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A molecular role for lysyl oxidase-like 2 enzyme in Snail regulation and tumor progression

Héctor Peinado[1,4], Maria del Carmen Iglesias-de la Cruz[1,4], David Olmeda[1], Katalin Csiszar[2], Keith SK Fong[2], Sonia Vega[3,5], Maria Angela Nieto[3,5], Amparo Cano[1,*] and Francisco Portillo[1,*]

[1]Departamento de Bioquímica, Instituto de Investigaciones Biomédicas 'Alberto Sols', Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Arturo Duperier, Madrid, Spain, [2]Cardiovascular Research Center, John A Burns School of Medicine, University of Hawaii, Honolulu, HI, USA and [3]Instituto Cajal, Avenida Doctor Arce, Madrid, Spain

The transcription factor Snail controls epithelial–mesenchymal transitions (EMT) by repressing *E-cadherin* expression and other epithelial genes. However, the mechanisms involved in the regulation of Snail function are not fully understood. Here we show that lysyl-oxidase-like 2 and 3 (LOXL2 and LOXL3), two members of the lysyl-oxidase gene family, interact and cooperate with Snail to downregulate *E-cadherin* expression. Snail's lysine residues 98 and 137 are essential for Snail stability, functional cooperation with LOXL2/3 and induction of EMT. Overexpression of LOXL2 or LOXL3 in epithelial cells induces an EMT process, supporting their implication in tumor progression. The biological importance of LOXL2 is further supported by RNA interference of LOXL2 in Snail-expressing metastatic carcinoma cells, which led to a strong decrease of tumor growth associated to increased apoptosis and reduced expression of mesenchymal and invasive/angiogenic markers. Taken together, these results establish a direct link between LOXL2 and Snail in carcinoma progression.

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Introduction

Epithelial tumors are thought to metastasize by initially invading the adjacent tissues, a process involving the loss of their cell–cell adhesions and the acquisition of migratory capabilities. These processes include phenotypical changes associated with epithelial–mesenchymal transitions (EMT), similar to those that take place during certain steps of embryonic development (Thiery, 2002). The invasive and metastatic phenotype is associated with downregulation of E-cadherin expression (Birchmeier and Behrens, 1994). Several mechanisms have been implicated in the regulation of *E-cadherin* expression during tumor progression, including genetic, epigenetic and transcriptional changes (Christofori and Semb, 1999; Peinado *et al*, 2004c). Snail transcription factor has been described as a direct repressor of *E-cadherin* expression in epithelial cells; the expression of Snail induces a full EMT and increases migration/invasion in different physiological and pathological situations (Batlle *et al*, 2000; Cano *et al*, 2000; Peinado *et al*, 2004b). Moreover, *Snail* expression has been detected in different invasive carcinoma and melanoma cell lines and, importantly, in invasive regions of squamous cell carcinomas and dedifferentiated ductal breast carcinomas and hepatocarcinomas (reviewed in Nieto, 2002; Peinado *et al*, 2004c). Recently, we have described the recruitment of the mSin3A corepressor complex with histone deacetylases (HDACs) by Snail, through the Snail and Gfi (SNAG) domain, to repress *E-cadherin* expression (Peinado *et al*, 2004a). In order to identify additional proteins that might interact with Snail to regulate *E-cadherin* expression, we carried out a yeast two-hybrid screen. Using Snail as bait, we found members of the lysyl oxidase (LOX) gene family to be potential interacting partners. Five LOX family genes have been identified so far in mammalian genomes encoding the prototypic LOX and four different LOX-like proteins (LOXL1, LOXL2, LOXL3 and LOXL4) (Kagan and Li, 2003; Molnar *et al*, 2003). LOX and LOX-like proteins are copper-containing enzymes that catalyze the oxidative deamination of the ε-amino group in certain peptidyl lysine residues promoting covalent protein crosslinkages (Kagan and Li, 2003; Molnar *et al*, 2003). All members of the LOX family show a highly conserved C-terminus region that contains the catalytic domain. The N-terminus of the LOX isoforms is less conserved among the different members and it is thought to determine the individual role and tissue distribution of each isoenzyme (Maki *et al*, 2001). The prototypic LOX plays a key role in the biogenesis of the connective tissue catalyzing crosslinkage formation in collagen and elastin components (Kagan and Li, 2003) and, recently, it has been shown that LOXL1 is required for proper elastic fiber homeostasis (Liu *et al*, 2004). The individual function of the remaining members of the family remains unclear, although recent evidences suggest the involvement of LOX, LOXL2 or LOXL4 in breast and head and neck squamous cell carcinoma progression (Kirschmann *et al*, 2002; Akiri *et al*, 2003; Holtmeier *et al*, 2003). In the present report, we show that LOXL2 and LOXL3 collaborate *in vivo* with Snail to repress *E-cadherin* transcription. Snail–LOXL2/3 physical interaction depends on the SNAG domain and Snail's

*Corresponding authors. A Cano, Instituto de Investigaciones Biomédicas 'Alberto Sols', CSIC-UAM, Arturo Duperier 4, 28029 Madrid, Spain. Tel.: +34 91 585 4411; Fax: +34 91 585 4401;
E-mail: acano@iib.uam.es or F Portillo, Instituto de Investigaciones Biomédicas 'Alberto Sols', CSIC-UAM, Arturo Duperier 4, 28029 Madrid, Spain. Tel.: +34 91 585 4457; Fax: +34 91 585 4401;
E-mail: fportillo@iib.uam.es
[4]These authors contributed equally to this work
[5]Present address: Instituto de Neurociencias, Apartado de Correos, 18, 03550 San Juan, Alicante, Spain

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lysine residues K98 and K137 are critical for Snail stability and functional cooperation with LOXL2/3. We also present evidence for a role of LOXL2 in tumor growth and progression.

