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SHORT COMMUNICATION

Poly(ADP-ribose)-dependent regulation of Snail1 protein stability

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Snail1 is a master regulator of the epithelial–mesenchymal transition (EMT) and has been implicated in key tumor biological processes such as invasion and metastasis. It has been previously shown that poly(ADP-ribose) polymerase-1 (PARP-1) knockdown, but not PARP inhibition, downregulates the expression of Snail1. In this study we have characterized a novel regulatory mechanism controlling Snail1 protein expression through poly(ADP-ribosyl)ation. The effect is not only limited to repression of Snail1 transcription but also to downregulated Snail1 protein stability. PARP-1 (but not PARP-2) poly(ADP) ribosylates Snail1, both *in vivo* and *in vitro*, and interacts with Snail1, an association that is sensitive to PARP inhibitors. PARP inhibition has also clear effects on EMT phenotype of different tumor cells, including Snail1 downregulation, E-cadherin upregulation, decreased cell elongation and invasiveness. Therefore, this study reveals a new regulatory mechanism of Snail1 activation through poly(ADP-ribosyl)ation with consequences in malignant transformation through EMT. *Oncogene* (2011) **30,** 4365–4372; doi:10.1038/onc.2011.153; published online 16 May 2011

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Introduction

Metastatic melanoma is a fatal malignancy, which is remarkably resistant to treatment, but the molecular and cellular events that determine transition from primary local to metastatic melanoma are not entirely clear. Epithelial–mesenchymal transition (EMT) is essential for correct organ development during embryogenesis by creating cells with the ability to move (Guarino, 1995, 2007). Furthermore, EMT can be associated with tumor cells invasion and metastasis (Cavallaro and Christofori, 2004; Sarrio *et al*., 2004). Molecular markers for EMT include E-cadherin downregulation, responsible for the loss of cell-cell adhesion, upregulation of matrix-degrading proteases and mesenchymal-related proteins such as Vimentin and N-cadherin, actin–cytoskeleton reorganization and upregulation and/or nuclear translocation of transcription factors responsible to turn-on the specific gene program of EMT, such as β-catenin and members of the SNAIL family (Thiery and Sleeman, 2006).

SNAIL family members, including Snail1 and Snail2, are the major EMT inducers during embryonic development (Barrallo-Gimeno and Nieto, 2005; De Craene *et al*., 2005; Peinado *et al*., 2007) and tumor progression (Nieto, 2002; Thiery, 2002; Peinado *et al*., 2005). Both genes encode transcriptional repressors capable of binding and inhibiting E-cadherin promoter activity (Batlle *et al*., 2000; Cano *et al*., 2000; Bolos *et al*., 2003) by the direct interaction of the Snail1 C-terminal domain with specific 5′-CACCTG-3′core sequences present in this promoter. Moreover, Snail1 represses E-cadherin expression indirectly, inducing the synthesis of Zeb1, a transcriptional repressor (Guaita *et al*., 2002). This factor also binds to the E-boxes in the E-cadherin promoter, inhibiting the expression of this gene (Grooteclaes and Frisch, 2000).

Snail1 expression is regulated at multiple levels. It has been reported that Snail1 protein stability and cellular localization are finely controlled by glycogen synthase kinase (GSK)-3β-dependent phosphorylation and subsequent ubiquitination. This degradation of Snail1 by GSK-3β can be attenuated by Loxl2, a member of the lysyl oxidase gene family (Peinado *et al*., 2005), resulting in Snail1 stabilization and promotion of EMT. Recently, the hypoxia-controlled Fbxl-14 ubiquitin ligase has been shown to modulate Snail1 protein stability independently of its previous phosphorylation (Vinas-Castells *et al*., 2010).

Snail1 transcription is also stimulated in conditions leading to EMT (Barbera *et al*., 2004). Recently it has been demonstrated that poly(ADP-ribose) polymerase-1 (PARP-1) is involved in the activation of *Snail1* gene transcription through binding to the integrin-linked kinase (ILK) promoter (McPhee *et al*., 2008). PARP-1 is the principal member of a family of enzymes with poly(ADP-ribosyl)ation catalytic capacity. PARP-1 is a conserved nuclear protein that binds rapidly and directly to both single- and double-strand breaks (Schreiber *et al*., 2006). Both processes activate the catalytic capacity of the enzyme, which in turn modulates the activity of a wide range of nuclear proteins by covalent attachment of branching chains of ADP–ribose moieties. Members of

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PARP share a conserved catalytic domain that contains the PARP signature motif, a highly conserved sequence that forms the active site. PARP-1 and PARP-2 are, so far, the only known members of the PARP family whose activity is stimulated by DNA strand breaks. PARP uses nicotinamide adenine dinucleotide (NAD) as substrate to synthesize poly(ADP-ribose) (PAR) (Hassa and Hottiger, 2008). At the cellular level, PARP-1 formation has been implicated in a number of cellular functions including maintenance of genomic stability, transcriptional regulation, energy metabolism and cell death (Schreiber *et al*., 2006).

