nature cell biology

Roles of sumoylation of a reptin chromatin-remodelling complex in cancer metastasis

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Defining the functional modules within transcriptional regulatory factors that govern switching between repression and activation events is a central issue in biology. Recently, we have reported the dynamic role of a β-catenin–reptin chromatin remodelling complex in regulating a metastasis suppressor gene *KAI1* (ref. 1), which is capable of inhibiting the progression of tumour metastasis[2–5]. Here, we identify signalling factors that confer repressive function on reptin and hence repress the expression of *KAI1*. Biochemical purification of a reptin-containing complex has revealed the presence of specific desumoylating enzymes that reverse the sumoylation of reptin that underlies its function as a repressor. Desumoylation of reptin alters the repressive function of reptin and its association with HDAC1. Furthermore, the sumoylation status of reptin modulates the invasive activity of cancer cells with metastatic potential. These data clearly define a functional model and provide a novel link for SUMO modification in cancer metastasis.

Deciphering the molecular strategies by which specific signalling pathways regulate biological processes by switching genes to the appropriate 'on' or 'off' state remains an important issue in biology[6–8]. The transcription of most genes in the nucleus is regulated by the coordinate action of cofactors and chromatin remodelling complexes. Reptin has been reported to be a component of ATP-dependent chromatin remodelling complexes that function as antagonistic regulators of the Wnt–β-catenin pathway[9,10]. Reptin is related to the RuvB protein in bacteria, the INO80 ATPase complex in yeast and the polycomb complex in *Drosophila*[11–13]. In mammals, reptin is a component of the Tip60 or p400 complex[14,15].

Recently, we have reported that the transcription of *KAI1* is regulated by Tip60 and β-catenin–reptin complexes[1]. Activation of the Wnt–β-catenin pathway can selectively downregulate a subset of NF-κB target genes, including *KAI1*, and thus provides a specific mechanism of crosstalk between the Wnt–β-catenin pathway and the NF-κB pathway[1]. Because the repressive function of β-catenin is conferred by a reptin chromatin remodelling complex, we asked which specific regulatory signalling factors induce the transcriptional repressive function of reptin and the selection of specific binding partners.

To investigate the function of reptin, a FLAG epitope-tag strategy was used to purify a reptin-containing complex, and liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) analysis was used to identify proteins in the complex (Fig. 1a and see Supplementary Information, Fig. S1a). In addition to pontin, a well-known binding partner of reptin, β-catenin and HDAC1 were also identified, consistent with previously reported components of the Wnt–β-catenin pathway[16,17] and the observation that they function together with reptin to modulate *KAI1* in prostate cancer[1]. Of the remaining proteins, the presence of two small ubiquitin-like modifier (SUMO)-processing enzymes (SUMO–sentrin-specific protease 1 (SENP1) and SUMO1-specific protease 1 (SUSP1)) wass particularly striking (Fig. 1a). The partially overlapping elution peaks of the individual components may suggest the existence of different reptin-containing complexes, rather than one multiprotein reptin complex (Fig. 1b). The association of these polypeptides with reptin was further confirmed by immunoblotting analysis from eluates, which revealed the presence of pontin, β-catenin, histone deaceyltase 1 (HDAC1), SENP1 and SUSP1 in the reptin-containing complex (Fig. 1c). Although reptin has been reported to be a component of the Tip60 or p400 complex[14,15], Tip60 or p400 were not detected in the reptin-containing complex, suggesting that multiple types of reptin-containing complexes may coexist in the cell (Fig. 1c). To validate the unexpected association of desumoylating enzymes with reptin, an immunoprecipitation assay was performed to confirm their association (Fig. 1d, e).

The existence of SUMO processing enzymes in the reptin-containing complex provided speculative information of a functional link between sumoylation and chromatin remodelling-mediated transcriptional regulatory processes. To examine whether SUMO modification is important for the functional regulation of reptin, both *in vivo* and *in vitro* SUMO modification systems were used[18,19]. Addition of SUMO induced the formation of reptin conjugates both *in vivo* and *in vitro* (Fig. 2a, b).

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Received 16 January 2006; accepted 18 April 2006; published online 14 May 2006; DOI: 10.1038/ncb1415