Results

LOXL2 and LOXL3 interact with Snail in vivo

To identify new proteins involved in Snail functionality, we performed a yeast two-hybrid screen. Using the N-terminus part of Snail (amino acids 1–150; Figure 1A) as bait, we identified the catalytic domain of LOX and LOXL1 enzymes as positive clones in the screen (Figure 1B). Since the C-terminus is a region of high conservation among all LOX family members, one or more of the LOX isoforms could be potential Snail interacting partner(s). Thus, we analyzed by reverse transcriptase–polymerase chain reaction (RT–PCR) the expression of the endogenous LOX gene family in a panel of both mouse epidermal keratinocyte (MCA3D, CarB and HaCa4) (Figure 2A) and human melanoma and carcinoma cell lines (MCF7, MDA-MB231 MDA-MB435 and A375P) (Figure 2B). The analysis included from poorly invasive/nonmetastatic cell lines with normal levels of E-cadherin expression and undetectable levels of *Snail* transcripts (MCA3D and MCF7) to cell lines that show high levels of *Snail* expression, E-cadherin loss and a highly invasive/metastatic phenotype (CarB, HaCa4, MDA-MB231, MDA-MB435 and A375P) (Cano *et al*, 2000). We detected expression of *LOXL2*, *LOXL3* and/or *LOXL4* in cell lines that were highly invasive and metastatic but not of *LOX* and *LOXL1* mRNAs (Figure 2A and B). Interestingly, we observed a direct correlation between the expression of at least one of the *LOXL2*, *LOXL3* and *LOXL4* genes, and the presence of *Snail* and the loss of *E-cadherin* transcripts. This result was further confirmed for LOXL2 and LOXL3 proteins by immunoblotting analysis using specific antibodies (Figure 2C and D). These results led us to pursue LOXL2, LOXL3 or LOXL4 as potential Snail's partners for collaborating in EMT.

To confirm the molecular interaction suggested by the two-hybrid screen, we carried out co-immunoprecipitation analyses in HEK293T cells transiently transfected with tagged versions of Snail and LOXL2, LOXL3 or LOXL4 isoforms. Co-immunoprecipitation of LOXL2 and LOXL3, but not LOXL4, by Snail (Figure 3A, left panels) indicated an *in vivo* interaction between Snail and either LOXL2 or LOXL3. Furthermore, inverse co-immunoprecipitation analysis reinforced this notion (Figure 3A, right panels). Additional co-immunoprecipitation experiments carried out with several versions of Snail-HA containing different functional domains showed that LOXL2 interacts with the full-length Snail protein, but not with mutants lacking the N-terminal region (ΔNt) or just the first 9 amino acids (ΔSNAG) (Figure 3B), indicating that Snail interaction with LOXL2 requires the repressor SNAG domain (Peinado *et al*, 2004a). Similar results were obtained in Madin Darby canine kidney (MDCK) cells and in pulldown assays with LOXL2 or LOXL3 (data not shown). Unfortunately, Snail-HA lacking the C-terminal domain (ΔZn-HA) was highly unstable (Figure 3B, left panel) precluding confirmation of the interaction between LOXL2/3 and Snail N-terminal domain detected in the two-hybrid screen. On the other hand, confocal analysis of MDCK cells transiently transfected with tagged

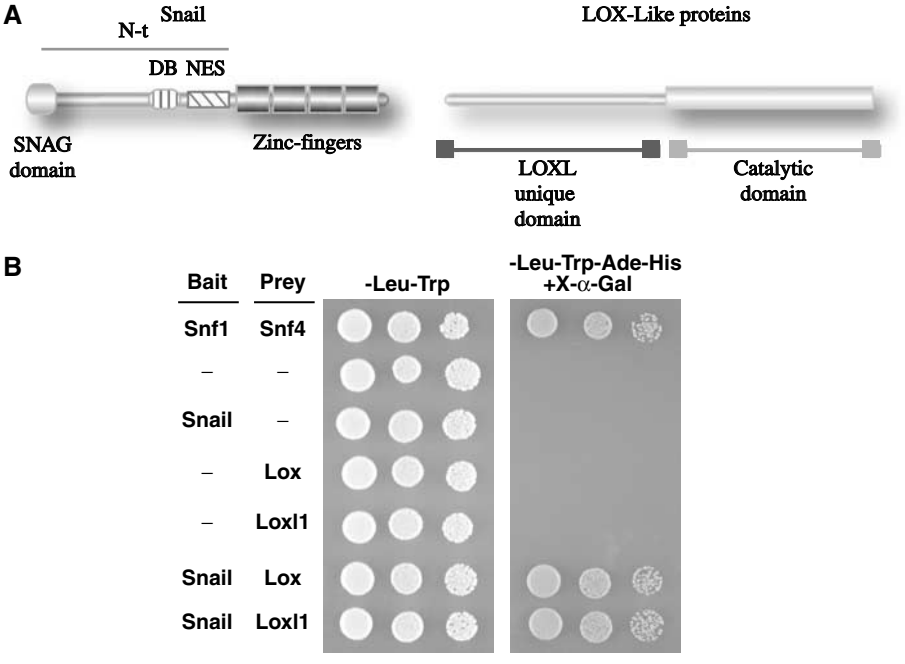


Figure 1 Snail interacts with LOX and LOXL1 in the two-hybrid screen. (**A**) Diagrammatic representation of the main functional domains of Snail and LOX-like proteins. (Left) Snail organization: N-half part (N-t) used as bait in the two-hybrid screen, containing the N-terminal SNAG domain, the destruction box (DB) and the NES domain. (Right) LOX-like proteins organization: N-terminal region specific to each family member and C-terminal catalytic region common to LOX and LOXL enzymes. (**B**) Specificity of interactions between Snail (N-t) and LOX and LOXL1 (catalytic domain) in the two-hybrid system. The isolated cDNAs from LOX and LOXL1 isoforms were tested for interaction with Snail in complete medium (middle) or in the absence of adenine and histidine and in the presence of X-αGal (right) at three serial dilutions. Interactions in the absence of bait and prey cDNAs and those between Snf1 and Snf4 cDNAs were tested in parallel as negative and positive controls, respectively.