ADP–ribose polymers are then subjected to degradation by poly(ADP-ribose)glycohydrolase (Lin *et al*., 1997). Poly(ADP-ribosyl)ation is, therefore, an immediate, covalent, but transient, post-translational mechanism of modification of cellular proteins (Schreiber *et al*., 2006). Poly(ADP-ribosyl)ation of histones induces chromatin relaxation, allowing transcription factors to access DNA; PARP also participates in promoter/enhancer-binding complexes (Jagtap and Szabo, 2005; Schreiber *et al*., 2006).

In this study, we have determined that PARP-1 interacts with and modifies Snail1 by increasing the protein half-life with functional consequences in the EMT-associated phenotype.

Results and discussion

Involvement of PARP-1 on Snail1 activation and E-cadherin expression

Previous results have shown that PARP-1 is implicated in the regulation of transcription at different levels (Kim *et al*., 2005), and its ability to modulate transcription factors involved in tumor development and angiogenesis has been repeatedly reported (Aguilar-Quesada *et al*., 2007). In the present study, we have analyzed the involvement of PARP-1 and its activity on the modulation of Snail1, a key transcription factor involved in cell migration during development and metastasis. We first analyzed the role of PARP activity in the regulation of Snail1 expression. In all experiments, inhibition of PARP was achieved by both PJ-34 and the more novel and potent PARP inhibitor KU0058948 with very similar results. The inhibition of PARP using [*N*-(6-Oxo-5,6-dihydro-phenanthridin-2-yl)*N*,*N*-dimethylacetamide] (PJ-34) or KU0058948 in the human melanoma cell lines A375 or G361 downregulated endogenous protein Snail1 as well as mRNA levels and Snail1-dependent transcriptional activation (Figures 1a (A375) and 1c (G361) and Supplementary Figures S1a and b

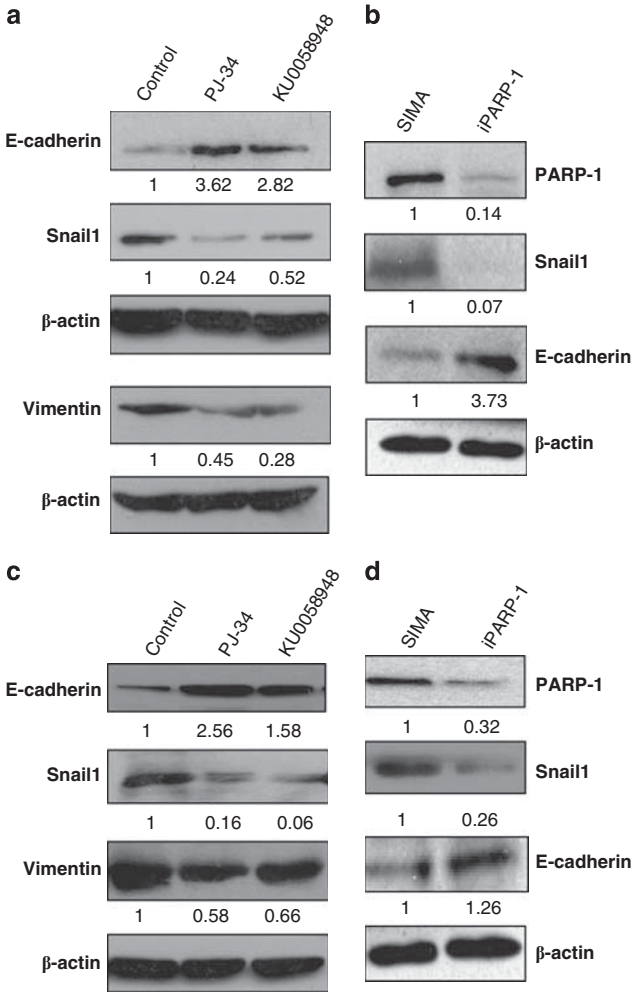

Figure 1 Snail1 downregulation and E-cadherin accumulation induced by PARP inhibition and PARP-1 knockdown. (**a**) Treatment with the PARP inhibitor PJ-34 (10 μM) or KU0058948 (100 nM) for 22 h (PARP-1 inhibitors) reduces Snail1 or Vimentin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) levels and increase the E-cadherin levels (Santa Cruz Biotechnology) in A375 human melanoma cells. (**b**) Knockdown of PARP-1 by siRNA in A375 human melanoma cells induces downregulation of Snail1 and subsequent increase in the expression of E-cadherin. (**c** and **d**) Treatment with the PARP inhibitor PJ-34 or KU0058948 (left panel), or knockdown of PARP-1 by siRNA (right panel) produces the same effect in G361 human melanoma cells than in A375 cells. Both cells were transiently transfected with an irrelevant siRNA (SIMA) or PARP-1 siRNA for 24 h using Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. At 48 h post-transfection, the expression of E-cadherin and Snail1 was measured. Cells were washed twice in phosphate-buffered saline (PBS) and scraped in Laemmli buffer (1 M Tris, 20% SDS and 10% glycerol) and sonicated. The protein concentration was determined using the Lowry assay. Levels of β-actin were monitored as a loading control. Immunoreactive bands were visualized with the ECL Plus system (Amersham Biosciences, Piscataway, NJ, USA). The G361 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS) and gentamicin (Gibco, Carlsbad, CA, USA). The A375 cells were given by Dr Bosserhoff (Institute of Pathology, University Regensburg, Germany). Cells were maintained in DMEM supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (300 μg/ml), MEM non-essential amino acids (Gibco) and 10% FBS. All cells were grown in a 5% CO_2 atmosphere at 37 °C. For treatment of the cells, we used different PARP inhibitors as follows: [*N*-(6-Oxo-5,6-dihydro-phenanthridin-2-yl)*N*,*N*-dimethylacetamide] (PJ-34, Alexis Biochemical, San Diego, CA, USA) (IC_{50} of 30 nM) (Virag and Szabo, 2002) was dissolved in water at a concentration of 10 μM and KU0058948 at a concentration of 100 nM. KU0058948 is a novel and very potent small-molecule inhibitor of both PARP-1 and PARP-2, exhibiting IC_{50} of 3.4 nM against PARP-1 and an IC_{50} of 6 nM against cellular PARP activity (Farmer *et al*., 2005).