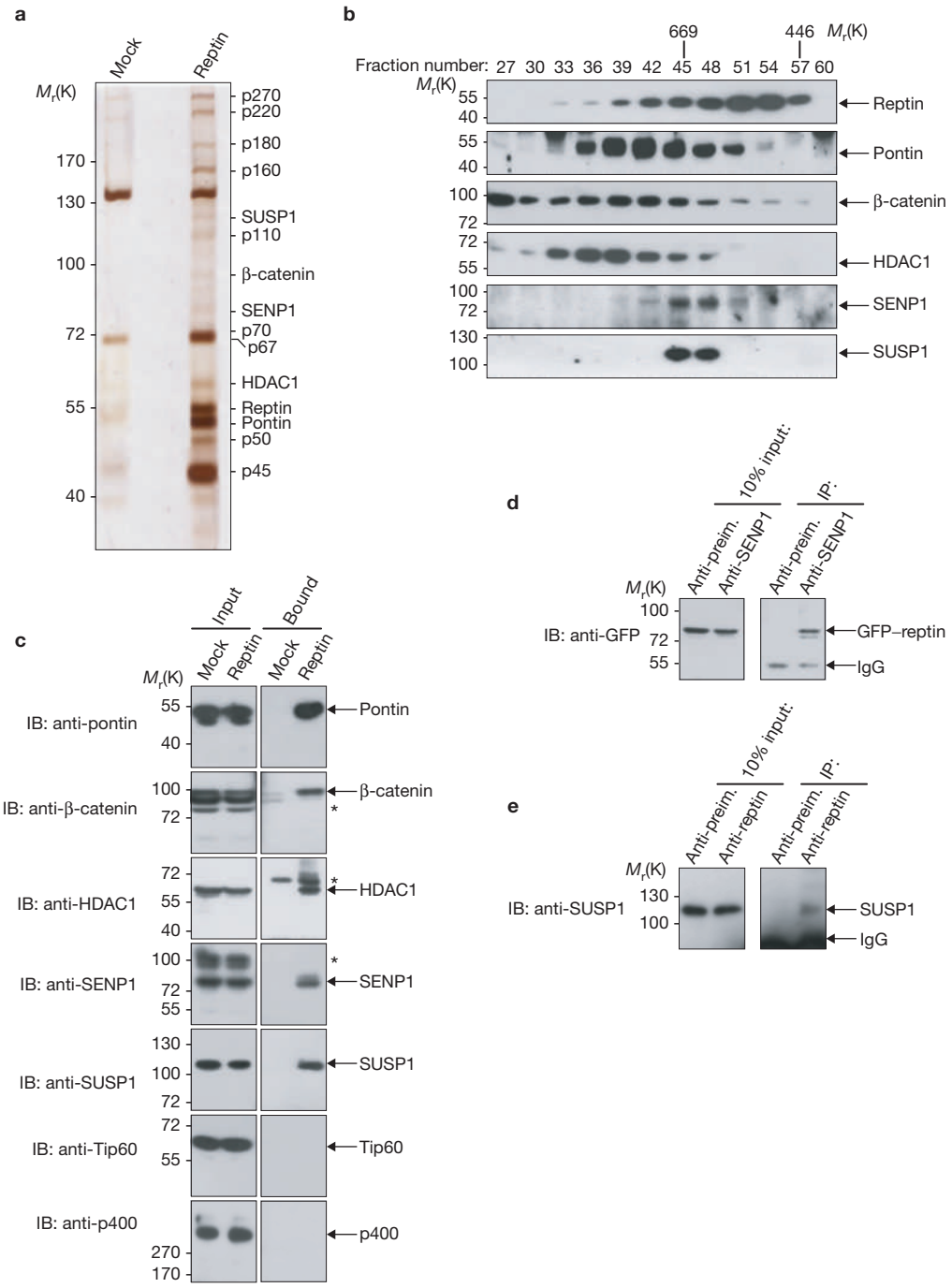

Figure 1 Desumoylating enzymes are components of a reptin-containing complex. (**a**) Purification of a reptin-containing complex. A reptin-containing complex was purified from extracts obtained from 293 cells stably expressing FLAG-tagged reptin. A mock purification from 293 cells stably expressing an empty vector was used as a negative control. Bound proteins were resolved by SDS–PAGE and prepared for LC-MS/MS analysis. (**b**) Sephacryl–S400 gel filtration analysis of the purified reptin-containing complex. Western blot analysis was performed using the indicated antibodies. The numbers represent a fraction number of elutes. (**c**) Immunoblot analysis was performed after purification of a reptin-containing complex. Pontin, β-catenin, HDAC1, SENP1 and SUSP1 were identified. Asterisks indicate non-specific bands. Preim. indicates preimmune IgG. (**d**) Reptin interacts with SENP1. Cell lysates were subjected to immunoprecipitation against either anti-SENP1 or control IgG, and the resultant precipitates were subjected to immunoblotting against anti-GFP. (**e**) Reptin interacts with SUSP1. 293T cell lysates were subjected to immunoprecipitation against either anti-reptin or control IgG, and the resultant precipitates were subjected to immunoblotting against anti-SUSP1.

SUMO modification was also observed with endogenous reptin in the presence of SUMO (Fig. 2c). The identity of the SUMO protein was confirmed by LC-MS/MS analysis (see Supplementary Information, Fig. S1b). To determine which lysine residue(s) are SUMO modified in reptin, we searched for the consensus SUMO targeting sequence, ψKxE (where ψ represents a large hydrophobic amino acid and x represents any amino acid)[20]. The four lysine residues within the identified consensus sequences were individually mutated to arginine (see Supplementary Information, Fig. S2). Among the mutations, K456R abrogated SUMO modification both *in vivo* and *in vitro* (Fig. 2d, e).

