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ORIGINAL ARTICLE

Akt2 interacts with Snail1 in the E-cadherin promoter

P Villagrasa[1,2], VM Díaz[1,3], R Viñas-Castells[1], S Peiró[1], B Del Valle-Pérez[2], N Dave[1], A Rodríguez-Asiain[4], JI Casal[5], JM Lizcano[4], M Duñach[2] and A García de Herreros[1,3]

[1]Programa de Recerca en Càncer, IMIM-Hospital del Mar, Barcelona, Spain; [2]Unitat de Biofísica-CEB, Facultat de Medicina, Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Bellaterra, Spain; [3]Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain; [4]Institut de Neurociència i Departament de Bioquímica i Biologia Molecular, Facultat de Medicina, Universitat Autònoma de Barcelona, Bellaterra, Spain and [5]Centro de Investigaciones Biológicas (CSIC), Madrid, Spain

Snail1 is a transcriptional factor essential for triggering epithelial-to-mesenchymal transition. Moreover, Snail1 promotes resistance to apoptosis, an effect associated to PTEN gene repression and Akt stimulation. In this article we demonstrate that Snail1 activates Akt at an additional level, as it directly binds to and activates this protein kinase. The interaction is observed in the nucleus and increases the intrinsic Akt activity. We determined that Akt2 is the isoform interacting with Snail1, an association that requires the pleckstrin homology domain in Akt2 and the C-terminal half in Snail1. Snail1 enhances the binding of Akt2 to the E-cadherin (CDH1) promoter and Akt2 interference prevents Snail1 repression of CDH1 gene. We also show that Snail1 binding increases Akt2 intrinsic activity on histone H3 and have identified Thr45 as a residue modified on this protein. Phosphorylation of Thr45 in histone H3 is sensitive to Snail1 and Akt2 cellular levels; moreover, Snail1 upregulates the binding of phosphoThr45 histone H3 to the CDH1 promoter. These results uncover an unexpected role of Akt2 in transcriptional control and point out to phosphorylation of Thr45 in histone H3 as a new epigenetic mark related to Snail1 and Akt2 action.
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Introduction

In the last years the essential role of the Snail1 transcriptional factor in the induction of epithelial–mesenchymal transition (EMT) has received a lot of experimental support (see Thiery *et al*., 2009 for a recent review). Elimination of Snail1 gene expression prevents gastrulation, the first embryonic process where an EMT takes place (Carver *et al*., 2001). Upregulated expression of Snail1 is detected in cell lines that have adopted a mesenchymal phenotype (De Craene *et al*., 2005). Moreover, ectopic expression of Snail1 promotes an EMT in epithelial tumor cell lines (Batlle *et al*., 2000; Cano *et al*., 2000), characterized by decreased expression of epithelial genes, such as E-cadherin, and upregulation of mesenchymal genes. Repression of epithelial genes by Snail1 requires the binding of the C-terminal domain of this protein to 5′-CACCTG-3′ elements present in these promoters and the interaction of the SNAG Snail1 sequence with co-repressors (Thiery *et al*., 2009; García de Herreros *et al*., 2010). Conversely, activation of mesenchymal genes (fibronectin, vimentin) is thought to be a more indirect effect, partially dependent on E-cadherin downregulation, and requiring stimulation of several transduction pathways, such as those involving extracellular signal-regulated kinase-2 (ERK2), Sp1/Ets-1, nuclear factor-κB and β-catenin (Ohkubo and Ozawa 2003; Jordà *et al*., 2005, 2007; Solanas *et al*., 2008; Stemmer *et al*., 2008).

The Ser/Thr protein kinase Akt/PKB plays a critical role in regulating different cellular properties such as growth, proliferation, survival and metabolism, through phosphorylation of specific substrates (Bellacosa *et al*., 2004, Woodgett, 2005). This protein kinase is activated by the phosphatidylinositol-3′-kinase products phosphoinositide-3,4-diphosphate and phosphoinositide-3,4,5-triphosphate, which facilitate its phosphorylation in Thr308 by PDK1 and in Ser473 by TOR-Rictor (Alessi *et al*., 1996). Therefore, phosphorylation of Akt Thr308 and Ser473 is commonly used to assess the activity of this enzyme. Akt can be localized both in the nucleus and the cytoplasm (Ahmed *et al*., 1993). Translocation of Akt to the nucleus occurs after activation and is reported to be enhanced by interaction with Akt-binding partners (Du and Tsichlis, 2005).

Akt activity has been related to EMT and Snail1 expression. Overexpression of Akt has been shown to induce EMT through nuclear factor-κB-dependent activation of Snail1 (Grille *et al*., 2003; Julien *et al*., 2007). Akt also participates in the Snail1 upregulation induced by epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF-1) in different cell lines,

Correspondence: Dr M Duñach, Unitat de Biofísica-CEB, Facultat de Medicina, Departament Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Bellaterra E-08193, Spain.
E-mail: mireia.dunach@uab.cat or Dr A García de Herreros, Institut Municipal d'Investigació Mèdica, Parc de Recerca Biomèdica de Barcelona, c/Doctor Aiguader, 88, Barcelona E-08003, Spain.
E-mail: agarcia@imim.es
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increasing both gene transcription and protein stability (Zhou *et al*., 2004; Gan *et al*., 2010). Besides these effects on Snail1 expression, Akt has also been positioned downstream from Snail1 in transforming growth factor-β-induced EMT (Cho *et al*., 2007). Snail1 overexpression stimulates this protein kinase as it increased the levels of phosphoThr308 (P-Thr308) in Akt, at least in part through direct repression of PTEN, an inhibitor of Akt activation (Vega *et al*., 2004; Escrivà *et al*., 2008). This upregulated Akt activity is probably the cause of the apoptosis resistance induced by Snail1 in several cell lines (Vega *et al*., 2004).

The Akt family is composed of three members, Akt1, Akt2 and Akt3, structurally homologous but which show distinct features. Akt1 and Akt2 are highly expressed in cells, whereas Akt3 is more limited (Bellacosa *et al*., 2004). Different results indicate isoform-specific functions in tumor cells. For instance, mutations and amplifications in epithelial tumors have been detected mainly in Akt2 and very rarely in Akt1 (Bellacosa *et al*., 2004; Parsons *et al*., 2005). Overexpression of Akt2, but not Akt1, increased the invasiveness of a tumor breast cell line *in vitro* and in animal models (Arboleda *et al*., 2003). Moreover, Akt1 downregulation cooperates with IGF-1 in promoting EMT, whereas Akt2 depletion suppresses this phenotypic conversion (Irie *et al*., 2005). Therefore, Akt1 activity seems to have a more prominent role in the maintenance of the epithelial characteristics, whereas Akt2 is required for the acquisition of mesenchymal traits.

In this report we have analyzed the activation of Akt by Snail1. Surprisingly, when we directly measured this protein kinase activity using Akt purified from cells expressing or not expressing Snail1, we detected that a transcriptionally inactive Snail1 mutant still activated Akt, although to a lesser extent than the wild-type protein, suggesting that part of the activation by Snail1 was not due to the transcriptional repression of PTEN or other targets. We have further investigated this result and demonstrate that Snail1 directly interacts with Akt2, enhancing its intrinsic protein kinase activity. This association is detected in the nucleus and in the E-cadherin (CDH1) promoter. Therefore Snail1 and Akt2 interact not only functionally but also physically.