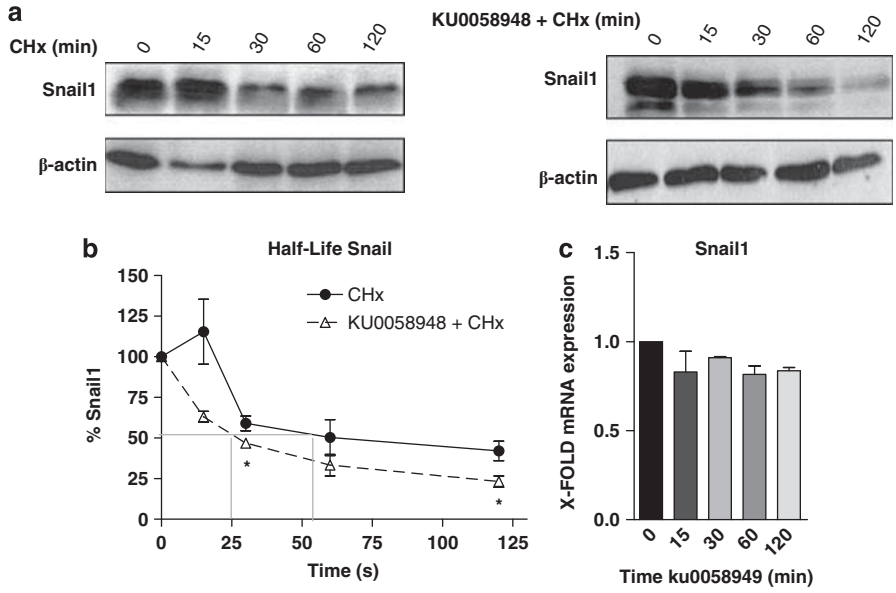

Figure 2 Reduced Snail1 half-life after PARP inhibition. (**a**) A375 cells treated with Cicloheximide (100 μg/ml), or KU0058948 + Cicloheximide for different time points as follows: 0, 15, 30, 60 and 120 min. Total cell lysates were prepared and analyzed by western blot using anti-Snail1 as primary antibody. (**b**) Graph showing band intensities corresponding to Snail1 and quantified using ImageQuant 5.2 software (GE Healthcare, Uppsala, Sweden, bottom left). Results (from three representative experiments) are expressed as the percentage of the signal of Snail1 compared with β-actin. $*P<0.05$ versus cycloheximide (CHX). The ratio was set to 100% at zero time. (**c**) mRNA expression of Snail1 after KU0058948 treatment in A375 cells (bottom right). For quantitative RT–PCR analysis, after 0, 15, 30, 60 and 120 min of treatment with KU0058948, cells were washed with phosphate-buffered saline (PBS) and total RNA were extracted using the RNeasy Mini Kit (Qiagen, Pleasonton, CA, USA) and reverse-transcribed by oligo(dT) priming using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative RT–PCR analyses were performed using the iCycler iQ detection system (Bio-Rad) and SYBR Green (Bio-Rad). The level of mRNA in each sample was normalized by the level of 36B4. The relative quantitation value for each target gene compared with the calibrator for that target is expressed as $2-(C_t\text{-}C_c)$ (where C_t and C_c are the mean threshold cycle differences after normalization to 36b4). The experiment was carried out in duplicate or triplicate, and repeated at least three times.

(G361)). Knockdown of PARP-1 also results in down-regulation of Snail1 accumulation and increases the levels of E-cadherin (Figures 1b and d). Similar results have been found in other cell lines both tumoral and non-tumoral (mouse melanoma B16F10, human primary endothelial cells, human umbilical vein endothelial cells —not shown). Levels of Vimentin (an important EMT marker) were also downregulated by PARP inhibition (Figures 1a and c).

We also checked Snail1 and E-cadherin levels, by indirect immunofluorescence after treatment with PJ-34 (Supplementary Figures S2a, e and f), in G361, MCF7 and V12Ras-transformed Madin–Darby canine kidney-f3 cells, respectively, or KU0058948 (Supplementary Figures S2e and f) in MCF7 and V12Ras-transformed MDCK-f3 cells, respectively. Compared with untreated cells, Snail1 expression was diminished and the levels of E-cadherin were augmented. We also observed that the effect of the knockdown of PARP-1 in Snail and E-cadherin levels was similar to that obtained with the PARP inhibitors (Supplementary Figure S2e). Moreover, cells treated with PARP inhibitors displayed an increased circularity and tendency to form clusters (Supplementary Figures S2a and f), which results in increase in cell-cell adhesion. Cell migration analyzed as hepatocyte growth factor-induced cell scattering was also notably decreased by PARP inhibition in Madin–Darby canine kidney cells (Supplementary Figure S2g). On the contrary, Snail1 expression decreased in both nuclear and cytosolic compartments (Supplementary Figure S2a, b, e and f) in the presence of PARP inhibitor, PJ-34 or KU0058948 (Supplementary Figure S2e and f).