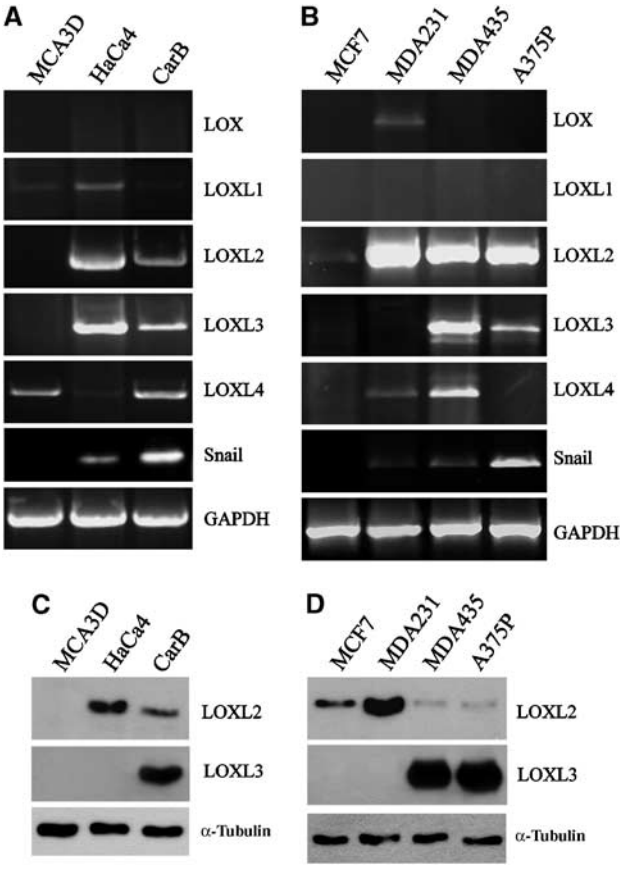


Figure 2 Expression of LOX and LOXL isoforms in mouse and human carcinoma cells. (**A, B**) The expression of *LOX*, the indicated *LOXL* isoforms and *Snail* was analyzed by RT–PCR in the indicated mouse (A) and (B) human cell lines. *GAPDH* mRNA levels were analyzed in parallel as a control of the amount of cDNAs. (**C, D**) LOXL2 and LOXL3 expression was analyzed by Western blot in the indicated mouse (C) and human (D) cell lines; α-tubulin levels were analyzed in parallel as a loading control.

versions of the corresponding genes showed that LOXL2/3 and Snail colocalize in the perinuclear compartment (Figure 3C). The perinuclear localization has also been recently observed for LOXL1 in cell cultures (Liu *et al*, 2004). Taken together, these results show that LOXL2 and LOXL3 interact with Snail through the SNAG domain.

LOXL2 and LOXL3 collaborate with Snail in E-cadherin repression

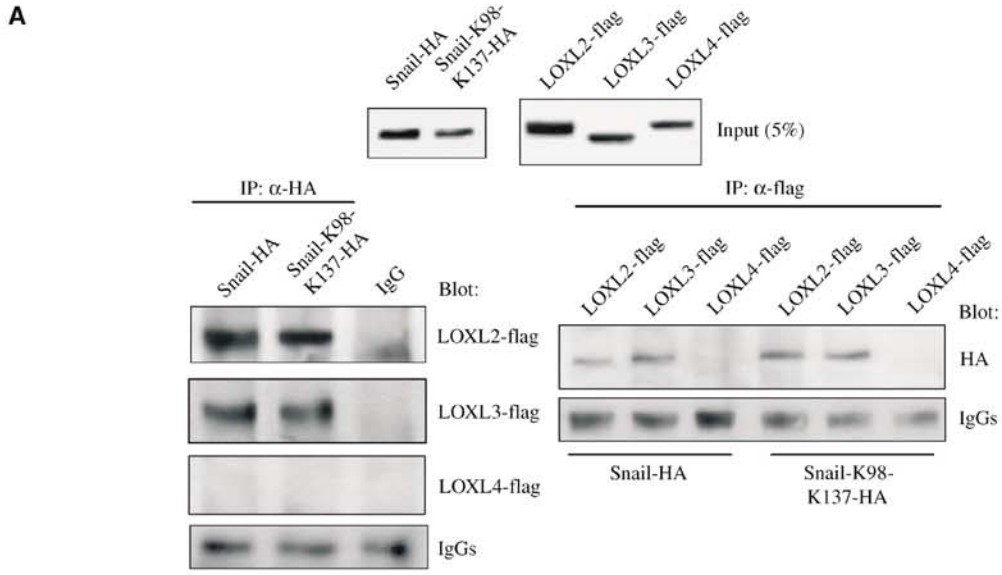
To get an insight into the functionality of the identified Snail–LOXL2/3 interactions, we next analyzed the effect of human LOXL2 and LOXL3 on *E-cadherin* promoter activity in MDCK cells in the absence or presence of Snail. To observe a potential cooperation, Snail was transfected under partial repression conditions (50 ng) (Peinado *et al*, 2004a) (Figure 4A, lane 2). Transfection of human *LOXL2* or *LOXL3* cDNAs (300 ng) induced a partial repression of the *E-cadherin* promoter (Figure 4A, lanes 3 and 5) and cotransfection of *Snail* with either *LOXL2* or *LOXL3* led to a significant increase, up to 70%, in the repression activity (Figure 4A, lanes 4 and 6), indicating that LOXL2/3 proteins collaborate with Snail in *E-cadherin* promoter repression. Cotransfection of the ΔSNAG mutant indicated the requirement of the N-terminal SNAG domain for Snail repression and functional collaboration with LOXL2/3 (Figure 4A, lanes 7–9).