Results

Snail1 induces Akt activation in tumor cell lines

As reported previously, tumor cell lines stably expressing Snail1 show elevated Akt activity, determined by measuring the extent of phosphorylation in Ser473 residues in the Akt protein (Figure 1a). Upregulated Akt phosphoSer473 (P-Ser473) was detected in all cell lines examined after ectopic expression of Snail1, and was more remarkable when the different cell lines (RWP-1, HT-29 M6, SW-480) were serum-starved for 16 h in order to decrease the endogenous activation of this kinase (Figure 1a).

Although Akt is mainly cytosolic, a relevant fraction is also found in the nucleus, particularly of the activated form (Ahmed *et al*., 1993; Andjelković *et al*., 1997). Both cytosolic and nuclear Akt activities were upregulated in Snail1-transfected cell lines (Figure 1b), an effect that, again, was more remarkable in serum-depleted cells.

We analyzed the phosphorylation of a direct Akt substrate, FoxO1 (Brunet *et al*., 1999). As shown in Figure 1c, FoxO1 phosphorylation was clearly upregulated in Snail1-transfected RWP-1 cells with respect to the control, as detected with a phosphospecific antibody.

We also directly measured the protein kinase activity of Akt in RWP-1 Snail1-transfected cells. Glutathione-*S*-transferase (GST)-Akt and Snail1 were co-transfected in RWP-1 cells; Akt was purified and its activity was assayed on Crosstide, a small peptide widely used as Akt substrate (Alessi *et al*., 1996). Akt purified from Snail1-expressing cells presented a higher activity than that obtained from control cells (Figure 1d). This upregulated activity was observed in serum-starved cells or in the presence of 10% fetal bovine serum. As expected, Akt activity was higher in the presence of fetal bovine serum. Surprisingly, the transcriptionally inactive P2A Snail1 mutant, unable to repress the PTEN and CDH1 genes (Batlle *et al*., 2000; Escrivà *et al*., 2008), also increased Akt activity although to a lower extent than the Snail1 wild-type. Similar results were obtained when Snail1 and Akt were co-expressed in another cell line, HEK-293T. As shown in Figure 1e, Snail1 wild-type, and to a lower extent the P2A mutant, enhanced the stimulation of Akt activity caused by IGF-1.

Therefore, these results indicate that Snail1 stimulates Akt activity, but this effect is not totally dependent on its activity as a transcriptional repressor.

An Akt inactive mutant prevents Snail1 repression of CDH1

In order to determine the relevance of Akt stimulation in Snail1-induced EMT, we analyzed whether ectopic expression of an inactive form of Akt prevented Snail1 repression of CDH1. In these experiments we used Akt K179M, a mutant devoid of protein kinase activity. Transfection of this mutant prevented the Snail1-induced repression of E-cadherin (CDH1) mRNA expression observed in RWP-1 cells (Figure 2a). Similar effects were observed on the downregulation of the E-cadherin protein (Figure 2b). Snail1 inhibition of CDH1 promoter activity was also affected: transfection of the kinase-dead form of Akt significantly prevented the repression of this promoter by Snail1 at the same time that it increased the basal promoter activity (Figure 2c), suggesting that Snail1 action requires Akt activity.

Akt and Snail1 interact

In our Akt activity assays, we incubated purified Akt with recombinant GST-Snail1. As shown in Figure 3a, addition of Snail1 increased Akt activity when assayed on the Crosstide peptide. This stimulation was observed upon incubation of Akt with recombinant Snail1 either

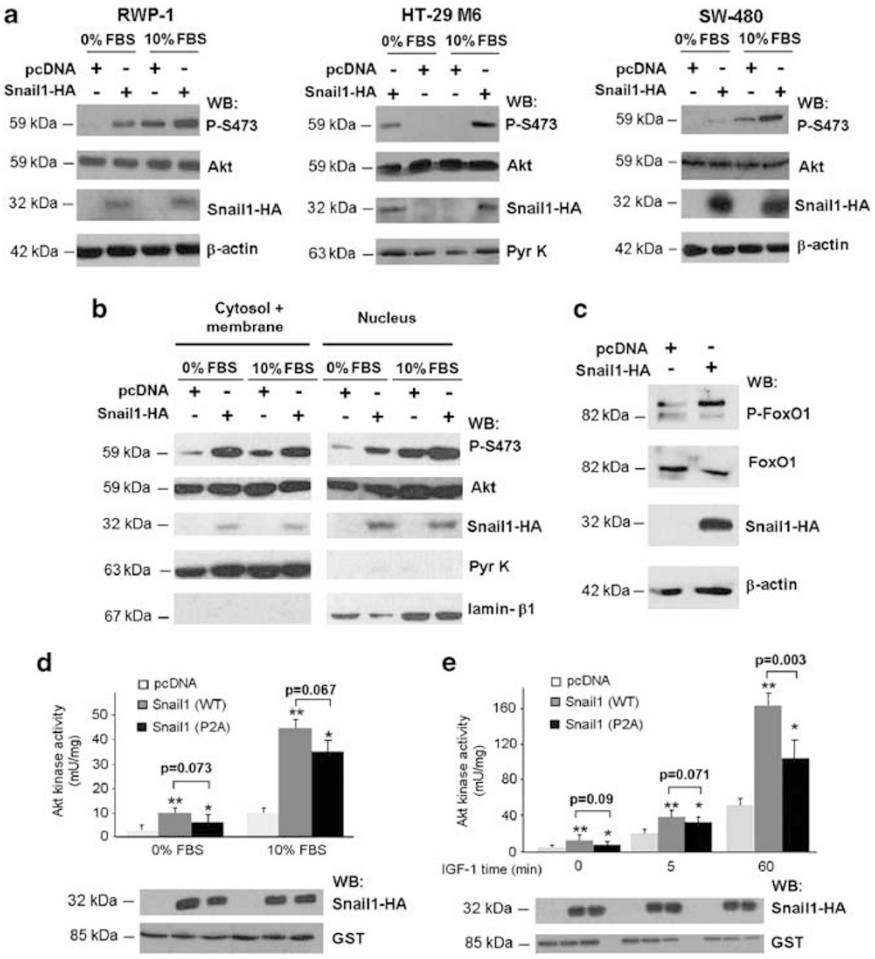

Figure 1 Snail1 transfection increases Akt activity. (**a**,**c**) Total extracts from the specified cell lines, serum-starved for 12 h when indicated, were analyzed by western blotting (WB) using the indicated antibodies. Pyruvate kinase (PyrK) or β-actin was used as loading controls. (**b**) Cytosolic (plus membrane) and nuclear fractions were prepared from control or Snail1-expressing RWP1 cells as detailed in the Supplementary information, serum-starved when indicated and analyzed by WB. PyrK and lamin-β1 were used as cytosolic or nuclear markers, respectively. (**d**, **e**) RWP-1 (**d**) or HEK-293 (**e**) were co-transfected with GST-Akt1 and Snail1 wild-type (WT) or a P2A mutant or empty pcDNA3 plasmid as control. After 36 h, the cell medium was depleted of serum for 12 h when indicated and incubated with IGF-1 for 5 or 60 min. GST-Akt1 was purified and the activity on the Crosstide peptide was determined as detailed under Materials and methods. The figure presents the average ± s.d. of three assays performed in duplicate. *P*-values were determined by analysis of variance. * indicates $P<0.05$; ** indicates $P<0.01$. A western blot analysis of the cell extract is shown at the bottom to demonstrate that similar amounts of Snail1 WT and P2A were expressed and that GST-Akt1 was not different in any of the experimental conditions.

containing the full-length protein or, to a lower extent, with the C-terminal domain. The increase in Akt activity was not due to modification of Ser473 or Thr308 phosphorylation on Akt protein (Figure 3b), discarding the possibility of an effect caused by the inhibition of a phosphatase co-purifying with Akt. Therefore, this result suggests that both proteins interact *in vitro* and that Snail1 binding stimulates intrinsic Akt activity.