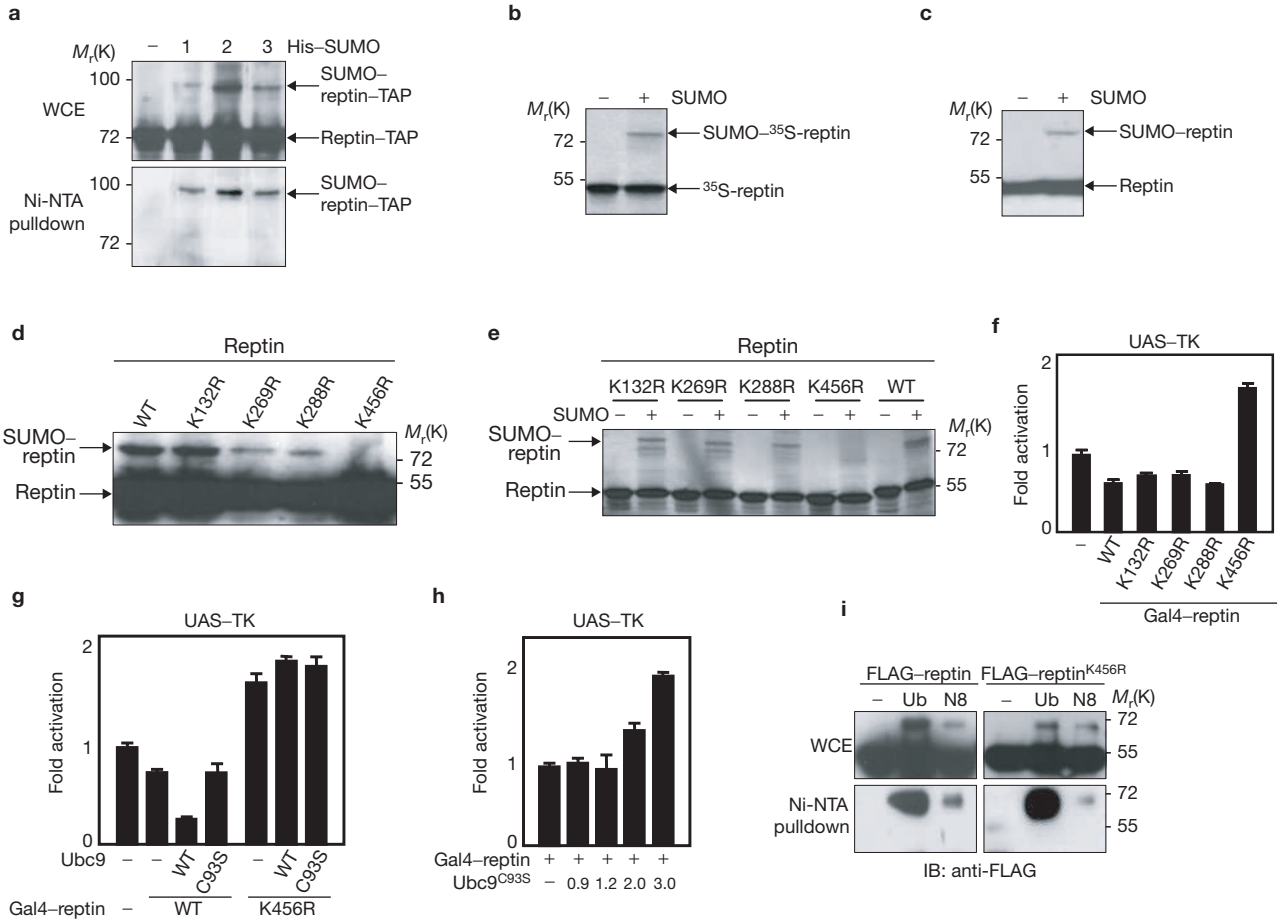

Figure 2 Lys 456 of reptin is critical for SUMO modification. (**a**) Reptin is modified by SUMO *in vivo*. 293T cells were cotransfected with plasmids expressing reptin–TAP, FLAG–Ubc9 and the indicated His–SUMO. Whole cell extracts (WCE) and Ni-NTA affinity-purified precipitates (Ni-NTA pulldown) were analysed by western blotting against anti-protein A to detect TAP. (**b**) *In vitro* modification of reptin by SUMO. ^{35}S-labelled *in vitro* translated reptin was incubated in a sumoylation mix containing purified SAE1–SAE2, Ubc9, and ATP in the absence or presence of SUMO. (**c**) Endogenous reptin is sumoylated in the presence of SUMO. Western blot analysis performed using anti-reptin revealed that overexpression of SUMO increased sumoylation of endogenous reptin protein. (**d**) Lys 456 of reptin is a major SUMO conjugation site. 293T cells were cotransfected with plasmids expressing either wild-type reptin or reptin^{K456R} in the presence of SUMO and Ubc9 followed by western blotting analysis. (**e**) *In vitro* sumoylation assay was conducted with ^{35}S-labelled *in vitro* translated wild-type reptin or reptin^{K456R} as in **b**. (**f**) Reptin^{K456R} relieves transcriptional repression on a UAS–TK–luciferase reporter. Luciferase activity was measured and normalized by β-galatosidase assay. Values are expressed as mean ± s.d. for five independent experiments. (**g**) Ubc9 enhances transcriptional repression of wild-type reptin but not reptin^{K456R} in a luciferase assay. Data are the mean ± s.d. for five independent experiments. (**h**) An excess amount of Ubc9^{C93S} slightly derepressed transcriptional repression of Gal4–reptin on a UAS–TK–luciferase reporter. Values are expressed as means ± s.d. for three independent experiments. (**i**) The K456 residue of reptin is not the major modification site for ubiquitin (Ub) or NEDD8 (N8).

In terms of transcriptional regulation, reptin has been reported to repress β-catenin-mediated transcriptional activation[9]. To gain insight into the role of SUMO modification of reptin, the effect of the modification on transcription by reptin was assessed (Fig. 2f). Gal-fused reptin^{K456R}, which lacks functional SUMO modification, relieved transcriptional repression compared with that of Gal4-fused wild-type reptin in a upstream activation sequence (UAS)–thymidine kinase (TK)–luciferase reporter-gene analysis (Fig. 2f). Furthermore, increase in sumoylation (by overexpressing the SUMO conjugating enzyme, Ubc9) enhanced the Gal4–UAS-mediated transcriptional repression by wild-type reptin, wheras an active-site mutant of Ubc9 (Ubc9^{C93S}) did not (Fig. 2g). Excess expression of Ubc9^{C93S} slightly derepressed Gal4–UAS-mediated transcriptional repression by reptin (Fig. 2h). To confirm that the K456 site was not modified by ubiquitin or Nedd8, ubiquitin and Nedd8 conjugation assays were performed. However, neither wild-type reptin, nor reptin^{K456R}, exhibited any difference in a ubiquitin or Nedd8 conjugation assay (Fig. 2i).

Sumoylation is a dynamic process and the removal of SUMO is conducted by various SUMO processing enzymes[21–24]. As SENP1 and SUSP1 had been identified from purification of a reptin-containing complex, their possible effects on reptin-mediated transcriptional repression was examined (Fig. 3a). SENP1 reversed the transcriptional repression of Gal4–reptin, whereas neither a mutated SENP1 (SENP1^{C603A}), nor other desumoylating enzymes, relieved reptin-mediated transcriptional repression (Fig. 3a). To evaluate the effects of the desumoylation of reptin on the well-known canonical β-catenin–T-cell factor (TCF) target genes[25], reporter gene analysis was performed using the *cyclin D1* promoter (Fig. 3b) and the TCF–lymphoid enhancer factor (LEF)-responsive (TOPFLASH) reporter (Fig. 3c). Coexpression of reptin with TCF and β-catenin reduced both the *cyclin D1* promoter-driven and the TOPFLASH-driven luciferase activity compared with that of TCF and β-catenin alone. Expression of SENP1 antagonized reptin-mediated repression, whereas SENP1^{C603A} did not (Fig. 3b, c).