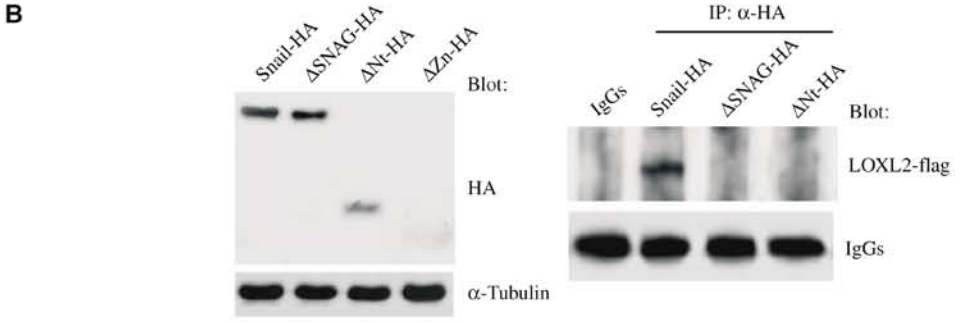
To confirm if the moderate *E-cadherin* promoter repression triggered by LOXL2 or LOXL3 might be caused by cooperation with the endogenous Snail (Peinado *et al*, 2003), *E-cadherin* promoter activity was assayed in MDCK cells stably transfected with either SnailshRNA or control EGFPshRNA. Expression of LOXL2 or LOXL3 in MDCK-SnailshRNA cells had no effect on *E-cadherin* promoter activity (Figure 4B, lanes 6 and 7, compare with lanes 2 and 3). Analysis of *E-cadherin* promoter in Snail-deficient MCA3D cells showed a very low repressive effect of LOXL2/3 (Supplementary data S2a). Together, these data suggest that LOXL2/3 enzymes can functionally cooperate with Snail in *E-cadherin* repression as a consequence of their physical interaction through the SNAG domain.

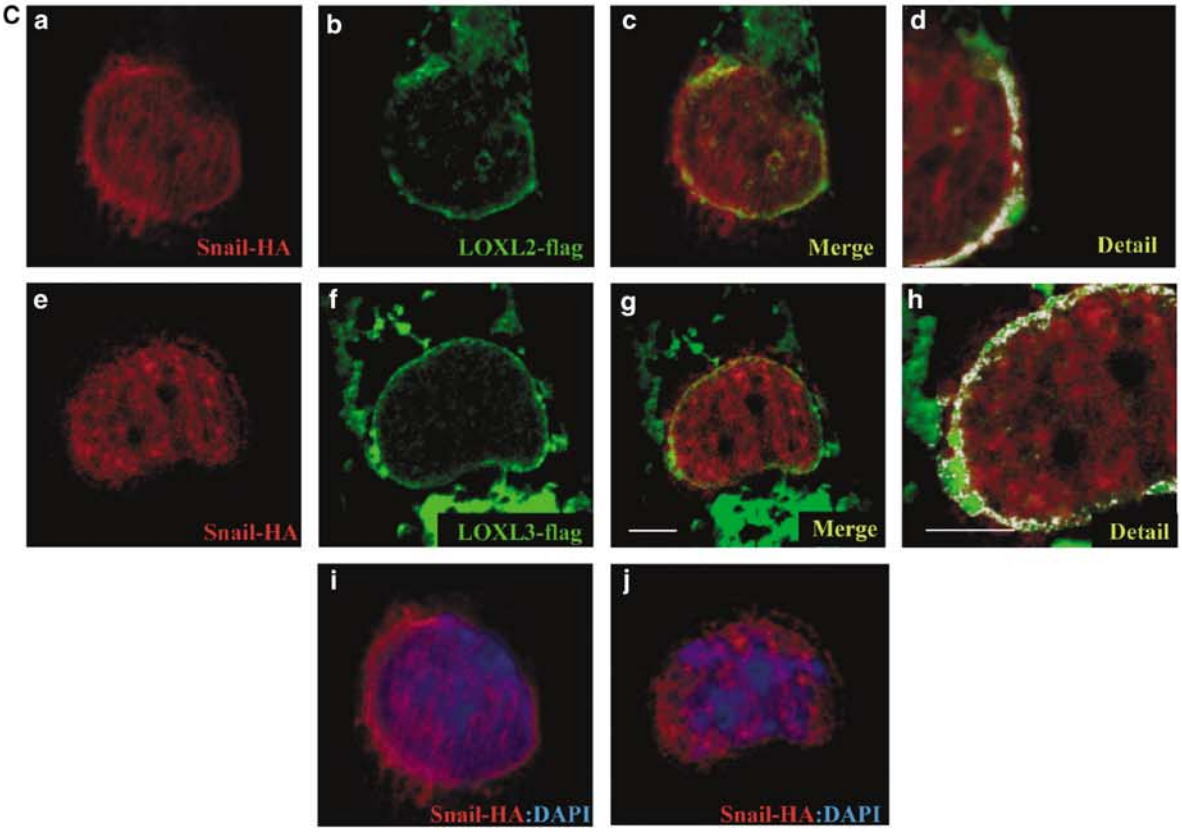
Snail Lys98 and Lys137 residues are essential for E-cadherin silencing, induction of EMT and Snail stability

Since LOXL enzymes exert their function by modification of specific peptidyl lysine residues, we analyzed the conserved lysine residues in the Snail subfamily of repressors (Sefton *et al*, 1998) and found that four of them (K9, K16, K98 and K137) are located within the N-terminus fragment used as bait in the protein interaction screen (Figure 4B). To determine if Snail's lysine residues could be required for collaboration with LOXL enzymes, we carried out site-directed mutagenesis of K9, K16, K98 and K137 residues that were replaced by arginine and the mutants were used in *E-cadherin* promoter assays. None of the individual mutations affected *E-cadherin* promoter repression mediated by Snail (Figure 4D, lanes 3–6, compare with lane 2) or the collaboration with LOXL2/3 (Supplementary data S1). Next, we analyzed the consequence of the double mutations K9R/K16R and K98R/K137R on Snail repressor activity. The K9R/K16R mutant exhibited a behavior similar to that of the wild-type Snail (Figure 4D, lane 7) and partly relieved the cooperation with LOXL2/3 (Supplementary data S1), probably because it altered the ability to recruit corepressor complexes. In contrast, the double mutant K98R/K137R, although with a conserved