The interaction was verified *in vivo* by co-immunoprecipitation experiments. Akt was detected in the immunocomplex obtained with an antibody that recognizes the hemagglutinin (HA) epitope tagging the Snail1 cDNA, only in cells expressing this protein (Figure 3c). A similar association was observed between Akt and the Snail1 P2A mutant (Supplementary Figure 1). The interaction with Akt was also confirmed with endogenous Snail1. As shown in Figure 3d, Snail1 was detected in Akt immunoprecipitates obtained from SW-620 or MiaPaca-2 cells, further confirming the association of these two proteins.

We also determined the subcellular compartment where this interaction takes place. As expected, Snail1 was detected mainly in the nucleus, although a significant part was observed in the cytosol; Akt showed a contrary pattern (Figure 3e). Akt co-immunoprecipitation with Snail1 was detected mainly in the nuclear fraction (Figure 3e). These results were validated using a Snail1 mutant (Snail1-HA SA), which is exclusively nuclear owing to mutation of the phosphorylation sequence required for nuclear export (Domínguez *et al*., 2003). This mutant also co-immunoprecipitated with Akt (Supplementary Figure 1), further confirming that the interaction takes place in the nucleus.

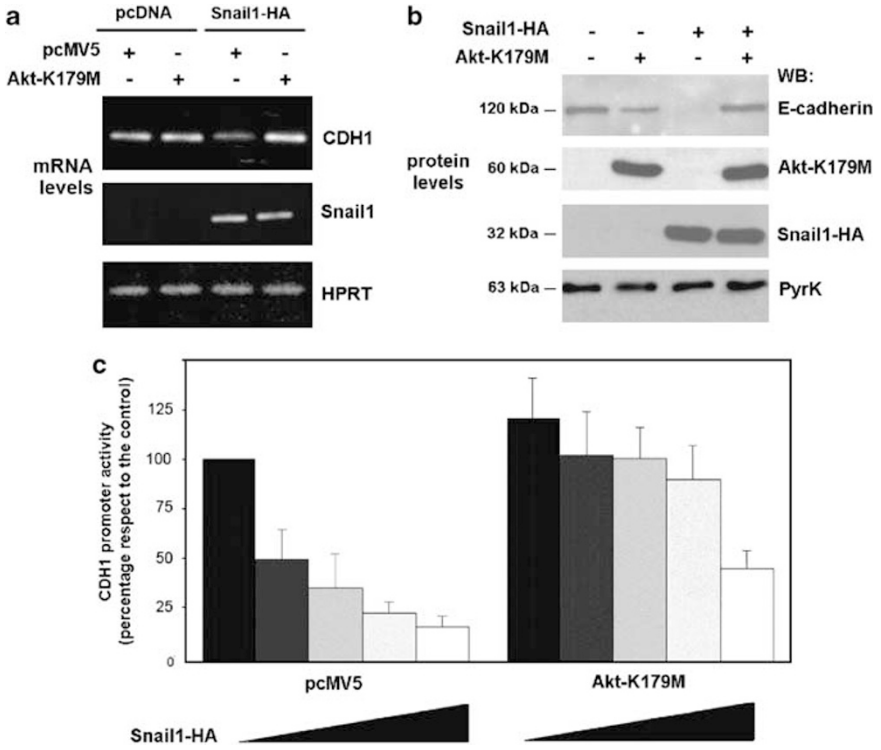

Figure 2 An Akt-inactive form prevents Snail1-induced repression of E-cadherin expression. RWP-1 cells expressing Snail1-HA or an empty plasmid were transfected with pCMV5-Akt1-K179M-HA. Cell transfectants were selected and analyzed by reverse transcription–PCR (**a**) or WB (**b**). The levels of the indicated RNAs or proteins are shown. In panel **c**, RWP-1 were transfected with a pGL3 plasmid containing the $-178/+92$ fragment of the E-cadherin (CDH1) promoter. Cells were co-transfected with growing amounts of pcDNA3-Snail1-HA or pcDNA3 as control, and pCMV5-Akt1-K179M-HA or pCMV5. The activity of Firefly and *Renilla* luciferase was determined as indicated under Materials and methods. The figure shows the average ± s.d. of the results of three experiments performed in triplicate.

The association between Akt and Snail1 was confirmed by pull-down assays. Both Akt1 and Akt2 isoforms were retained by GST-Snail1 and not by GST (Figure 3f). Although, when overexpressed, the Akt1 isoform interacts with Snail1, co-immunoprecipitation experiments indicated that Akt2 was the isoform preferentially associated to this transcriptional factor (see below). Therefore, we focused on the characterization of the elements involved in this interaction. Snail1 was retained by GST-Akt2, further demonstrating the interaction between these two proteins. This association requires the C-terminal part of Snail1 as it was detected with the C-terminal and not the N-terminal Snail1 domain (Figure 3g). The same Snail1 sequence was involved in binding to GST-Akt1 (not shown).

We also mapped the Akt2 sequence involved in Snail1 binding. As shown, full-length Akt2 was retained by a GST-Snail1 fusion protein (Figure 3h). Deletion of the last 72 amino acids (the C-terminal hydrophobic tail) in Akt2 did not affect binding (see F3 mutant in Figure 3h). However, elimination of the first 152 residues (the pleckstrin homology (PH) domain, F1 mutant) totally prevented it (Figure 3h). A protein fragment comprising this sequence was efficiently bound by GST-Snail1, suggesting that Snail1 association is confined to the PH domain.

Akt2 associates with Snail1

GST pull-down experiments showed that both Akt1 and Akt2 can bind to Snail1 *in vitro*. We analyzed which Akt isoform interacts with Snail1 in the nucleus using monoclonal antibodies specific for Akt1 and Akt2. Both isoforms were detected in the cell lines used in this study and their levels were not modified by expression of Snail1; on the contrary, Akt3 was not detected (not shown). Experiments of co-immunoprecipitation were performed either using endogenous or ectopically expressed Snail1. As shown in Figure 4a, Akt2 but not Akt1 was detected in Snail1 immunocomplexes in MiaPaca-2 cells. Results from RWP-1 cells expressing ectopic Snail1-HA were similar. In these cells, Akt1 was also detected in the Snail1-HA immunoprecipitates, although at lower levels than Akt2 (Figure 4b).

The Snail1–Akt2 association was also verified by immunofluorescence. In order to eliminate most Akt reactivity, cells were treated with CSK buffer before fixation, a condition that removes cellular components apart from the cytoskeleton and nuclear proteins. As shown in Figure 4c, Akt2 clearly colocalized with Snail1 in the nucleus of MiaPaca-2, RWP-1 Snail1-HA or HT-29 M6 Snail1-HA cells.