We noticed that the effects of PARP-1 knockdown or inhibition was more pronounced on Snail1 protein than in mRNA levels (compare Figures 1a–d with Supplementary Figure S1a). Also, we analyzed the effect of PARP-1 inhibition on Snail1 protein stability in A375 cells. PARP inhibition quickly downregulated Snail1 protein levels without affecting Snail1 mRNA levels (Figures 2a–c and Supplementary Figure S3). An analysis of Snail1 protein half-life showed that KU0058948 decreased up to 25 min, compared with that detected with cycloheximide (CHX) alone in these cells (52 min) (Figure 2b). Snail1 mRNA levels, however, decreased much slower after PARP inhibition (Figure 2c), suggesting a post-translational effect of poly(ADP-ribose) on Snail1.

Snail1 protein is polyADP-ribosylated

In view of the previous results, we next evaluated the *in vitro* ability of PARP-1 to poly(ADP-ribosyl)ate HA-Snail1 in Cos1 cells. Trapped proteins on the beads were incubated with PARP-1, PARP-2 or no protein, in the presence of [^{32}P]NAD$^+$ and DNase I-activated DNA

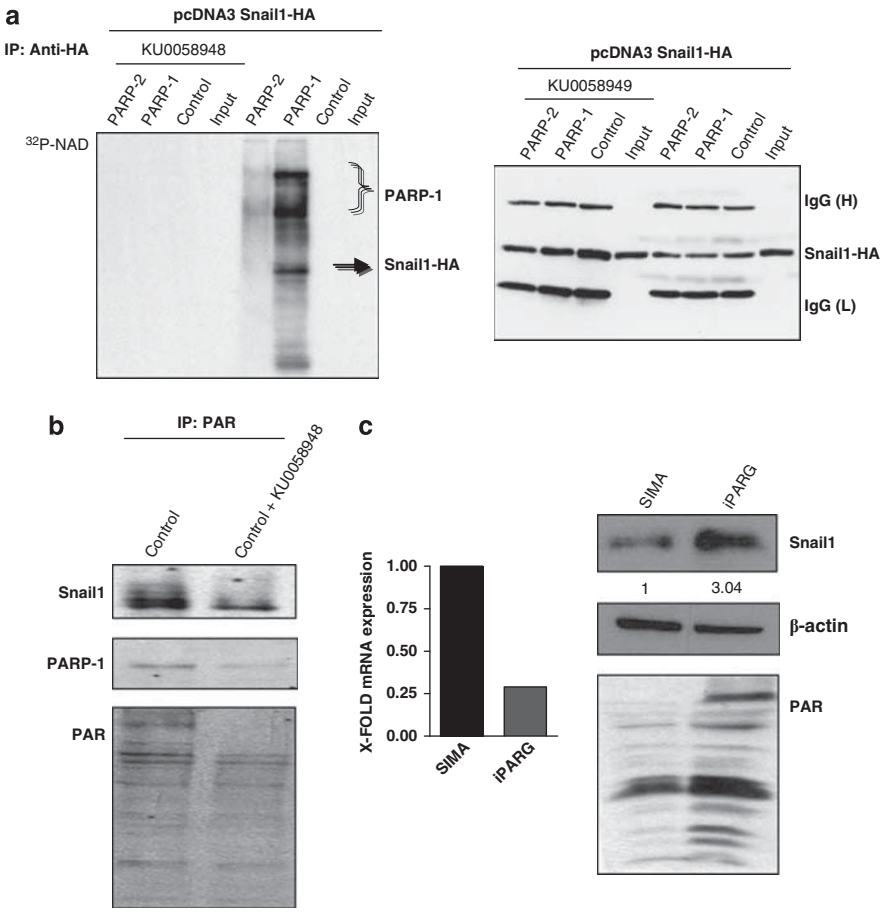

Figure 3 Snail1 poly(ADP-ribosyl)ated *in vitro* and *in vivo*. (**a**) *In vitro*, poly(ADP-ribosyl)ation of Snail1. A plasmid-expressing Snail1, tagged with the HA epitope, was inserted into plasmid pcDNA3 and transfected into Cos1 cells. Cos1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% activated fetal bovine serum (FBS) and gentamicin. Snail1 was immunoprecipitated with anti-HA antibody and revealed by western blot of the HA-Snail1 (human influenza hemagglutinin (HA) Tag, Santa Cruz Biotechnology). To analyze the heteromodification of Snail1 protein by PARP-1, Cos1 cells were transfected with HA-Snail1 and protein were immunoprecipitated using HA antibody and then collected using 50 µl of Protein A-Sepharose beads (GE Healthcare, Piscataway, NJ, USA) and bound immune complexes were washed two times with lysis buffer (20 mM Tris–HCl, pH 7.5, 400 mM NaCl, 20% glycerol, 5 mM DTT, 0.5 mM Pefabloc, 0.1% NP-40, PIC, KU0058948) and two times with DB (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, PIC components). After a last wash with activity buffer (50 mM Tris–HCl, pH 8, 0.2 mM DTT, 4 mM $MgCl_2$, 0.1 µg/µl bovine serum albumin), each sample was split into three, the beads were pelleted and resuspended in 300 µl of activity buffer containing either 300 pmol of hPARP-1, 600 pmol of mPARP-2 or no PARP (Ame *et al*., 1999). Reaction was started by the addition of 180 µl of activity buffer containing DNase I-activated calf thymus DNA, 0.1 µM NAD for PARP-1 and 1 µM for control and PARP-2 samples. In addition, each sample contained 1 pmol