Figure 3 Snail interacts with LOXL2 and LOXL3 isoforms. (**A**) HA-tagged Snail-wt (wild type) or Snail-K98R/K137R constructs were transiently coexpressed with LOXL2-, LOXL3- or LOXL4-flag isoforms in HEK293T cells. (Left panel) Snail immunoprecipitation with anti-HA and detection of LOXL isoforms by Western with anti-flag antibodies. Control IgG immunoprecipitation is also shown. Reversal immunoprecipitation (right panel) with anti-flag and detection of Snail-wt or Snail-K98R-K137R with anti-HA antibodies was performed. IgGs were used to confirm equal immunoprecipitation. The expression of Snail and LOXL isoforms was detected by Western blot in 5% of cell lysates (upper panel). (**B**) (Right) Co-immunoprecipitation analyses performed after transfection of LOXL2-flag and Snail-HA, or the indicated Snail deletion mutants, with anti-HA and detection of associated LOXL2 with anti-flag antibodies. (Left) Input fractions showing Snail-HA and mutants levels; α-tubulin was used as a loading control. Note the low levels of ΔZn-HA expression precluding its analyses in co-immunoprecipitation. (**C**) Confocal analyses of MDCK cells transiently transfected with Snail-HA (a, e) and either LOXL2- (b) or LOXL3-flag (f), showing the colocalization of Snail with LOXL2/3 in the perinuclear region (merge images, c, g; and d and h). Snail nuclear localization was confirmed by DAPI staining (i, j). Bar, 5 μm.







intact SNAG domain, was unable to repress the *E-cadherin* promoter activity (Figure 4D, lane 8) and failed to collaborate with LOXL2/3 (Figure 4D, lanes 9 and 10). Analysis of the effect on endogenous *E-cadherin* mRNA levels confirmed the collaboration of Snail and LOXL2/3 and the strict requirement of Snail's K98 and K137 residues for *E-cadherin* repression (Figure 4E). The unsuccessful collaboration of Snail K98R/K137R mutant with LOXL2/3 is not due to a lack of interaction, since the double mutant maintains interaction with either LOXL2 or LOXL3 (Figure 3A). However, the Snail K98R/K137R mutant has impaired ability to recruit corepressor complexes; decreased interaction with mSin3A and HDAC1/2 components has been detected (Supplementary data S2b, and data not shown). Altogether, these results indicate that K98 and K137 residues are essential for Snail to achieve its full repressor capability and suggest that these residues could be the substrates of LOXL2/3 enzymes.

Since both Snail and Slug members of the Snail superfamily have been described as repressors of *E-cadherin*, it is possible that Slug could also be modified by LOXL2/3. Interestingly, K9 and K16 residues are fully conserved in the Snail superfamily, but K98 is replaced by arginine in the Slug subfamily and K137, although conserved, is located in a very different sequence context being embedded in the first zinc-finger domain of Slug (Sefton *et al*, 1998) (Figure 4C), suggesting that Slug members would not collaborate with LOXL2/3 in silencing *E-cadherin* promoter. To confirm this assumption, we carried out *E-cadherin* promoter assays with mouse Slug in the absence and presence of LOXL2/3. Transfection of Slug led to a moderate level of *E-cadherin* promoter repression in MDCK cells even when used at higher doses (100 ng) than Snail (50 ng) (Figure 4D, compare lanes 11 and 2), in agreement with previous observations (Bolos *et al*, 2003). No collaboration of LOX2/3 with Slug could be detected over a range of Slug concentration (50–250 ng) (Figure 4C, lanes 11–13, and unpublished data), supporting that, in contrast to Snail, Slug would not require interaction/modification by LOXL2/3 to be active. These data indicate that Snail and Slug use different mechanisms to repress *E-cadherin* transcription, unveiling the existence of functional differences between the Snail and Slug subfamilies.

To further explore whether the Snail K98R/K137R mutation has any *in vivo* consequence, we evaluated the competence of the mutant Snail to achieve EMT. To this end, MDCK cells were stably transfected with HA-tagged variants of Snail and Snail-K98R/K137R. MDCK cells expressing Snail-HA suffered EMT with complete loss of E-cadherin (Figure 5A and B), while cells expressing the double mutant exhibited an unaltered epithelial phenotype (95% of the clones) similar to that of the mock-control cells (Figure 5A, compare panels e and f with i and j) and maintained the expression of E-cadherin (Figure 5B) organized in cell–cell junctions (Figure 5A, compare panels g and h with k and l). These results reinforce the requirement of intact K98 and K137 residues for Snail-mediated EMT.

The K98 and K137 residues are flanking the Snail NES domain (Dominguez *et al*, 2003) and the K98 residue (K99 in human Snail) is located inside the conserved destruction box (DSGKSS) recently reported to be required for GSK3β-dependent phosphorylation and proteasome degradation of Snail (Zhou *et al*, 2004). We, therefore, analyzed the stability of wild-type and variant K98R/K137R Snail proteins after transient transfection in HEK293T cells. The mutant K98R/K137R protein exhibits a slightly lower stability than wild-type Snail (Figure 6A and C), which is in agreement with recent reports (Yook *et al*, 2005). Strikingly, coexpression of LOXL2 led to an increased stability of wild-type Snail while it strongly decreased the stability of the mutant K98R/K137R Snail (Figure 6B and D), an effect that can be prevented by pretreatment with GSK3β and proteasome inhibitors (data not shown). We next evaluated the interaction of wild-type Snail and mutant K98R/K137R protein with GSK3β and their ubiquitination degree. The K98R/K137R mutant protein exhibited a higher degree of interaction with GSK33β and ubiquitination than the wild-type Snail (Figure 6E), in agreement with its highest instability. These data indicate that K98 and K137 residues are crucial for Snail stability and suggest that interaction/modification with LOXL2/3 might prevent its degradation and/or nuclear export, therefore increasing its functional transcription activity.