As Snail1 binds to the CDH1 promoter (Batlle *et al*., 2000), we determined whether Akt proteins were also located in this promoter. Chromatin immunoprecipita-

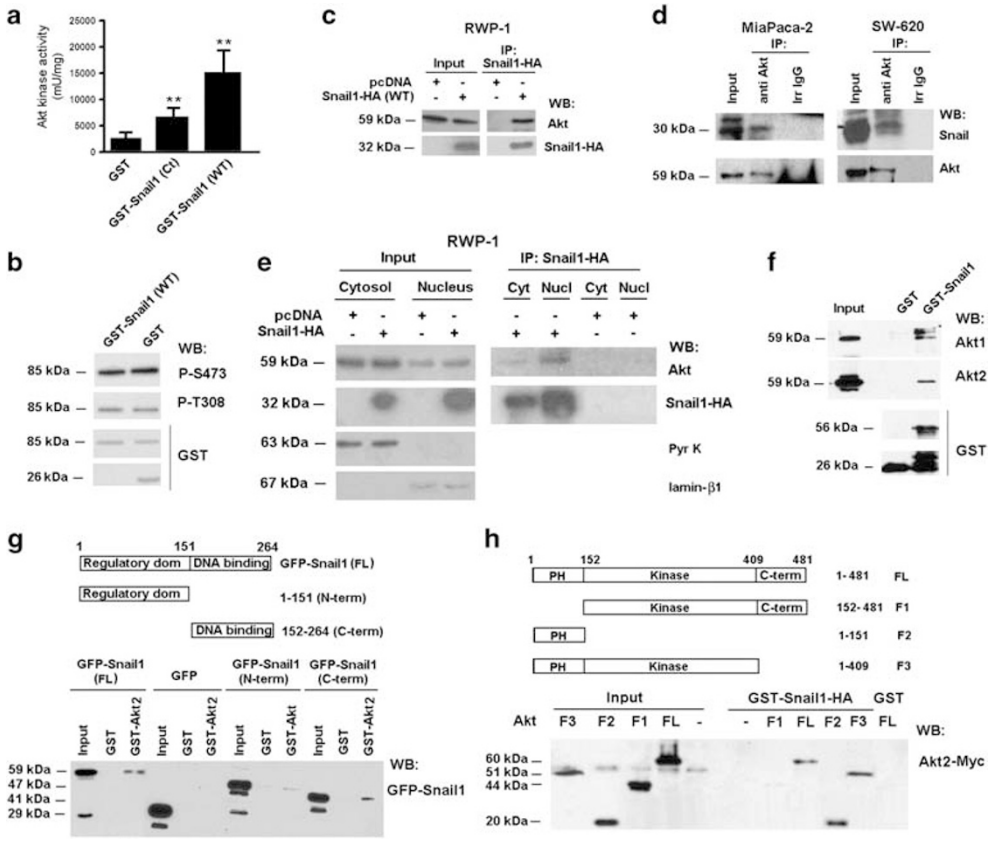

Figure 3 Snail1 and Akt interact. (**a**, **b**) GST-Akt1 was purified from HEK-293 cells serum-starved for 12 h and incubated with IGF-1 for 5 min. The protein kinase activity of purified GST-Akt1 was determined as above in the presence of 1 μg of recombinant GST-Snail1 (full-length, WT), the C-terminal Snail1 domain (GST-Snail1-Ct) or GST. The average ± s.d. of the results of three experiments performed in triplicate is shown in panel **a**. The *P*-values were lower than 0.01 (**). (**b**) The protein kinase samples were analyzed by WB using the indicated antibodies to check if the phosphorylation status of Thr308 and Ser473 was affected by Snail1. (**c**–**e**) Total (**c**, **d**), or cytosolic and nuclear extracts (**e**), from the indicated cells were immunoprecipitated with anti-HA (**c**, **e**) or anti-Akt (**d**) antibodies. Immunocomplexes were analyzed using anti-Akt or anti-Snail1 antibodies. (**f**–**h**) Pull-down assays were performed using recombinant GST-Snail1 (**f**, **h**), GST-Akt2 (**g**) or GST as bait and cell extracts from RWP-1 cells control or expressing the indicated proteins tagged with GFP (for Snail1, **g**) or Myc (for Akt2, **h**). A 5% of the total amount used in the assays was loaded in the input. Glutathione–-sepharose bound proteins were analyzed using antibodies against Akt1 or Akt2 (**f**), GFP (**g**) or Myc tag (**h**).

tion (ChIP) assays showed that Akt binds to an amplicon placed in the proximal CDH1 promoter, independently of Snail1 expression (Figure 5a). However, when the binding of the different isoforms was determined, we observed that Akt2 associated to the CDH1 promoter only in the presence of Snail1, both in RWP-1 (Figure 5b) and HT-29 M6 cells (Figure 5c). On the contrary, Akt1 bound to this promoter only in epithelial cells, which do not express Snail1. Therefore, Snail1 promotes a switch in the Akt isoform bound to CDH1, Akt1 being the one found in epithelial cells and Akt2 the one in mesenchymal cells.

Presence of the Snail1–Akt2 complex in the CDH1 promoter was also verified by sequential ChIP assays. As shown in Figure 5d, the CDH1 promoter amplicon was detected in the immunocomplex obtained after sequential immunoprecipitation using anti-Akt2 and HA antibodies in RWP-1 Snai11-HA cells.

Finally, binding of Akt2 to the CDH1 promoter was also verified by biotinylated DNA pull-down assays. As shown in Figure 5e, Akt2 co-purified with a fragment of DNA promoter containing the three E-boxes, the binding site for Snail1 protein (Batlle *et al*., 2000). Association of Akt2 was not detected to a CDH1 promoter with mutated E-boxes. Binding of Akt2 also required the presence of Snail1, as it was observed only when we used extracts from Snail1-expressing cells (Figure 5e).

Snail1 increases Akt2 activity on Thr45 in histone H3

We analyzed the presence of Akt2 substrates in the CDH1 promoter. Akt has been reported to bind to and phosphorylate histone H3 (He *et al*., 2003). We confirmed these results in *in vitro* assays using recombinant Akt2, which efficiently phosphorylates histone H3 (Figure 6a). A proteomic analysis identified Ser10, Ser28 and Thr45 as the modified residues in this protein. Taking advantage of the availability of antibodies specific for the phosphorylated forms of these amino acids in histone H3, we determined the effect of Snail1 in histone H3 phosphorylation by Akt2. As shown in Figure 6b, supplementation with GST-Snail1 increased the phosphorylation by recombinant Akt2 of the three residues analyzed, Ser10, Ser28 and Thr45, in histone

