cells and Flag-Chfr proteins were purified from HEK293T cells with anti-M2 resin. Purified Flag-Chfr (4 µg) and GST-HDAC1 (4 µg) were incubated with glutathione Sepharose for 1 h at 4 °C. The resins were washed three times with buffer A to remove Chfr not bound to HDAC1. Bound proteins were eluted using 0.2% SDS and subjected to SDS-PAGE followed by immunoblotting with appropriate antibodies.

To analyse competition between HDAC1, Plk1 and Aurora A for binding to Chfr *in vitro*, GST-HDAC1 (1 µg, purified from *E. coli*) and His-Chfr (1 µg, purified from insect cell) were incubated with increasing quantities of Flag-PLK1

or Flag-Aurora A (0.5, 1, 2 and 4 µg) for 1 h at 4 °C and pulled down by NTA resin. The resins were washed three times with buffer A to remove HDAC1, Plk1 and Aurora A not bound to Chfr. Precipitates of bound proteins were eluted with 0.2% SDS and subjected to SDS-PAGE followed by immunoblotting with the appropriate antibodies.

Assays for ubiquitylation and pulse-chase analysis. For *in vitro* ubiquitylation assays, purified Flag-HDAC1 (1 µg) from HeLa cells was incubated at 37 °C for the indicated times with E1 (0.5 µg), UbcH5b (0.5 µg), Ub (5 µg), ATP-regenerating system (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 10 mM creatine phosphate, 5 U ml⁻¹ of phosphocreatine kinase and 5 mM ATP) and 1× protease inhibitor cocktail in the presence or absence of His-Chfr (3 µg) purified from insect cells. After incubation, each sample was subjected to SDS-PAGE and immunoblotted with anti-Flag (HDAC1) antibody.

For *in vivo* ubiquitylation assays, HeLa cells were transfected with plasmids encoding Flag-HDAC1, HA-ubiquitin and Myc-Chfr (wild-type, I306A mutant or ΔCR mutant). After incubation for 24 h, cells were treated with MG132 (2 µM) for 12 h. Cells were lysed with buffer A and incubated with anti-M2 resin for 2 h at 4 °C. The resins were collected by centrifugation and washed three times with buffer A. The samples were then subjected to SDS-PAGE followed by immunoblotting analysis.

For pulse-chase analysis, HeLa cells that had been transfected with the appropriate vectors were cultured for 24 h. After incubation, the cells were treated with cycloheximide (200 µg ml⁻¹) for 8 h. Cell lysates then were subjected to immunoblotting with the indicated antibodies.

Reporter assays. Luciferase activity was measured in a luminometer 48 h after transfection and normalized by β-galactosidase expression with a luciferase system (Promega). Values are expressed as mean ± s.d. for at least three independent experiments.

RT-PCR and real-time PCR. Total RNA was extracted from HEK293T and HeLa cells with Ultraspec (BioTex). Reverse transcription reactions were performed using RevertAid M-MuLV reverse transcriptase and oligo (dT) primer, according to the manufacturer's instructions. Real-time PCR was performed using platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and the 7300 real-time PCR system (Applied Biosystems). *Chfr* and *p21* mRNA was amplified with primers against the unique region of *Chfr* and *p21*. *GAPDH* mRNA was amplified as an expression control. We used the following primers: *p21*^{CIP1/WAF1}, forward, 5'-GCAGACCAGCATGACAGATTTC-3'; reverse, 5'-GGATTAGGGCTTCTCTTGGGA-3'; *KAI1*: forward, 5'-TTCTACTTCAACATGGGCAAGC-3', reverse, 5'-AGGTGACCTCAGGGCGATTTC-3'; *E-cadherin*: forward, 5'-TGAAGGTGACAGAGCCTCTGGAT-3', reverse, 5'-TGGGTGAATTCGGGCTTGT-3'.

Flow cytometry. HeLa cells were transfected with an empty vector, Flag-Chfr or Flag-HDAC1^{H141A} in 60-mm dishes. After incubation, cells were collected and washed twice with PBS. The cells were fixed by treatment with 1 ml of 70% ethanol, gently vortexed and kept at 4 °C until used. Fixed cells were washed once with PBS and resuspended in a propidium iodide solution (10 µg ml⁻¹) containing 4% NP-40 buffer and RNase A (250 µg ml⁻¹). Propidium iodide-stained cells were then analysed for their DNA content by using a FACS instrument (BD Biosciences).

Immunocytochemistry. HeLa cells plated on gelatin-coated coverslips were fixed with 2% formaldehyde in PBS for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS. All subsequent dilutions and washes were performed with PBS containing 0.1% Triton X-100 (PBS-T). Non-specific binding sites were saturated by incubation for 30 min with a blocking solution consisting of 10% goat serum, 1% BSA and 1% gelatin in PBS. Cells were incubated with primary antibody for 1 h and washed with PBS-T four times at 10-min intervals. They were then incubated with FITC- or TRITC-conjugated secondary antibody for 1 h and washed four times. DAPI was used to counterstain the nuclei. The coverslips were mounted in Vectashield and cells were visualized under a Zeiss AxioPlan II microscope.

Matrigel invasion assay. MCF7, MCF10A or HeLa cells were loaded into the top of a 24-well Matrigel invasion chamber assay plate (BD Biocoat). Conditioned

DMEM medium containing 15% FBS was added to the bottom chamber as a chemottractant. After 22 h incubation, the cells that had migrated to the lower chamber of the filter were fixed with 100% methanol, stained with Giemsa and quantified by counting the total number of cells in four independent areas. All experimental studies were performed in accordance with the manufacturers' protocols.

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

We thank S. H. Baek (SNU) for reagents. This work was supported by grants from the Korea Science and Engineering Foundation (M10533010001-07N3301-00110), the SRC program (R11-2005-009-02002-0), the Korea Research Foundation (KRF-2002-015-CS0069) and the BK21 program. Y.E.K. was supported by the Seoul Science Fellowship.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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