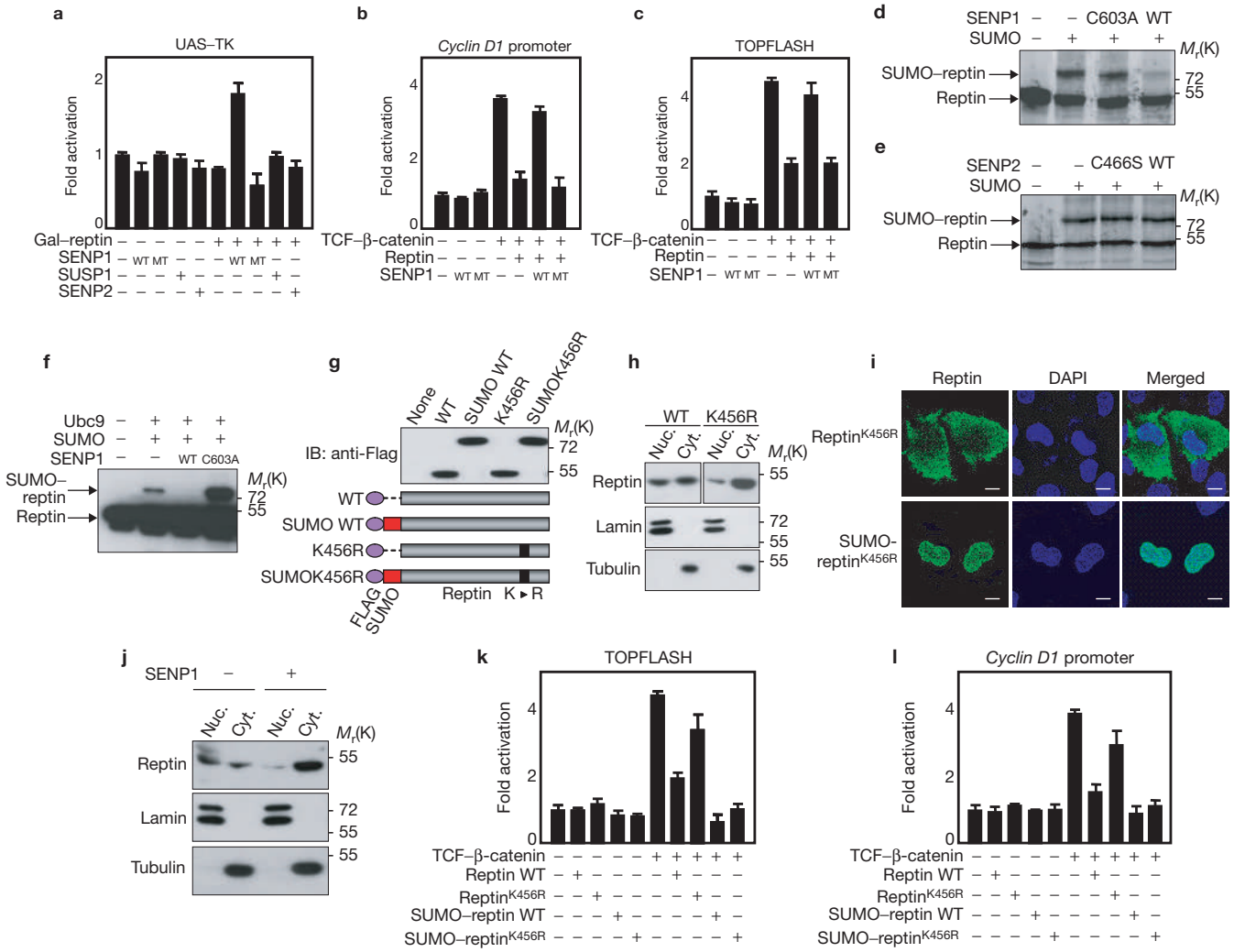

Figure 3 Desumoylation of reptin by SENP1 both *in vitro* and *in vivo*. (**a**) The modulation of the transcriptional repressive function of reptin by SENP1, SUSP1 or SENP2 was assessed by luciferase reporter assays. 293T cells were cotransfected with a UAS–TK–luciferase reporter, Gal4–reptin, SENP1, SUSP1 or SENP2. SENP1^{C603A} (MT, mutant) indicates the active site mutant that has impaired enzymatic activity. Values are expressed as mean ± s.d. for five independent experiments. (**b**) 293T cells were cotransfected with a *cyclin D1* promoter reporter, TCF, β-catenin, reptin, and SENP1 (WT) or SENP1^{C603A} (MT) expression plasmids. A luciferase assay was performed and the values are expressed as means ± s.d. for five independent experiments. (**c**) A luciferase reporter assay was conducted with the TOPFLASH reporter. Wild-type SENP1 but not SENP1^{C603A} relieved the reptin-mediated repression. Data are the mean ± s.d. for five independent experiments. (**d**) SENP1 desumoylates reptin *in vitro*. 293T cells were transfected with Myc–SENP1, Myc–SENP1^{C603A} or empty vector and then immunoprecipitated with anti-cMyc antibody. Each immunoprecipitate was incubated with ^{35}S-labelled *in vitro*-sumoylated reptin. (**e**) *In vitro* sumoylated reptin was not desumoylated by SENP2. (**f**) SENP1 desumoylates reptin *in vivo*. Whole cell extracts were assessed by western blotting analysis against anti-reptin after transfection of the indicated plasmids. (**g**) Immunoblot analysis indicates expression of FLAG-tagged reptin, SUMO-fused reptin, reptin^{K456R} or SUMO-fused reptin^{K456R}. Their schematic representations are also indicated. (**h**) Nuclear and cytoplasmic fractionation of wild-type reptin and reptin^{K456R}. Anti-lamin was used as a nuclear marker and anti-tubulin was used as a cytoplasmic marker. (**i**) Subcellular localization of FLAG-tagged reptin^{K456R} and SUMO-fused reptin^{K456R} (green). Nuclei were visualized by DAPI staining (blue). The scale bar represents 10 μm. (**j**) Biochemical fractionation of reptin in the presence or absence of SENP1. (**k**, **l**) An amino-terminal fusion of SUMO enhances the transcriptional repression function of reptin. Luciferase reporter assays were conducted after cotransfection of a TOPFLASH luciferase reporter (**k**) or a *cyclin D1* promoter reporter (**l**) with TCF, β-catenin and each reptin mutant. Values are expressed as mean ± s.d. for five independent experiments.

To determine whether the increase in transcriptional activation mediated by SUMO processing enzymes was due to the removal of SUMO from reptin, a desumoylation assay was performed *in vitro* and *in vivo* (Fig. 3d–f). SENP1 hydrolysed the SUMO-modified reptin, whereas neither SENP1^{C603A} (Fig. 3d) nor SENP2 (Fig. 3e) were able to hydrolyse SUMO from reptin *in vitro*. Expression of wild-type SENP1 also led to the loss of the SUMO-conjugated forms of reptin *in vivo*, demonstrating that reptin is an *in vivo* substrate for the desumoylating activity of SENP1 (Fig. 3f). To further prove that SENP1 interacts with reptin and modulates the sumoylation status of reptin, a SENP1-containing complex was purified and shown to contain reptin (see Supplementary Information, Fig. S3).