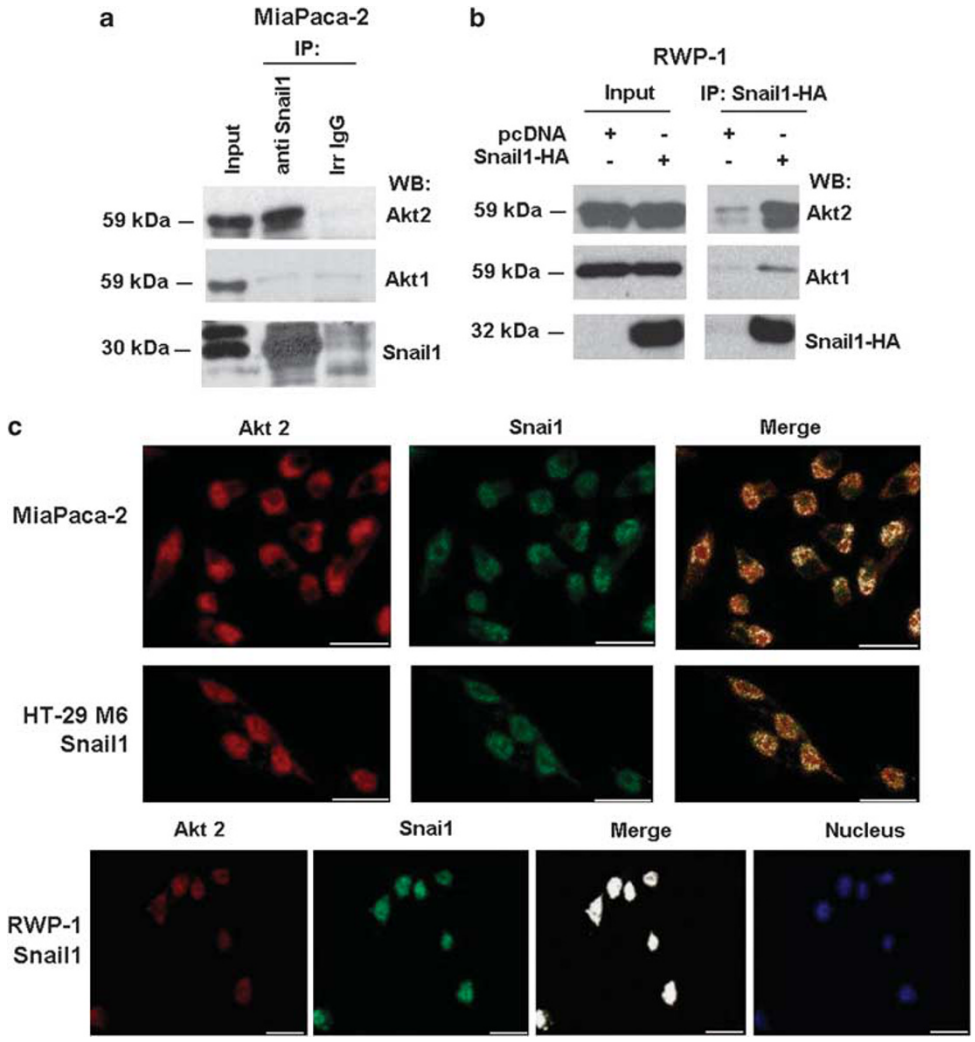

Figure 4 Snail1 binds to the Akt2 isoform. (**a**, **b**) Total extracts from MiaPaca-2 cells or RWP-1 cells transfected with Snail1-HA or a control plasmid were immunoprecipitated using anti-HA or Snail1 antibodies. The immunocomplexes were analyzed with specific antibodies against Akt1 or Akt2. A 5% of the extract used in the immunoprecipitation was loaded as control. (**c**) MiaPaca-2, RWP-1 Snail1 or HT-29 M6 Snail1 cells were analyzed for expression of Snail1-HA, Snail1 and Akt2 by immunofluorescence using specific antibodies. Akt2 is shown in red, Snail1 or Snail1-HA in green, and points of colocalization are shown in white. Nuclei were localized with Hoechst dye. The figure shows the results of a representative experiment of three performed. The bar corresponds to 20 μM.

H3. As expected, the phosphospecific antibodies did not recognize the unmodified protein in the absence of Akt2. Therefore, these results indicate that direct binding between both recombinant proteins increases the kinase activity of Akt2 on histone H3.

Binding of the modified forms of histone H3 to the CDH1 promoter was determined by ChIP assays. We did not observe a significant interaction of P-Ser28 histone H3 with the CDH1 promoter, either in control or in Snail1-HA-transfected RWP-1 cells. Binding of P-Ser10 H3 was low and only very slightly modified by Snail1 (Figure 6c). On the contrary, we detected the association of P-Thr45 histone H3 to the CDH1 promoter. The interaction was significantly upregulated in cells expressing Snail1 (Figure 6c). These results suggest that Akt2 interaction with Snail1 enhances its activity on histone H3 and the association of phosphorylated Thr45 histone H3 to the CDH1 promoter.

We also evaluated if the effects of Akt2 on histone H3 Thr45 phosphorylation were limited to the CDH1 promoter. As shown in Figure 6d, a specific Akt2 small interfering RNA (siRNA) efficiently downregulated cellular Akt2 levels. This siRNA also decreased the total cellular levels of histone H3 phosphorylated in Thr45 but not in Ser10 or Ser28, indicating that, at least in these cells, Akt2 is the protein kinase specifically controlling this epigenetic mark.

These results indicate that P-Thr45 histone H3 is an Akt2 substrate and a surrogate marker for nuclear Akt2 activity. Therefore, as expected and as Snail1 upregulates Akt2 in this compartment, P-Thr45 was sensitive to Snail1 protein levels. In cells with endogenous expression of Snail1, such as MiaPaca-2, Snail1 depletion downregulated P-Thr45 histone H3 (Figure 6e). Conversely, Snail1 ectopic expression in RWP-1 cells increased this epigenetic mark (Figure 6f).

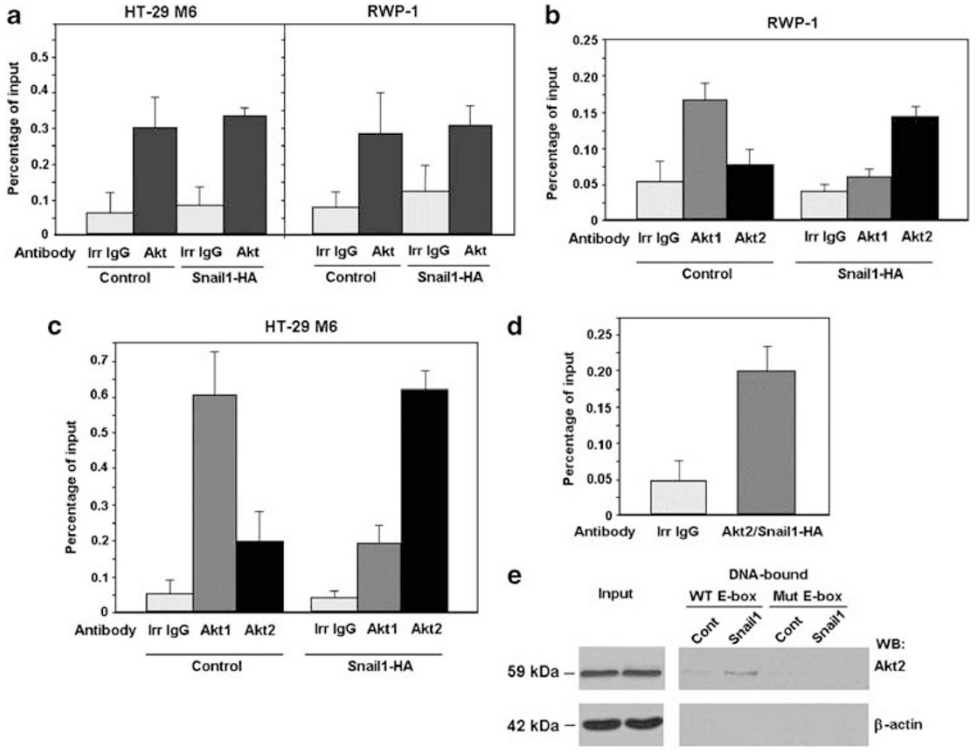

Figure 5 Snail1 recruits Akt2 to the CDH1 promoter. (**a**–**c**) ChIP assays were performed immunoprecipitating total Akt, Akt1 or Akt2, and analyzing the presence of specific sequences corresponding to the CDH1 promoter in the immunoprecipitates as indicated under Materials and methods. An irrelevant IgG was used as control for immunoprecipitation. Results were referred as percentage of input and presented as the average ± s.d. of the results of three experiments performed in triplicate. (**d**) A successive ChIP was performed using anti-Akt2 and anti-Snail1-HA antibodies in RWP-1 Snail1 cells. Results were represented as above. (**e**) A 250-ng weight of a biotinylated DNA corresponding to the CDH1 promoter and containing the three E-boxes either wild-type (WT) or mutated (MUT) were incubated with 200 μg of cell lysates from RWP-1 or RWP-1 Snail1-HA cells. The biotinylated DNA was purified and associated Akt2 was analyzed by western blotting. The input corresponds to 20% of the extract used in the assay.