of [^{32}P]NAD^+. After 10 min at 25 °C, the reaction was stopped by the addition of 500 µl of cold DB on ice, and beads were washed three times with DB, resuspended in 20 µl of charge buffer and analysis was carried out by autoradiography and western blot. (**b**) *In vivo*, poly(ADP-ribosyl)ation in A375 cells using an antibody against PAR-modified proteins; pre-treatment with the PARP inhibitor KU0058948 (100 nM). (**c**) Effect of poly(ADP-ribose)glycohydrolase (PARP) silencing on Snail-1 accumulation in A375 cells. Cells were transfected with PARG or scrambled siRNA; at 48 h after transfection, total RNA was extracted and poly(ADP-ribose)glycohydrolase (PARG) mRNA levels were assessed by quantitative real-time PCR. In this situation, Snail1 and PAR (Trevigen, Gaithersburg, MD, USA) expression protein were analyzed by western blot.

(Figure 3a). Autoradiography revealed that Snail1 is poly(ADP-ribosyl)ated by PARP-1. In the presence of PARP-2, no poly(ADP-ribosyl)ation was noticed (Figure 3a). Moreover, an *in vivo* experiment using PAR immunoprecipitation, with a monoclonal antibody specific to poly(ADP-ribosyl)ated proteins, show that Snail1 is poly(ADP-ribosyl)ated (Figure 3b) and the addition of the PARP inhibitor KU0058948 decreased the levels of poly(ADP-ribosyl)ated Snail1 (Figure 3b).

Poly(ADP-ribose) glycohydrolase is the enzyme responsible for PAR degradation. To test the consequences of PAR accumulation in Snail1 protein levels we knocked down poly(ADP-ribose)glycohydrolase in A375 cells and, as shown in Figure 3c, although PAR accumulated in poly(ADP-ribose)glycohydrolase siRNA cells, Snail1 levels were elevated with respect to control cells, suggesting that PAR is actively involved in the regulation of Snail1 protein levels.

Snail1 and PARP-1 form a complex that needs poly(ADP-ribosyl)ation

In order to get mechanistic information on how PARP or poly(ADP-ribose) could participate in the regulation of Snail1, we decided to investigate the possible interaction between PARP-1 and Snail1. Cos1 cells

were transfected with pcDNA$_3$-Snail1-HA together with glutathione S-transferase (GST), GST-fused PARP1 or GST-fused PARP-2 (Figure 4a). GST-fusion proteins were trapped on glutathione-sepharose beads and Snail1-HA was analyzed by western blot analysis using anti-HA antibody. Figure 4a show that PARP-1, but not PARP-2, interacted with Snail1 in Cos1 cells. In the presence of the PARP-1 inhibitor KU0058948, this interaction faded, indicating that PAR synthesis is a pre-requisite for Snail1 binding to PARP-1 (Figure 4b). Co-immunoprecipitation of Snail1 and PARP-1 in both senses was also evident with the endogenous proteins