We next examined whether SUMO conjugation of reptin is associated with modulation of subcellular localization. A constitutively sumoylated form of reptin was generated and expression was confirmed by immunoblot analysis (Fig. 3g). Reptin was observed in both nuclear and cytoplasmic locations, whereas reptin^{K456R} displayed more prominent cytoplasmic localization than wild-type reptin (Fig. 3h). However,

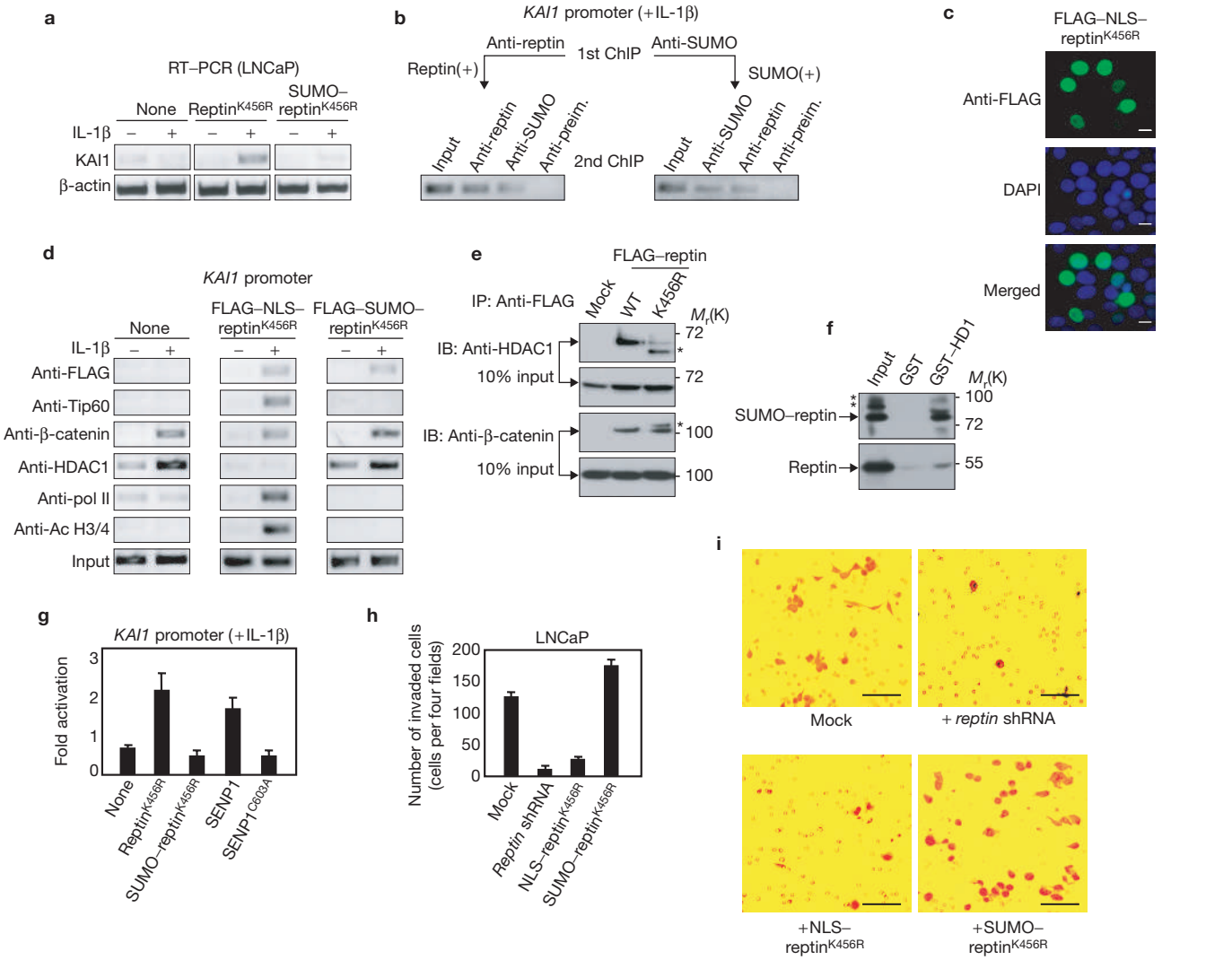

Figure 4 Sumoylation status of reptin affects the metastatic potential of KAI1. (**a**) RT–PCR of the *KAI1* transcript following IL-1β treatment and after overexpressing either reptin^{K456R} or SUMO-reptin^{K456R} in LNCaP cells. (**b**) Two-step ChIP assay with anti-reptin and anti-SUMO indicates that SUMO-modified reptin is recruited onto the *KAI1* promoter where it confers a repressive function. (**c**) Targeting of reptin^{K456R} by a heterologous NLS into the nucleus. NLS-reptin^{K456R} exhibited an almost exclusive nuclear staining pattern. The scale bar represents 10 μm. (**d**) ChIP assay on the *KAI1* promoter in untransfected cells (none) and cells transfected with either FLAG–NLS–reptin^{K456R} or Flag–SUMO–reptin^{K456R} in the presence or absence of IL-1β. (**e**) Coimmunoprecipitation assay to verify interaction of reptin or reptin^{K456R} with HDAC1 or β-catenin. The ssterisk indicates a non-specific band. (**f**) *In vitro* association experiment between sumoylated or non-sumoylated reptin and HDAC1. Sumoylated reptin exhibited stronger binding to HDAC1. The asterisk indicates a non-specific band. (**g**) Cotransfection of reptin^{K456R} activated the *KAI1* promoter reporter aproximately 2.5-fold, whereas SUMO-reptin repressed the *KAI1* promoter reporter in LNCaP cells in the presence of IL-1β. Values are expressed as mean ± s.d. for five independent experiments. (**h**, **i**) The invasive activity of LNCaP cells expressing shRNA against *reptin*, NLS–reptin^{K456R} or SUMO–reptin^{K456R} assayed in Matrigel chambers. Values are expressed as mean ± s.d. for three independent experiments.

SUMO-fused reptin^{K456R} resulted in exclusive nuclear localization (Fig. 3i). Overexpression of SENP1 resulted in increased cytoplasmic accumulation of reptin, which mimics the localization pattern of reptin^{K456R} in cells (Fig. 3j). Together, these data indicate that SUMO conjugation of reptin seems to be an active control mechanism for the transcriptional repressive function of reptin, by recruiting reptin to the nucleus.

Next, reporter-gene analysis was used to examine whether wild-type reptin, reptin^{K456R}, SUMO-fused reptin or SUMO-fused reptin^{K456R} influence the transactivation potential of the β-catenin (Fig. 3k, l). Expression of reptin^{K456R} reversed the inhibitory effect of reptin on β-catenin-mediated transactivation potential, whereas SUMO-fused reptin (which mimics constitutive sumoylation[26–28]) repressed the TCF–β-catenin-mediated transactivation potential almost completely (Fig. 3k, l). A FOPFLASH reporter containing a mutated TCF4-consensus binding site was used as a negative control (see Supplementary Information, Fig. S4). These data indicate that covalent attachment of SUMO is sufficient, at least in part, for nuclear retention of reptin and for maintaining the β-catenin–reptin-mediated transcriptional repression on Wnt–β-catenin target promoters.