Snail1 repression of CDH1 gene expression requires Akt2

Finally, we evaluated the relevance of Akt2 in Snail1 transcriptional activity. Akt2 protein levels were selectively downregulated in RWP-1 and RWP-1 Snail1 cells using a specific short-hairpin RNA (shRNA) that did not affect Akt1 levels (Figure 7a). Similar results were obtained with an siRNA (not shown). Snail1 protein levels were not modified by this shAkt2 RNA. On the contrary, shAkt1 decreased Snail1 ectopic protein (Supplementary Figure 2), suggesting that Akt1 controls Snail1 protein stability in these cells. As shown in Figure 7a, Akt2 depletion decreased basal E-cadherin levels and totally prevented the effect of Snail1 on the repression of this protein; as a consequence E-cadherin levels were markedly higher when RWP-1 Snail1 was transfected with the Akt2 shRNA than when the same cells were transfected with the shRNA control. A very similar effect on RNA levels was observed for Akt2 depletion on control or RWP1-Snail1 cells (Figure 7b). We also analyzed the repression of the vitamin D receptor (VDR), another direct target of Snail1 (Pálmer *et al*., 2004). Differently to control cells, Snail1 did not downregulate the VDR in Akt2-depleted cells (Figure 7a). The upregulation by Snail1 of fibronectin (FN1) and vimentin, two specific markers of mesenchymal cells, was also partially prevented by Akt2 interference (Figures 7a and b), probably as a consequence of the impaired downregulation of E-cadherin, as expression of these genes is inhibited by the E-cadherin protein (Ohkubo and Ozawa, 2003, Solanas *et al*., 2008).

Discussion

Although Akt proteins localize predominantly in the cytoplasm, they also reside in the nucleus or translocate to this compartment upon stimulation (Ahmed *et al*., 1993; Andjelković *et al*., 1997). In the nucleus Akt phosphorylates or interacts with proteins implicated in apoptosis resistance, a pathway where Akt has a key role (Andjelković *et al*., 1997; Masuyama *et al*., 2001; Ahn *et al*., 2006; Lee *et al*., 2008). Moreover, Akt proteins have been involved in the process of EMT (Thiery *et al*., 2009). For instance, Akt1 overexpression induces EMT, increasing the expression of the Snail1 transcriptional factor and other E-cadherin repressors (Julien *et al*., 2007). Snail1 upregulation is a consequence of increased transcription and enhanced protein stability as Akt controls both processes (García de Herreros *et al*., 2010). Akt proteins are also important downstream effectors of Snail1 and are

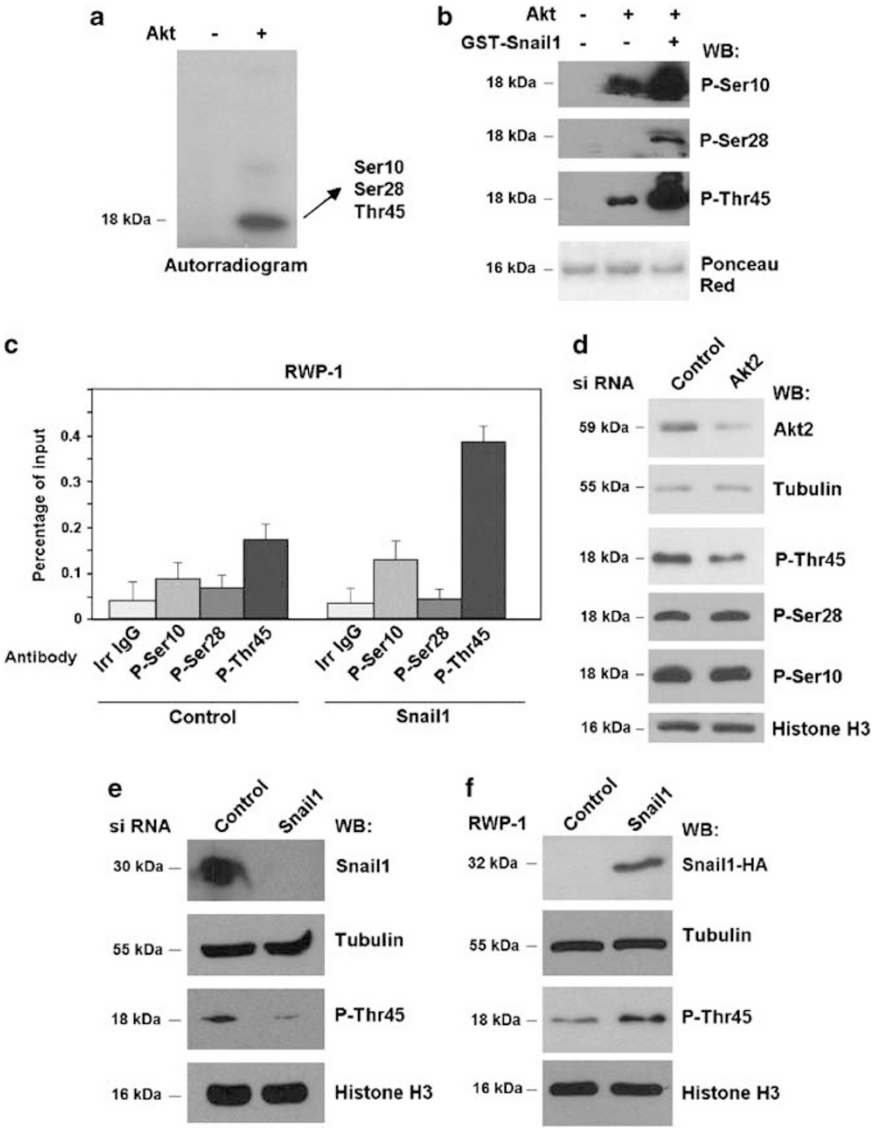

Figure 6 Akt2 increases histone H3 phosphorylation in Thr45 and binding of P-Thr45 H3 to the CDH1 promoter. (**a**) Recombinant H3 was phosphorylated *in vitro* with recombinant Akt2 and γ-^{32}P-ATP. A representative autoradiogram is shown. In parallel, a reaction was performed with non-radioactive ATP. The phosphorylated protein was analyzed by mass spectrometry as detailed in the Supplementary information. The identified residues were Ser10, Ser28 and Thr45. (**b**) Recombinant histone H3 was incubated with recombinant Akt2, and GST-Snail1 or GST, when indicated. Phosphorylation was analyzed using antibodies against P-Ser10, P-Ser28 or P-Thr45 in histone H3. The figure shows the result of a representative experiment of two performed with identical results. (**c**) Association of modified histone H3 to the CDH1 promoter was analyzed by ChIP using antibodies specific for P-Ser10, P-Ser28 or P-Thr45, or an irrelevant IgG as control. The analysis was performed in RWP-1 control or RWP-1 Snail1 cells. The figure shows the average ± s.d. of the results of three experiments performed in duplicate. (**d**, **e**) Akt2 (**d**) or Snail1 (**e**) was downregulated by transfection of specific siRNAs (20 nM) in MiaPaca-2 cells for 48 h. Akt2, Snail1, tubulin (as loading control), P-Thr45, P-Ser10, P-Ser28 in histone H3 or histone H3 levels were determined by western blotting. As control, cells were transfected with an irrelevant siRNA. A similar analysis was also performed in RWP-1 control and RWP-1 Snail1 cells (**f**).

activated by expression of this EMT inductor (this report; see also Vega *et al*., 2004; Escrivà *et al*., 2008). Activation of Akt has been related to another very relevant consequence of Snail1 expression in cell lines: acquisition of resistance to apoptotic insults (Vega *et al*., 2004).