LOXL2 and LOXL3 induce EMT

To further analyze the role of LOXL2 and LOXL3 in E-cadherin downregulation *in vivo*, we examined the phenotype of MDCK cells stably expressing each of the human enzymes. As a control, we analyzed MDCK cells either transfected with the empty vector (CMV) (Cano *et al*, 2000) or expressing the human LOXL4 that exhibit an unaltered epithelial phenotype, maintaining growth in an epithelial monolayer (Figure 7Ac) and the expression of E-cadherin (Figure 7B and C) in organized cell–cell junctions (Figure 7Af). No expression of the mesenchymal marker fibronectin was observed in MDCK-hLOXL4 cells (Figure 7B) and vimentin exhibited a distribution (Figure 7Al) typical of control MDCK cells in culture (Cano *et al*, 2000). In striking contrast, stable expression of hLOXL2 or hLOXL3 in MDCK cells induced a conversion to a fibroblastic/spindle phenotype (Figure 7Aa and b) and vimentin exhibited an organization typical of mesenchymal cells (Figure 7Aj and k). Although both hLOXL2 and hLOXL3 showed a similar expression pattern (Figure 7Ag and h), the EMT effect seems to be stronger in hLOXL2-transfected cells than in hLOXL3-transfected cells. While MDCK-hLOXL2 cells do not express E-cadherin (Figure 7Ad, B and C) and show an induction of fibronectin (Figure 7B), MDCK-hLOXL3 cells still express E-cadherin mRNA and protein, although at reduced levels (Figure 7B and C) and with a disorganized distribution (Figure 7Ae), and do not express fibronectin (Figure 7B). No changes in the expression level of endogenous *Snail* transcripts were observed in the MDCK-LOX2/3 transfectants (data not shown). To discard a post-transcriptional regulation of E-cadherin by LOXL2/3, we investigated the effect of proteasome inhibition in the different cell lines (Figure 7D) finding no significant differences in the E-cadherin protein levels.

Although changes in cell phenotype induced by hLOXL2 and hLOXL3 in MDCK cells could be simply explained by expression levels, the observed differences could also be attributed to variations in the interaction degree with Snail, differential modification of K98/K137 residues or differences in the spectrum of targets modified by each enzyme that we cannot exclude at the moment. Consequently, because of the partial phenotypic changes caused by LOXL3, we decided to focus our next studies on LOXL2.

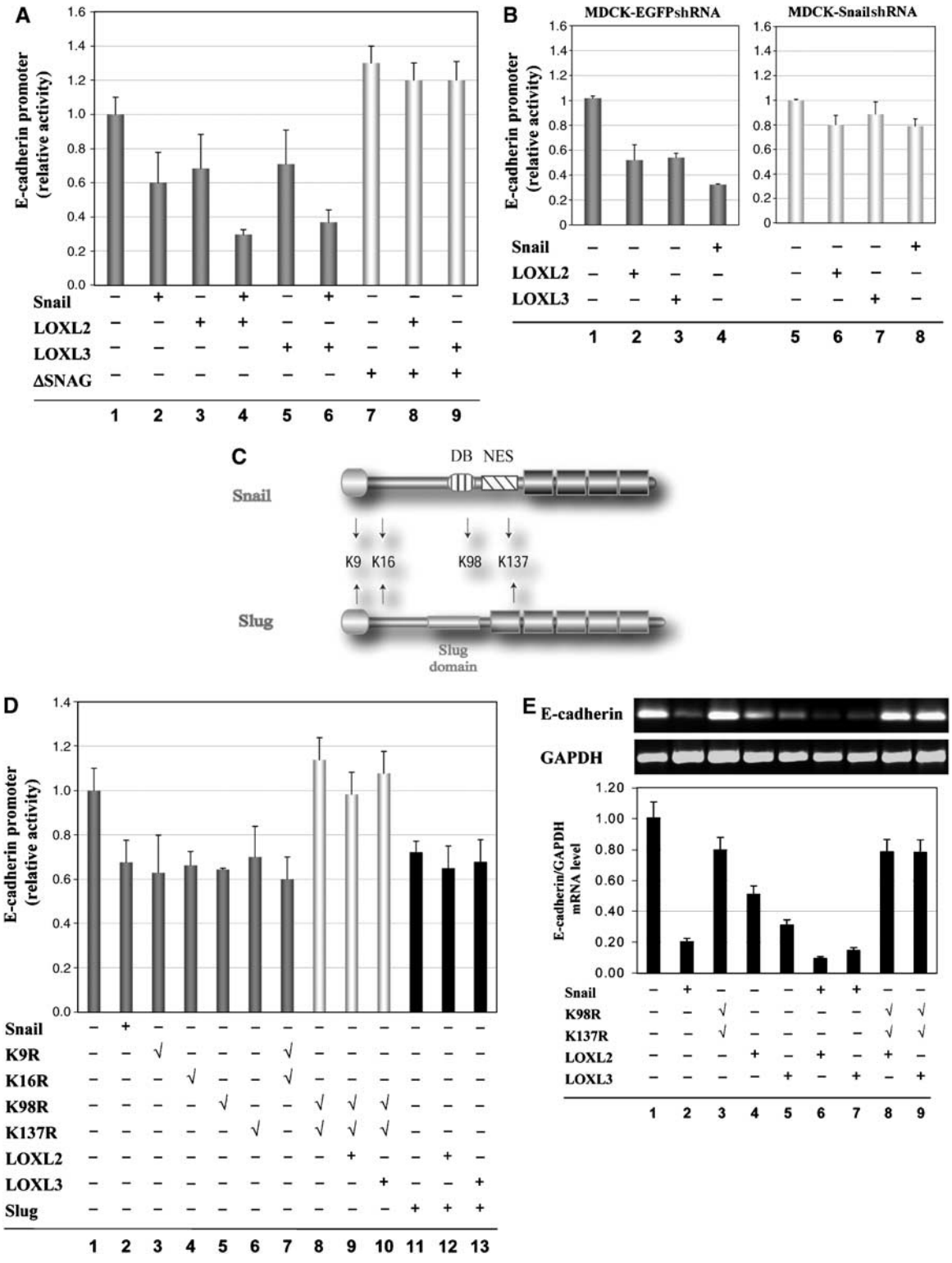


Figure 4 K98 and K137 residues of Snail are required for its collaboration with LOXL2/3 in *E-cadherin* promoter repression. (**A**) *E-cadherin* promoter was transiently transfected in MDCK cells and the activity was measured in the presence of wild-type Snail or the ΔSNAG mutant (50 ng) in the absence or presence of LOXL2 or LOXL3 cDNAs (300 ng). (**B**) *E-cadherin* promoter activity was measured as above in MDCK-EGFPshRNA or MDCK-SnailshRNA stable cell lines in the absence or presence of Snail, LOXL2 or LOXL3. (**C**) Schematic diagrams of Snail and Slug proteins showing the localization of lysine residues K9, K16, K98 and K137; note the absence of K98 and the location of K137 in the first zing-finger domain of Slug. (**D**) The activity of the *E-cadherin* promoter in MDCK cells was measured as above in the absence or presence of the indicated individual mutant forms of Snail (50 ng) (lanes 3–6) or double mutants in the absence or presence of LOXL2 and LOXL3 cDNAs (300 ng), as indicated. The effect of Slug (100 ng) in the absence or presence of LOXL2/3 isoforms (300 ng) was also tested (lanes 11–13). (**E**) RT–PCR analyses for detection of endogenous *E-cadherin* transcripts after transient transfection of the indicated plasmids as above. Levels of *GAPDH* transcript were used as a control of cDNA loading; densitometry of *E-cadherin*/*GAPDH* ratio of two independent experiments is shown.