A number of oncogenes undergo sumoylation, but the functional significance of SUMO modification of these molecules in cancer has not yet been extensively studied[29]. To address the potential effects of the sumoylation of reptin on the invasion step of cancer metastasis, *KAI1*, a metastasis suppressor gene, was used to

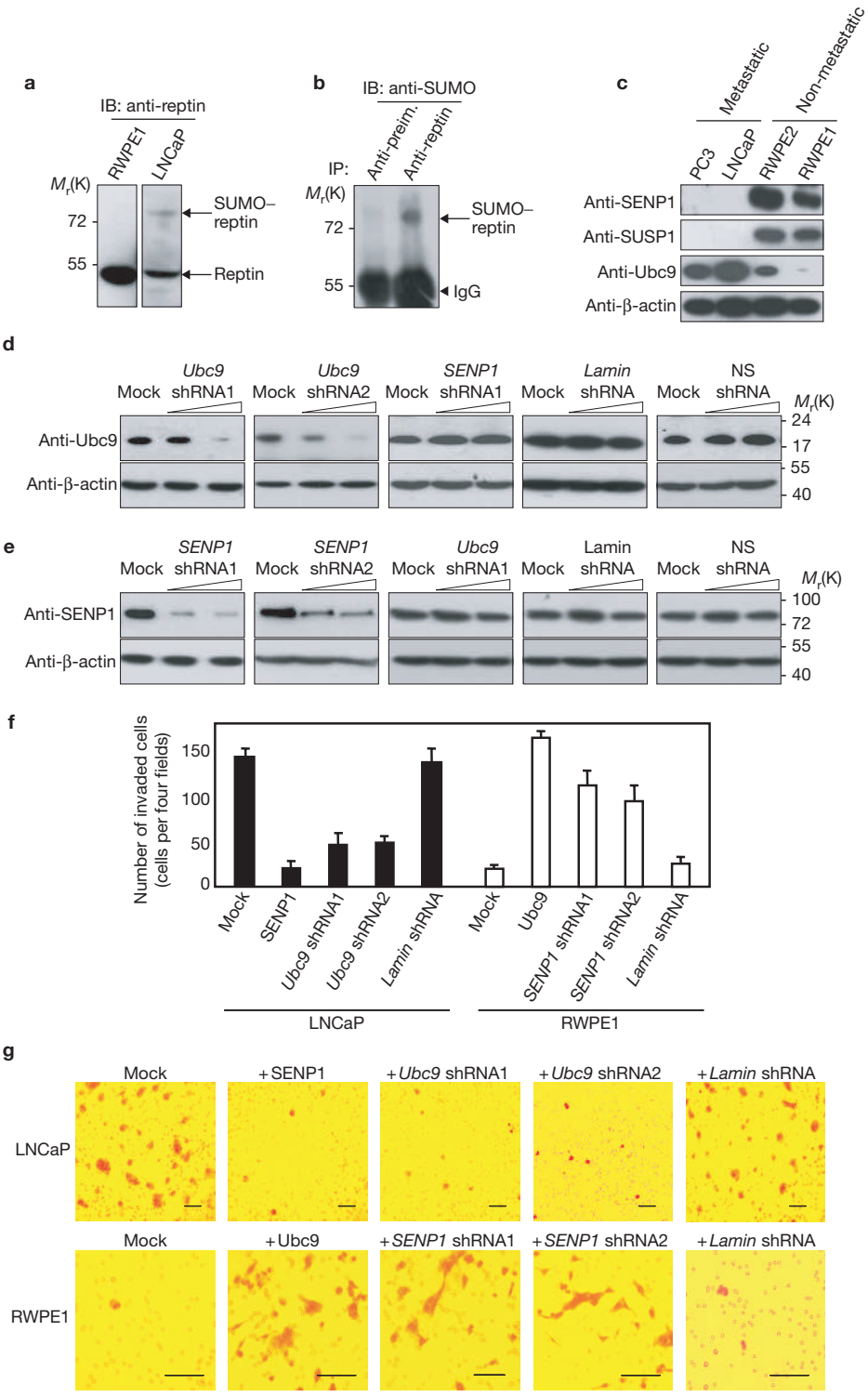

Figure 5 SENP1 and Ubc9 modulate the invasive activity of LNCaP and RWPE1 cells. (**a**) Immunoblot analysis with anti-reptin indicates that endogenous reptin is highly sumoylated in LNCaP metastatic cancer cells compared with RWPE1 cells. (**b**) *In vivo* coimmunoprecipitation assay revealed that endogenous reptin and SUMO are associated in LNCaP cells. (**c**) Immunoblot analysis of SENP1, SUSP1 and Ubc9 in various non-metastatic and metastatic cancer cell lines. (**d**, **e**) The ablation of *Ubc9* (**d**) or *SENP1* (**e**) by each shRNA, together with negative control shRNAs and confirmation of its knockdown effect by immunoblot analysis. (**f**, **g**) Overexpression of either SENP1 or shRNAs against *Ubc9* in IL-1β-treated LNCaP cells decreased Matrigel invasion, whereas RWPE1 cells expressing Ubc9 or shRNAs against *SENP1* increased the Matrigel invasion, as assayed in Matrigel chambers. Lamin A/C shRNA was used as a negative control. Values are expressed as mean ± s.d. for three independent experiments in **f**. The cells that had migrated to the lower chamber of the filter were stained with Giemsa as shown in **g**. The scale bar represents 100 μm.

examine the transcriptional regulation mechanism and metastatic potential. Analysis of *KAI1* mRNA by RT–PCR revealed that increasing reptin^{K456R} expression restored *KAI1* expression, whereas increasing SUMO-fused reptin levels maintained down regulation of *KAI1* in the presence of IL-1β in LNCaP cells (prostate cells with metastatic behaviour; Fig. 4a). To address the detailed molecular mechanism of differential expression of *KAI1* in metastatic cancer cells depending on the SUMOylation status of reptin, a two-step chromatin immunoprecipitation (ChIP) assay was performed to monitor whether SUMO-modified reptin is present on the *KAI1* promoter under repressive conditions *in vivo* (Fig. 4b). In a reciprocal experiment, the *KAI1* promoter was immunoprecipitated — clearly indicating that SUMO-modified reptin is present on the *KAI1* promoter under repressive conditions (Fig. 4b).