In this article, we have analyzed Akt activation by not only determining the phosphorylation of key residues in the Akt molecule, but also by directly measuring its kinase activity on Crosstide, a small peptide widely used as an Akt substrate (Alessi *et al*., 1996). Surprisingly, part of Akt activation by Snail1 is independent of Snail1 repressive activity, as a Snail1 mutant unable to recruit co-repressors stimulated Akt kinase activity, although to a lower extent than the wild-type protein (see Figure 1). This mutant, P2A, does not bind to the different cofactors necessary for repression of CDH1 or PTEN (Escrivà *et al*., 2008, García de Herreros *et al*., 2010). These experiments indicated that, although part of the Akt activation by Snail1 is consequence of repression of

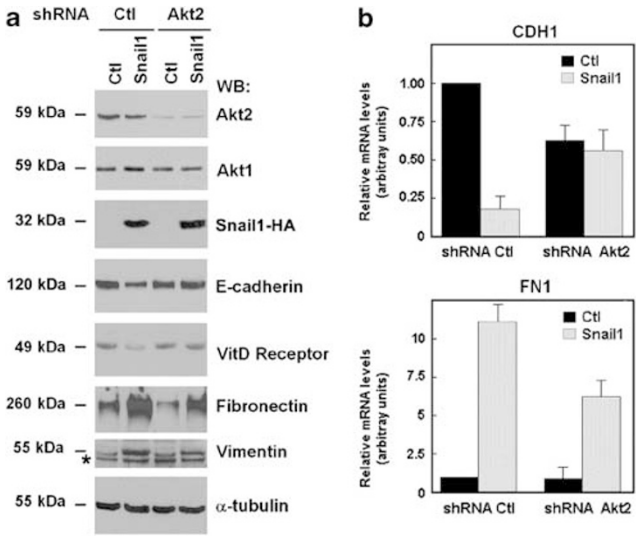

Figure 7 Akt2 interference prevents Snail1-induced CDH1 repression and FN1 activation. RWP-1 control and RWP-1 Snail1-HA cell were stably transfected with control and Akt2 shRNA as indicated under Materials and methods. Total cell extracts were analyzed by western blotting using the indicated antibodies (**a**) or RNA was obtained and analyzed by quantitative reverse transcription–PCR (see Supplementary information) (**b**). The asterisk in panel **a** corresponds to a non-specific band. The results in panel **b** are presented relative to the values obtained in RWP-1 cell transfected with a control shRNA and correspond to the average ± s.d. of the results of 3–4 experiments performed.

PTEN (Escrivà *et al*., 2008), other mechanisms also contribute to the full stimulation of this kinase.

We also show that Snail1 binds to Akt and the interaction stimulates the activity of this kinase. Other proteins also interact with Akt and upregulate its activity (Pekarsky *et al*., 2000; Sato *et al*., 2000; Jahn *et al*., 2002). Binding of Snail1 requires the PH domain of Akt, similarly to other reported interactors (Sato *et al*., 2000; Jahn *et al*., 2002). It is possible that this interaction stabilizes the active Akt conformer, preventing the intramolecular association between the PH and the kinase domains (Calleja *et al*., 2007). Regarding Snaill1, and in contrast to its binding with other co-repressors, the association with Akt is independent of the SNAG sequence but requires the C-terminal domain of Snail1 protein. *In vitro*, Snail1 binds to both Akt1 and Akt2; however, in cells Snail1 preferentially co-immunoprecipitates with Akt2. It is likely that additional proteins control the specificity of this interaction; for instance, associating to Akt1 and precluding Snail1–Akt1 binding. These proteins might be titrated out by Akt1 overexpression, and would be unable to prevent the Snail1–Akt1 interaction when this protein kinase is ectopically expressed. Moreover, they might vary in different cell lines, explaining the basal Snail1–Akt1 binding detected in some cells, in all cases much lower than the Snail1–Akt2 interaction.

Ectopic Snail1 expression promotes the Akt isoform switch in the CDH1 promoter as it enhances the binding of Akt2 kinase to the CDH1 promoter at the same time that it decreases Akt1 interaction with this DNA fragment. This result suggests that the two Akt isoforms have different roles on CDH1 transcription when bound to the CDH1 promoter. Akt proteins have been shown to interact and phosphorylate factors involved in the modulation of epigenetic signals, such as SETDB1 (Gao *et al*., 2007), EzH2 (Cha *et al*., 2005) or even histone H3 (Brunet *et al*., 1999). The final effect of this phosphorylation is variable as it can promote gene silencing (Jahn *et al*., 2002) or activation (Gao *et al*., 2007). It is possible that these discrepant effects are due to the contrary effects of the Akt isoforms on gene expression when bound to a promoter, Akt1 being involved in activation and Akt2 in repression.

A different role for the two Akt isoforms in EMT and metastasis has been reported by several researchers. In most cells, Akt1 seems to have a role in the maintenance of the epithelial phenotype (Irie *et al*., 2005; Iliopoulos *et al*., 2009). However, in some cells its downregulation decreases the formation of metastasis (Ericson *et al*., 2010). On the other hand, Akt2 is required for EMT as it is activated during this transition and its downregulation prevents the acquisition of the mesenchymal phenotype or the formation of metastasis (Irie *et al*., 2005; Cheng *et al*., 2007; Rychahou *et al*., 2008; Dillon *et al*., 2009; Iliopoulos *et al*., 2009; Ericson *et al*., 2010). However, these effects are cell-dependent as in some cells Akt2 depletion by itself does not promote any effect but prevents the EMT induced by Akt1 depletion. It is possible that these different effects are due to the distinct contribution of Snail1 to the maintenance of the mesenchymal phenotype in the cell lines studied. Although the role of this factor in triggering EMT and repressing CDH1 expression is well established, its contribution to CDH1 silencing is variable, as CDH1 is also controlled by Zeb proteins. These transcriptional factors require Snail1 for their induction but not for sustained expression (García de Herreros *et al*., 2010). Differently to this pro-mesenchymal action of Akt2, Akt1 might have a more complex role as, in addition to the pro-epithelial effects, it also contributes to Snail1 expression, acting both on gene expression and protein stabilization (see above, and also Supplementary Figure 2). This suggests that the contribution of Akt1 to EMT might be positive or negative depending on the cell line. Anyway, the finding that Snail1 interacts with Akt2 in the CDH1 promoter provides a mechanism to explain the pro-mesenchymal activity of this protein kinase.