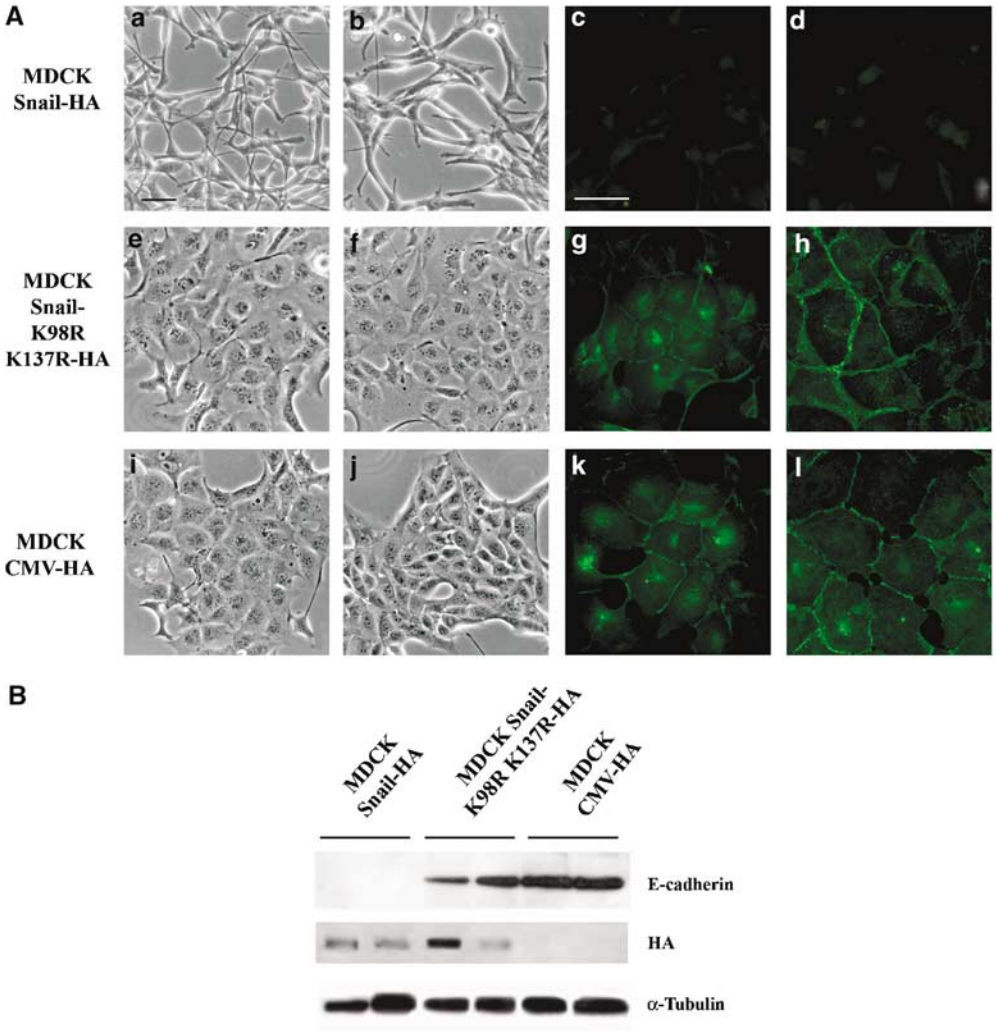


Figure 5 Lysine residues K98 and K137 of Snail are required for EMT induction. (**A**) MDCK transfectants obtained after stable expression of Snail-HA (upper panels), Snail-K98R/K137R-HA (middle panels) or pcDNA3-HA control vector (lower panels) were characterized by phase contrast of subconfluent cultures (left panels) and immunofluorescence of E-cadherin (right panels). Two independent clones, out of 20, from each transfection are shown. Bars, 40 μm. (**B**) Western blot analyses performed on whole cell extracts for the expression of E-cadherin and Snail-HA proteins in the indicated MDCK clones. Detection of α-tubulin was used as a loading control.

Knocking down LOXL2 by RNA interference strongly influences tumor growth and progression

To further confirm if LOXL2 plays a functional role in Snail-mediated silencing of *E-cadherin* expression and, therefore, in tumor progression, we used RNA interference to reduce LOXL2 expression. We used HaCa4 and CarB cell lines, derived from mouse squamous and spindle cell carcinomas, respectively, that are E-cadherin deficient, express high levels of *Snail* and are highly tumorigenic and metastatic (Navarro *et al*, 1991; Cano *et al*, 2000). CarB cells express both LOXL2 and LOXL3, while HaCa4 cells mainly express LOXL2 protein (Figure 2C). Stable transfection of CarB and HaCa4 cells with LOXL2shRNA resulted in about 80% reduction of *LOXL2* levels, while transcript levels of the homologous *LOXL3* remained unaltered (Figure 8A and B, and data not shown). Re-expression of E-cadherin at the mRNA and protein level was observed in HaCa4-LOXL2shRNA cells, but not in CarB-LOXL2shRNA cells (Figure 8A and B). As a control, we used stable transfectants with shRNA against EGFP whose sequence did not match any mouse gene (Caplen *et al*, 2001) and found no differences with parental CarB or HaCa4 cells (Figure 8A and B, and data not shown). The *in vitro* proliferation of CarB and HaCa4 cells stably expressing LOXL2shRNA showed no changes as compared with control EGFPshRNA or parental cells (unpublished data), suggesting that LOXL2 interference does not affect proliferation in culture. We then analyzed the tumorigenic potential of CarB and HaCa4 transfectants by injection into nude mice. CarB- and HaCa4-LOXL2shRNA cells formed primary tumors at all injection sites (eight tumors/eight injection sites), similar to the parental and/or control cells carrying EGFPshRNA. However, tumors induced by CarB-LOXL2shRNA cells grew at much lower rates than those induced by control CarB-EGFPshRNA or parental CarB cells (Figure 8C). Indeed, a 95% reduction in the volume of tumors induced by CarB-LOXL2shRNA cells was detected at 15 days postinjection. Remarkably, HaCa4-LOXL2shRNA cells also showed a significant decrease in the growth of tumors at 7- and 10-day postinjection, with 70 and 50% reduction of tumor volume, respectively, as compared with control cells (Figure 8D) and