ChIP assays were also used to monitor cofactor occupancy on the *KAI1* promoter during transcriptional regulation. As reptin^{K456R} was predominantly localized in the cytoplasm, reptin^{K456R} was targeted to the nucleus using a nuclear localization signal (NLS) sequence (Fig. 4c). ChIP assays were performed using either NLS-targeted reptin^{K456R} or SUMO-fused reptin^{K456R} (Fig. 4d). Coincident with the downregulation of *KAI1* mRNA levels, the β-catenin–reptin complex is recruited onto the promoter, together with HDAC1 (which underlies repression), and thus the *KAI1* promoter remains inactive. Surprisingly, overexpression of NLS-reptin^{K456R} converted the *KAI1* promoter from a repressed to activated state with concomitant recruitment of the coactivator Tip60, whereas SUMO-fused reptin^{K456R} resulted in the downregulation of *KAI1* by maintaining the recruitment of the β-catenin–reptin–HDAC1 repressor complex, suggesting that sumoylation of reptin is important for maintaining its repressive function (Fig. 4d). Interestingly, expression of NLS-reptin^{K456R} resulted in failure to recruit HDAC1 and activated the *KAI1* promoter (Fig. 4d). As it has been reported that SUMO attachment changes the binding specificity of proteins[30,31], we examined whether reptin^{K456R} exhibits altered binding specificity towards HDAC1 or β-catenin using an immunoprecipitation assay (Fig. 4e). Both wild-type reptin and reptin^{K456R} bound to β-catenin, whereas reptin^{K456R} exhibited very weak binding to HDAC1 compared with that of wild-type reptin (Fig. 4e). To confirm these observations, GST-pulldown experiments were performed with purified sumoylated or non-sumoylated reptin proteins and HDAC1 *in vitro*, and it was found that sumoylated reptin bound more strongly to HDAC1 than non-sumoylated reptin (Fig. 4f). Also, a luciferase assay, using the *KAI1* promoter as a reporter, was performed after transfection of either reptin^{K456R} or SUMO-fused reptin in the presence of IL-1β (Fig. 4g). The introduction of either reptin^{K456R} or SENP1 activated the *KAI1* promoter reporter in LNCaP cells, whereas SUMO-fused reptin did not (Fig. 4g). These data provide evidence that SUMO modification of reptin is important for maintaining and exerting β-catenin–reptin-mediated repression. A Matrigel invasion assay was then used to examine whether the expression of reptin^{K456R} or SUMO-fused reptin^{K456R} would actually modulate the metastatic potential of invasive prostate cancer cells (Fig. 4h, i). LNCaP metastatic prostate cancer cell lines that stably expressed either small hairpin RNA (shRNA) against *reptin*, NLS-reptin^{K456R} or SUMO-fused reptin^{K456R} were generated. Overexpression of *reptin* shRNA decreased Matrigel invasion of LNCaP cells by approximately 80% compared with control cells (Fig. 4h, i). Expression of NLS-reptin^{K456R} significantly decreased Matrigel invasion (up to 80%), whereas expression of SUMO-fused reptin^{K456R} increased Matrigel invasion (Fig. 4h, i). These data indicate that changes in the sumoylation status of reptin may affect the metastatic potential caused by KAI1.

The levels of endogenously sumoylated reptin proteins were compared using immunoblot analysis against anti-reptin IgG in RWPE1 (normal prostate epithelial cells) and LNCaP cell extracts. This analysis revealed higher levels of reptin sumoylation in LNCaP cells compared with RWPE1 cells (Fig. 5a). *In vivo* coimmunoprecipitation assays indicated that endogenously expressed reptin is sumoylated in LNCaP cells (Fig. 5b). To test whether changes in the expression and/or activities of SUMO-conjugating enzymes and desumoylating enzymes would have a significant impact on the sumoylation status of reptin, as well as the functional regulation of reptin, immunoblot analysis was used to compare the expression levels of Ubc9, SENP1 and SUSP1 in RWPE1 and LNCaP cells (Fig. 5c). Immunoblot analysis revealed that SENP1 and SUSP1 expression was decreased, whereas Ubc9 expression levels were increased in metastatic cancer cells (Fig. 5c).

To further examine whether Ubc9 and SENP1 modulate the invasive activity of LNCaP and RWPE1 cells in a Matrigel invasion assay, expression of Ubc9 or SENP1 was silenced using shRNAs (Fig. 5d, e). Overexpression of either SENP1 or *Ubc9* shRNAs in LNCaP cells decreased Matrigel invasion, whereas RWPE1 cells expressing Ubc9 or *SENP1* shRNAs increased invasion into the Matrigel (Fig. 5f, g). These data indicate that changes in the levels of Ubc9 and SENP1 expression seem to affect the KAI1-mediated metastatic potential, at least in part, by changing the sumoylation status of reptin.

Taken together our data identify a new signal recognition pathway, SUMO modification of reptin, in the regulation of the metastasis suppressor gene *KAI1*. Abrogation of SUMO modification by mutation of the SUMO targeting site, or by cotransfection with the desumoylating enzyme SENP1, leads to a switch from repression to activation in transcriptional regulation by reptin. This switch was almost completely prevented when SUMO was covalently attached to reptin. Desumoylation of reptin seems to favour recruitment of the coactivator, Tip60, to the promoter, which is responsible for transcriptional activation of *KAI1*. Desumoylation of reptin may change the binding affinity of proteins for promoters.

SUMO modification is a dynamic process, and SUMO conjugation and desumoylation are required for the coordinate regulation of this process. Given that Ubc9 has been identified as the single E2 SUMO-conjugating enzyme, whereas desumoylation has been ascribed to a number of enzymes, it is tempting to speculate that the changes in the expression of desumoylating enzymes may have an important effects on protein sumoylation, including sumoylation of reptin. Identification of desumoylating enzymes in the reptin complex and elucidation of functional and regulatory roles provides a molecular mechanism for SUMO modification-dependent control of the reptin complex and links this regulatory machinery to homeostasis and disease, including control of cancer metastases. As a number of oncogenes and tumour suppressor genes have been defined as targets for sumoylation[29], we speculate that the sumoylation status of proteins involved in tumorigenesis and cancer metastasis may be altered in cancer and determining the upstream signal for SUMO modification of these proteins may begin to address the functional significance of sumoylation in the context of human cancer.