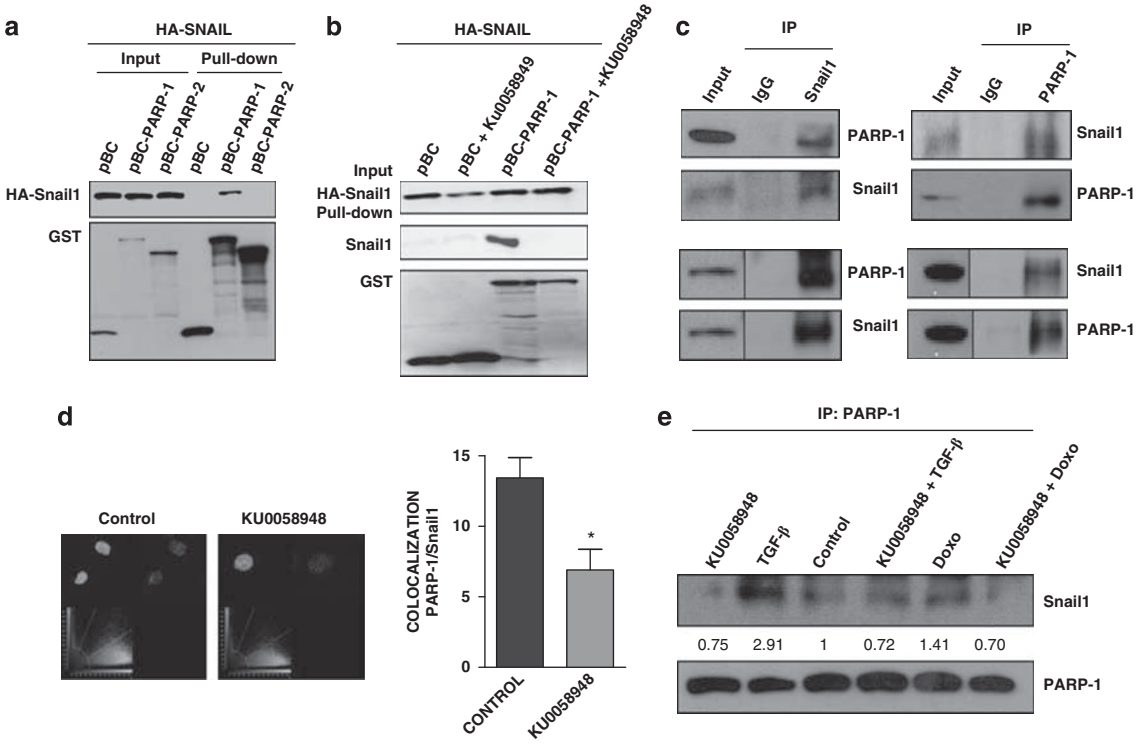

Figure 4 Dissection of the interaction domains between PARP-1 and Snail1. (**a**) Pull-down in Cos 1 cells was performed to study the association between PARP-1 and Snail1. Cos1 cells were transfected with JetPEI (Polyplus, Illkirch, France) co-precipitation with the plasmids expressing HA-Snail1 and GST fusion proteins (pBC, pBC-PARP-1 and pBC-PARP-2). At 48 h later, cells were lysed in 20 nM Tris-HCl (pH 7.5), 400 mM NaCl, 20% glycerol, 5 mM DTT, 0,5 mM pefabloc and protease inhibitors (Complete Mini; Roche, Mannheim, Germany). Lysates were cleared by centrifugation and incubated for 2 h with glutathione-sepharose 4B (Amersham). Beads were washed three times with 20 nM Tris–HCl (pH 7.5), 150 to 500 nm NaCl, 0.1% NP-40 and protease inhibitors. All samples were resuspended in charge buffer, boiled for 5 min and analyzed by western blot. Blots were subsequently incubated with anti-GST antibody (IGBMC, Illkirch, France) and anti-HA (Santa Cruz Biotechnology). (**b**) Interaction between PARP-1 and Snail1 in the presence of the PARP inhibitor, KU0058948 for 2 h. Complexes were bound to glutathione-sepharose and analyzed by western blot with HA and GST antibodies. (**c**) Co-immunoprecipitation of Snail1 and PARP-1 endogenous proteins in melanoma A375 cells (upper panel) and G361 cells (lower panel); an irrelevant antibody was used as negative control (middle lane). (**d**) Confocal microscopy to detect the colocalization between PARP-1 and Snail1. A375 cells were grown in coverslips and treated with 5 mM lithium chloride for 24 h. Cells fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, after washing three times with PBS, cells were permeabilized with 0.2% Triton in PBS for 10 min at room temperature. Incubation with primary antibodies anti-Snail1 and anti-PARP-1 (Alexis Biochemical) were performed in 2% BSA:PBS for 30 min at 37 °C, and second antibodies (FITC-labeled anti-mouse, Cy3-labeled anti-rabbit IgG, both from Sigma (St Louis, MO, USA)) in 2% BSA:PBS for 20 min at 37 °C. After extensive washing with PBS, coverslips were mounted with Vectashield (Burlingame, CA, USA) mounting medium. Images were captured on a Leica (Leica, Wetzlar, Germany) LCS SP5 confocal microscope. Colocalization percentages were quantifying the immunoflorescence obtained from the z-stacks on every image. The bar chart shows the percentage of cells in which colocalization foci were observed at control or upon treatment with KU0058948. $*P<0.05$. (**e**) Effect of Snail1 and PARP-1 activation on complex Snail1/PARP-1 formation in A375 cells. These cells were treated with KU0058948 (100 nM) for 2 h and then with 5 ng/ml TGF-β1 (Peprotech, Rocky Hill, NJ, USA) for 5 h to stimulate the pathway of Snail1 or with DNA-damaging agents (doxorubicin, 10 μg/ml). PARP-1 immunoprecipitation were carried out and endogenous Snail1 was analyzed by western blotting with an anti-Snail1 polyclonal antibody (Abcam, Cambridge, UK). (**f**) Mapping of hPARP-1 to observe association between PARP-1 and Snail1 by pull-down analyses in Cos1 cells. Complexes were bound to glutathione-sepharose and analyzed by western blotting with HA and GST antibody. All the GST fusion constructs were previously published (Dantzer *et al*., 2004). (**g**) Mapping of C-terminal and N-terminal domain of Snail1 with hPARP-1. Cos1 cells were also transfected using JetPEI and GFP-fused C- and N-terminal SNAIL domains and pBC-PARP-1. Pull-down experiments were performed and the interactions were determined by western blot using an anti-GFP and anti-GST antibody from Sigma. (**h**) Effect of siRNA-mediated depletion of ILK and the overexpression of PARP-1 in Snail1 expression. Cos1 cells were co-precipitated with the plasmids expressing HA-Snail1 and GST fusion proteins (pBC and PBC-PARP-1) and siRNA ILK or scrambled siRNA. At 24 h after transfection, the medium was replaced and TGF-β was added 24 h. The expression of exogenous Snail1 was analyzed by western blot with HA, GST and ILK (Millipore, Billerica, MA, USA) antibodies. (**i**) Lithium chloride (LiCl) stabilization of Snail1 in the presence of exogenous PARP-1. Co-transfection of Cos1 with the plasmids expressing HA-Snail1 and GST fusion proteins (pBC and PBC-PARP-1) were performed. At 24 h after transfection, cells were treated with KU0058948 for 2 h and then with 5 mM LiCl for 24 h. Complexes were bound to glutathione-sepharose and analyzed by western blot with HA and GST antibodies. A full colour version of this figure is available at the *Oncogene* journal online.