Finally, we have analyzed the consequence of the Snail1-driven Akt2 interaction with the CDH1 promoter. Snail1 binding increases the *in vitro* Akt2 kinase activity on histone H3, phosphorylating Ser10, Ser28 and Thr45. Among these, Thr45 is a bona fide substrate of the Akt2–Snail1 complex as its phosphorylation is dependent on the cellular levels of both proteins. Snail1 also upregulates the binding of P-Thr45 histone H3 to the CDH1 promoter. The role of this phosphorylation has not been investigated yet; in mammalian cells the only report associates it with apoptosis (Hurd *et al*., 2009). It is possible that the modification is not linked to apoptosis *per se* but it is the consequence of the

upregulated Snail1 expression detected in many cells in response to apoptotic insults, as Snail1 protects epithelial cells from cell death (Vega *et al*., 2004; Escrivà *et al*., 2008). More studies will be required to determine the specific role of this histone modification in gene expression. In any case, our results suggest that this new epigenetic mark is associated to Snail1 action and might contribute to CDH1 repression by this transcriptional factor.

Materials and methods

Cell culture and reagents

Cell lines were obtained from our institute's cell bank and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10 mM glutamine (Gibco BRL, San Francisco, CA, USA) and penicillin–streptomycin (Sigma, St Louis, MO, USA) unless otherwise specified. To ensure reproducibility, cultures were grown no longer than 3 months and monitored by PCR to prevent Mycoplasma contamination. The generation and properties of RWP-1, SW-480 or HT-29 M6 cells stably transfected with Snail1-HA have been described previously (Batlle *et al*., 2000; Pálmer *et al*., 2004; Peiró *et al*., 2006). For generation of RWP-1 and RWP-1 Snail1-HA deficient in Akt2, cells were infected with lentivirus expressing shRNAs specific for Akt2 (from Sigma, TRCN 39968, 39969, 39970, 39971 and 39972), Akt1 (from Sigma, TRCN 39793, 39794, 39795, 39796 and 39797) or the corresponding shRNA control (Herranz *et al*., 2008). Cells were selected with puromycin (2.5 μg/ml). Alternatively, in order to deplete Akt2, cells were transfected with specific synthetic siRNAs (from Dharmacon, Lafayette, CO, USA, ref D003001-21-0010) or the corresponding siRNA control. For human Snail1 depletion, an siRNA specific for human Snail1 was used (L-010847-01; Dharmacon).

Antibodies used in this study were the following: mouse monoclonal anti-Snail1 (Francí *et al*., 2006); rabbit anti-Akt, Akt1, Akt2, Akt3, P-Ser473 or P-Thr308 in Akt, FoxO1, phosphoFoxO1 (from Cell Signaling); rabbit anti-P-Ser10 or P-Ser28 in histone H3 (Millipore, Billerica, MA, USA and Upstate, Charlottesville, VA, USA respectively); mouse monoclonal anti-P-Thr45 in histone H3 or anti-histone H3, rabbit anti-lamin B1 (from Abcam, Cambridge, UK); rat anti-VDR (from Millipore); goat anti-pyruvate kinase (from Chemicon, Temecula, CA, USA); goat anti-vimentin (from Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-E-cadherin (from BD Biosciences, Franklin Lakes, NJ, USA); rabbit anti-fibronectin (from Dako, Golstrup, Denmark); and mouse anti-β-actin, rabbit anti-HA tag and rabbit anti-tubulin (from Sigma).

ChIP assays

ChIP assays were performed as described previously (Peiró *et al*., 2006) using antibodies against Akt, Akt1, Akt2, P-Ser10, P-Ser28 or P-Thr45 histone H3 or an irrelevant IgG (Sigma). For sequential ChIP assays, chromatin was divided in two aliquots, which were immunoprecipitated (first ChIP) using antibodies against HA and the irrelevant IgG. Immunocomplexes were eluted with 1% sodium dodecyl sulfate, diluted with dilution buffer (Peiró *et al*., 2006), and then soluble chromatin fractions obtained with anti-HA and IgG were immunoprecipitated again (second ChIP) using antibodies against Akt2 and the irrelevant IgG, respectively. More details are provided in the Supplementary information.

Analysis of protein distribution by immunofluorescence

Cells were cultured on coverslips for 24 h. Before fixation, cells were washed for 5 min on ice in CSK buffer (20 mM Hepes (pH 7.8), 100 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose) plus 0.5% Triton X-100. Cells were washed once with CSK buffer and fixed with 4% paraformaldehyde for 30 min. After washing three times with phosphate-buffered saline, cells were blocked with 3% bovine serum albumin in phosphate-buffered saline plus 0.3% Triton X-100 for 1 h at 20 °C. Akt1 or Akt2 antibodies were used at 1/50 dilution; the Snail1 hybridoma supernatant was used at 1/2 dilution. Secondary antibodies were goat anti-rabbit labeled with Alexa-555 and goat anti-mouse with Alexa-488. Coverslips were mounted on glass slides with Mowiol and immunofluorescence was viewed with a Leica confocal microscope (Leica spectral confocal TCS-SL, Leica Microsystems, Wetzlar, Germany). The acquisition software was from Leica Confocal Software (Leica Microsystems).

Biotinylated DNA pull-down assays

Biotinylated DNA pull-down assays were performed as described (Raurell *et al*., 2006) using a biotinylated DNA fragment corresponding to the $-178/+92$ sequence of the CDH1 promoter, or a control DNA fragment with the three E-boxes mutated (Batlle *et al*., 2000).

Akt protein kinase activity

Cells expressing GST-Akt1 fusion proteins were lysed in buffer-L (50 mM Tris–HCl (pH 7.5), 270 mM sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM 1% Triton X-100, 5 mM β-mercaptoethanol) plus protease inhibitors. After centrifugation, lysates were incubated with glutathione–sepharose-4B; the beads were washed with buffer-L and buffer-L plus 500 mM NaCl. Purified GST-Akt1 was incubated with 30 μM Crosstide peptide (GRPRTSSFAEG), 100 μM ^{32}P-γ-ATP (3000 c.p.m./pmol) in the presence of 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM EGTA for 10 to 30 min at 30 °C (Alessi *et al*., 1996). Samples were spotted onto P81 phosphocellulose paper (Whatman, Maidstone, UK), rinsed with 50 mM H_3PO_4 and dried, and radioactivity was determined on a Wallac counter. Experiments were performed in triplicates and repeated three times.

In vitro *phosphorylation of histone H3*

When indicated, histone H3 (1 μg) was phosphorylated using 0.4 μg of recombinant protein kinase Akt2 (160 mU; from Sigma) in a final volume of 50 μl in kinase buffer (25 mM Tris–HCl (pH 7.5), 5 mM β-mercaptoethanol, 0.01 mM EGTA, 10 mM $MgCl_2$, 100 μM ATP). Reactions were performed for 20 min at 30 °C. When indicated GST or GST-Snail1 (1 μg) were added to the reaction. Alternatively, radioactive γ-^{32}P-ATP (3000 c.p.m./pmol) was used. Samples were analyzed by autoradiography or western blotting using antibodies specific for the phosphorylated residues in H3 or processed for mass spectrometry.

Other methods, including preparation of fusion proteins, oligonucleotides used for RNA analysis, and ChIP assays and mass spectrometry analysis, are detailed in the Supplementary information.

Conflict of interest

The authors declare no conflict of interest.

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