METHODS

Materials and reagents. The following commercially available antibodies were used: anti-Tip60, HDAC1, β-catenin and lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA); anti-acetylated histone H3, acetylated histone H4 and Tip60 (Upstate Biotechnology, Charlottesville, VA); anti-RNA polymerase II (Berkeley Antibody Company, Richmond, CA); anti-Flag (Sigma, St Louis, MO); anti-tubulin (Lab Frontier, Suwon, South Korea); anti-SENP1 and SUSP1 (ABGENT, San Diego, CA). Human IL-1β was purchased from Calbiochem (San Diego, CA). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfection experiments.

***In vivo* sumoylation assay.** 293T cells were transfected with pcDNA–reptin–TAP and His_6–SUMO1, 2 or 3. After transfection (36 h), the cells were lysed in lysis buffer (150 mM NaCl, 25 mM Tri–HCl at pH 7.8, 0.1% Nonidet P40 and 1 mM EDTA) supplemented with complex protease inhibitor cocktail (Roche, Indianapolis, IN) and 0.2% SDS, sonicated briefly and then centrifuged. Clarified extracts were incubated in 15 μl of Ni-NTA agarose beads (Qiagen, Valencia, CA) for 2 h. The slurry was washed with lysis buffer. After denaturation, proteins were separated by SDS–PAGE and the existence of reptin was confirmed by immunoblotting.

***In vitro* sumoylation assay.** The *in vitro* sumoylation assays were performed in a 10 μl reaction volumes containing 1 μg recombinant SUMO, 150 ng E1 (SAE1–SAE2), 10 ng E2 (Ubc9) and an ATP regeneration system (50 mM Tris–HCl at pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U ml^{-1} creatin kinase and 0.6 U ml^{-1} inorganic pyrophosphatase) with 1 μl of ^{35}S-methionine-labelled *in vitro*-translated reptin prepared from the TNT T7 Quick-coupled reticulocyte lysate kit (Promega, Madison, WI). Reaction products were analysed by autoradiography.

Chromatin immunoprecipitation assays. The chromatin immunoprecipitation assay was conducted as previously described[32], with an average size of sheared fragments of approximately 300–1000 base pairs. For PCR, 1 μl from a 50 μl DNA extraction and 25–30 cycles of amplification were used.

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

RNA interference by shRNA. The target sequence for shRNA again *reptin* was previously reported[1]. Target sequences for shRNAs against *SENP1* and *Ubc9* are as follows: *SENP1* shRNA1, 5′-GGAUCAGACUCUGUGAUUU-3′; *SENP1* shRNA2, 5′-GUGACUUUAGUGAACCACA-3′ (ref. 33); *Ubc9* shRNA1, 5′-GAAGUUUGCGCCCUCAUAA-3′; *Ubc9* shRNA2, 5′-GGAACUUCUAAAUGAACCA-3′ (ref. 34); *lamin A/C* shRNA, 5′-CUGGACUUCCAGAAGAACAUC-3′ (ref. 35).

Matrigel invasion assay. Cultured cells were pretreated with 5 ng ml^{-1} IL-1β for 24 h. LNCaP (2.5×10^4) or RWPE1 (1.25×10^4) cells were loaded into the top of a 24-well Matrigel invasion chamber assay plate (BD Biocoat, BD Biosciences). Conditioned RPMI 1640 medium containing 15% fetal bovine serum was added to the bottom chamber as a chemoattractant. 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 manufacturer's protocols.

Gel filtration. Cell extracts (15 mg) obtained from 3×-FLAG-tagged reptin expressing cells were loaded on Sepharcryl-S400 chromatography column (1.7 × 67 cm, Sigma) equilibrated with buffer (20 mM Tris–HCl at pH 8.0, 5% glycerol, 150 mM NaCl). Fractions were collected and analysed by western blotting against each antibody. The molecular weight (M_r) markers (Amersham Biosciences, Piscataway, NJ) used were thyroglobulin (669 K), ferritin (440 K) and catalase (232 K).

Nuclear–cytoplasmic fractionation. Cells were harvested and resuspended in 800 μl buffer A (25 mM Tris–HCl at pH 8.0, 10 mM KCl, 1 mM DTT and 0.5 mM PMSF) supplemented with complete protease inhibitor cocktail (Boehringer Mannheim). After incubation on ice for 15 min, 50 μl of 10% Nonidet P40 was added to the cells and vortexed for 10 sec. After centrifugation at 1,400*g* for 30 sec, the supernatants were collected and subsequently referred to as cytoplasmic extracts. The pellet was resuspended in 100 μl of ice-cold buffer C (50 mM Tris–HCl at pH 8.0, 400 mM NaCl, 1 mM DTT and 1 mM PMSF) supplemented with complete protease inhibitor cocktail (Roche). After centrifugation at 15,000*g* for 30 min, the supernatants were collectd and subsequently referred to as nuclear extracts.

***In vitro* desumoylation assay.** Myc–SENP1 or Myc-$SENP1^{C603A}$ was transfected into 293T cells and immunoprecipitated with anti-cMyc (9E10) followed by protein A–Sepharose (Sigma). Precipitated materials were added to the *in vitro* sumoylated ^{35}S-methionine-labelled reptin in cleavage buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1 mM DTT) and incubated at 37 °C. Products were analysed by autoradiography.

GST pulldown assays. To examine the effect of the sumoylation of reptin on the binding to HDAC1, GST–HDAC1 bound to glutathione–Sepharose beads was prepared. The beads were incubated with the isolated sumoylated reptin proteins, or non-sumoylated reptin proteins, in a buffer containing 20 mM Tris–HCl at pH 7.5, 150 mM NaCl, 0.2% Nonidet P40 and 10% glycerol, respectively. After extensive washing, the bound materials were subjected to western blot analysis.

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

ACKNOWLEDGMENTS

We thank M. G. Rosenfeld (Howard Hughes Medical Institute), C. K. Glass (University of California at San Diego), C. Y. Choi (Sung Kyun Kwan University) and E. Park (Seoul National University) for critical reading of our manuscript. This work was supported by Korea Research Foundation Grant (MOEHRD, Basic Research Promotion Fund), the National Research and Development programme for cancer control from the Ministry of Health & Welfare, and Science Research Center (SRC) programme of the Ministry of Science and Technology (MOST) and the Koreas Science and Engineering Foundation (KOSEF), Brain Koreas 21 (BK21) and Seoul Science Fellowship (H.J.C and B.K), and Ubiquitome Research Program from MOST/KOSEF (C.H.C and K.I.K.).

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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