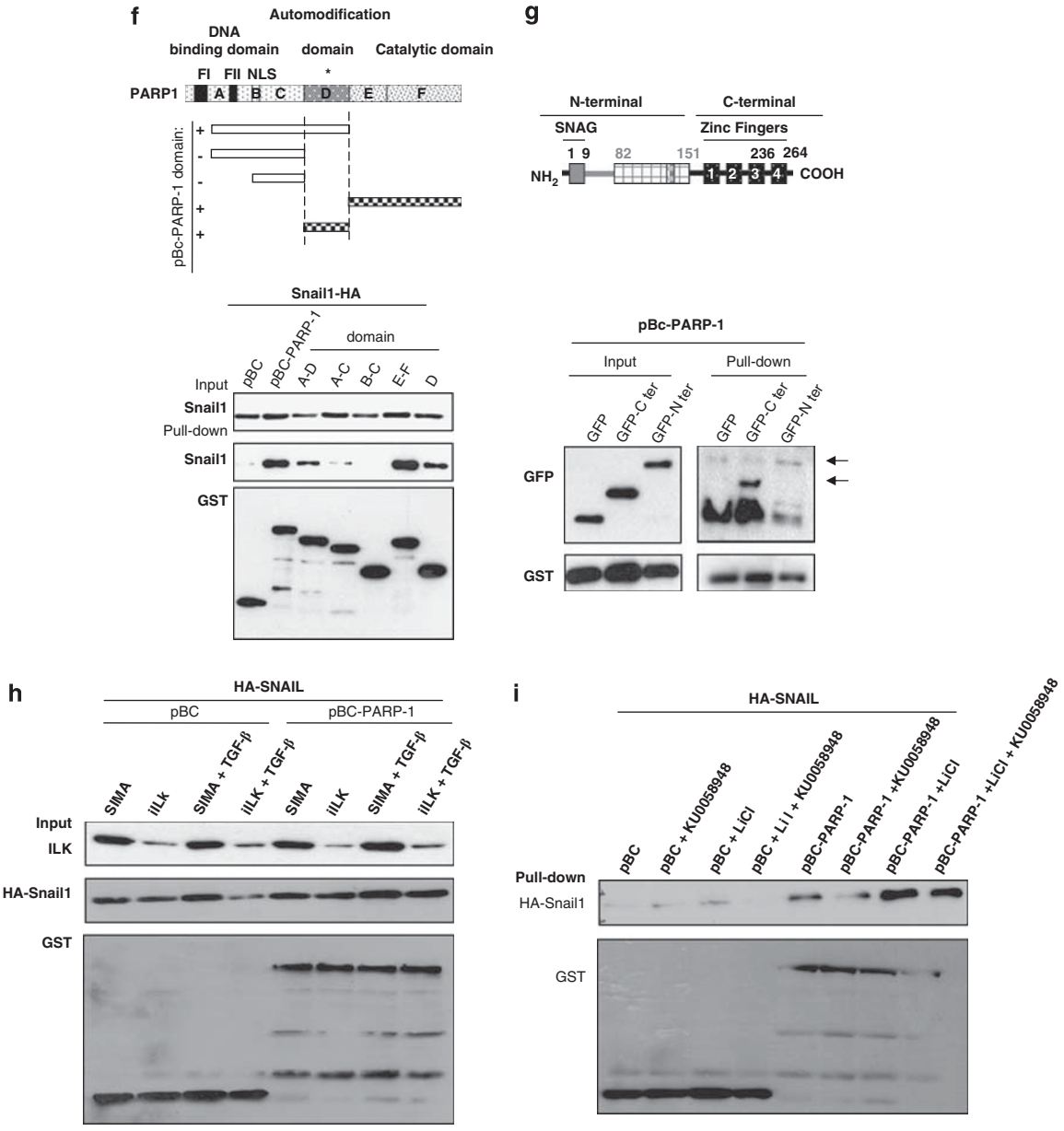

Figure 4 Continued.

in non-transfected melanoma cells A375, G361 cell (Figure 4c, upper panel and lower panel, respectively) and SK-Mel28 cells (Supplementary Figure S2d, upper panel) and also in another tumor cell line as HepG2 (Supplementary Figure S2d, lower panel). Confocal microscopy studies confirmed the previous results: PARP-1 and Snail-1 colocalize in the nucleus (white dots) and inhibition of PARP decreased the rate of colocalization (Figure 4d in A375 cells and Supplementary Figure S2c in A375 and G361, respectively).

As this complex was observed under conditions not involving PARP-1 or Snail-1 activation, we next performed co-immunoprecipitation using transforming growth factor-β (TGF-β) or DNA damage to activate Snail1 or PARP-1, respectively, (Figure 4e). TGF-β has dual roles during tumor progression: functioning as a tumor suppressor during early stages of tumor development and a promoter of invasion at later stages (Ikushima and Miyazono, 2010; Lasfar and Cohen-Solal, 2010). TGF-β also functions as a promoter of tumor cell invasion and metastasis in advanced states of tumorigenesis (Pardali and Moustakas, 2007). The presence of TGF-β together with the PARP inhibitor blunted TGF-β-induced Snail1 accumulation and diminished the complex between PARP-1 and Snail1 (Figure 4e), suggesting that PAR is actively involved in signaling TGF-β-induced Snail1 upregulation and in PARP-1/Snail1 complex formation. Moreover, under conditions involving PARP activation, after treatment with the DNA damaging agent doxorubicin, we observed an increased complex formation between PARP-1 and Snail1 (Figure 4e).

To get more insight information about the interaction between Snail1 and PARP-1, we mapped the structural

domains involved in this interaction. PARP-1 can be dissected into five domains (domains A–F, see Figure 4f). To map the domain of interaction within PARP-1 and Snail1, GST fusion proteins were generated expressing truncated versions of hPARP-1: amino acids 1–523 (A–D, the DNA-binding domain and automodification domain); amino acids 1–371 (A–C, the DNA-binding domain); amino acids 174–366 (B and C); amino acids 524–1014 (E and F, the catalytic domain); amino acids 372–524 (D, the automodification domain) (Masson *et al*., 1998). These fusions proteins were overexpressed in Cos1 cells together with Snail1-HA. Co-precipitating proteins were analyzed by GST pull-down followed by western blot. The results in Figure 4f indicate that the domain of PARP-1 involved in the association with Snail1 is the domain E and F and in a lesser extent the D domain, the catalytic and the automodification domains, respectively. Earlier studies suggested that domain D is likely to function as an interface for protein–protein interaction between PARP-1 and its targets, and is also the primary region for automodification.

The reverse experiment was also performed to investigate which domains of Snail1 interact with PARP-1 using GST pull-down experiments (Figure(4(g). Two different functional regions of Snail1 have been described: the C-terminal DNA-binding domain (amino acids 152 to 264) and the N-terminal regulatory domain (amino acids 1 to 151). The N- and C-terminal Snail1 domains were fused to GFP and co-transfected with GST-fused hPARP1 in Cos1 cells. Pull-down experiments showed that PARP-1 interacts mainly with the C-terminal domain of Snail1 (Figure(4(g). This finding agrees with the '*in silico*' results in which we distinguished two hypothetic PAR-binding sites in the C-terminal domain (Supplementary Figure S4).

Previously it has been reported that ILK-dependent activation of Snail1 transcription required PARP-1 binding to ILK promoter (McPhee *et al*., 2008). To analyze the involvement of ILK in the interaction between PARP-1 and Snail1, ILK was knocked down using siRNA (Figure 4h). Under basal conditions (without TGF-β treatment), siRNA of ILK slightly decreased Snail1 levels (Figure 4h, lane 2), whereas the combination of siRNA of ILK and TGF-β treatment strongly decreased Snail1 (Figure 4h, lane 4). Of note, when PARP-1 is overexpressed (first four right lanes in Figure 4h), Snail1 levels remained elevated, irrespective of the presence of ILK, and TGF-β activated Snail1 accumulation in a manner that is independent of ILK. On the contrary, under normal PARP-1 levels, TGF-β requires ILK to regulate Snail1, as reflected by Snail1 levels. In summary, in the presence of PARP-1 overexpression, the stability of Snail1 is maintained, irrespective of the presence or absence of ILK.

Glycogen synthase kinase phosphorylation regulates the rate of Snail1 protein degradation, and inhibition of GSK-3β activity leads to Snail1 transcription, E-cadherin repression and EMT (Bachelder *et al*., 2005). We have addressed the implication of GSK-3β activation on the association between PARP-1 and Snail1. Lithium chloride is a well-known inhibitor of GSK-3β and prevented the Snail1 translocation to the nucleus in A375 cells (result not shown). Inhibiting GSK-3β alone or the concomitant inhibition of PARP activity and GSK-3β increased PARP-1/Snail11 interaction (Figure 4i), implying that non-phosphorylated (active) Snail1 is needed to form the complex with PARP-1.

In summary, the results of this study reveal a new regulatory mechanism of Snail1 by PARP-1, involving the post-translational modification of Snail1 by PAR, with changes in Snail1 stabilization. Moreover, signals that activate either Snail1 (as TGFβ) or PARP-1 (as DNA damage) result in an increased complex formation between these two proteins, suggesting a close functional interaction between Snail1 and PARP-1 signaling pathways. This data highlight the importance of PARP-1 and PARP activity in the control of Snail1 function and EMT, and suggest new avenues for the use of PARP inhibitors as a potential therapeutic target to impede melanoma cancer cell invasion and metastasis.

Conflict of interest

The authors declare no conflict of interest.

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