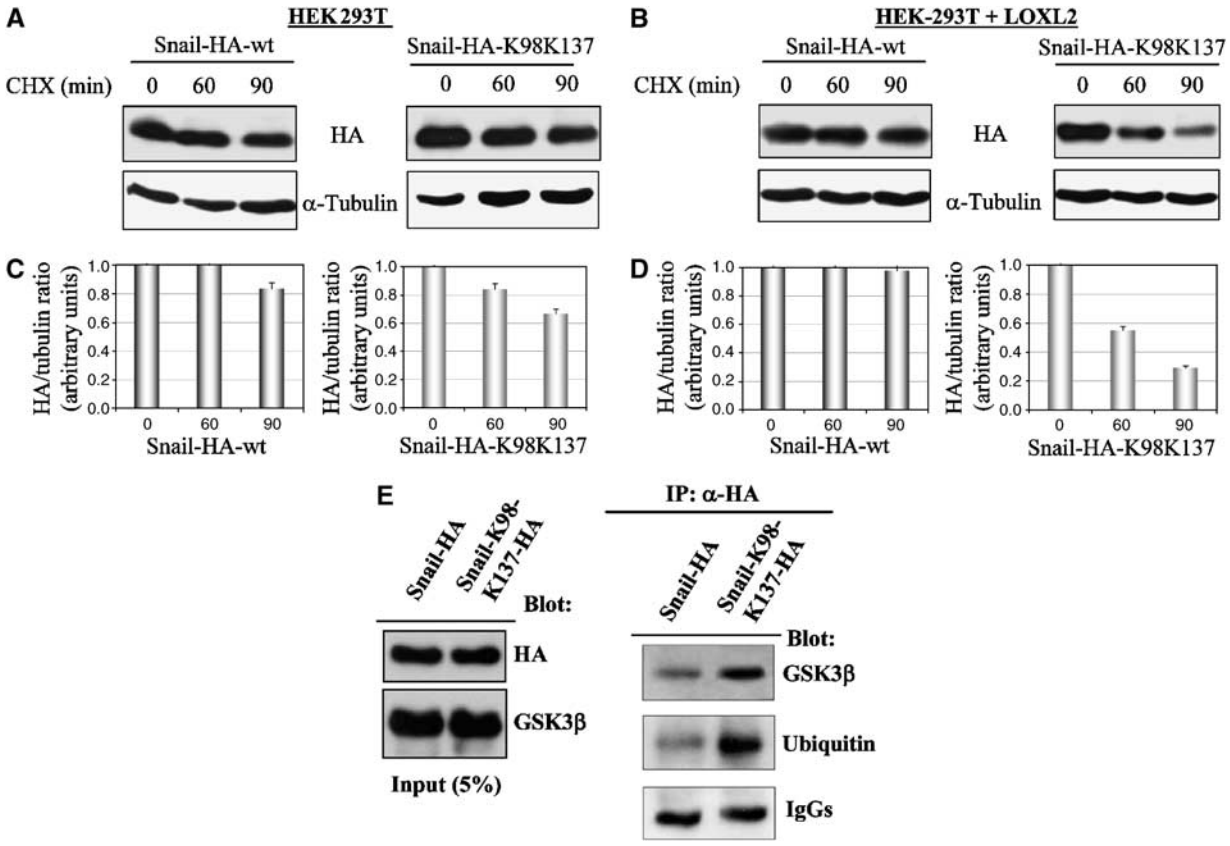


Figure 6 Lysine residues K98 and 137 are crucial for Snail protein stability. HEK293T cells transiently transfected with wild-type or variant K98R/K137R Snail in the absence (**A**) or presence (**B**) of LOXL2 were treated with 20 μM cycloheximide for the indicated time intervals and whole cell extracts analyzed by Western blotting. (**C, D**) Densitometric analysis of blots shown in panels A and B, respectively. Results show the mean ± s.d. of two independent experiments. (**E**) (Right) Immunoprecipitation analysis of Snail wild type or K98R/K137R-HA performed with anti-HA and detection of associated GSK3β or the ubiquitination level by Western blot. IgGs were used as a control to confirm equal immunoprecipitation. The expression level of Snail and GSK3β proteins was detected in 5% of cell lysates (left panel).

parental HaCa4 cell line, which are extremely tumorigenic generating very large tumors in just 9–11 days (Navarro *et al*, 1991). Tumors from HaCa4-LOXL2shRNA cells indeed maintain LOXL2 silenced (Figure 8F) indicating that loss of LOXL2 strongly diminishes the tumorigenic ability of HaCa4 cells.

To further analyze the consequences of *LOXL2* silencing, tumors induced after 15 and 35 days by CarB-derived cells were collected and the levels of *E-cadherin*, *Snail* and *LOXL2* were analyzed by RT–PCR. In tumors induced by CarB-LOXL2shRNA cells, the expression of *LOXL2* was reduced and, significantly, re-expression of *E-cadherin* was observed, while tumors induced by CarB-EGFPshRNA cells did not express *E-cadherin* and a high level of *LOXL2* transcripts was detected (Figure 8E). The levels of *Snail* expression showed no correlation with *E-cadherin* in the tumors analyzed (Figure 8E). Histological analysis of CarB-derived tumors showed that CarB-EGFPshRNA-induced tumors exhibit a fusiform pattern of growth typical of parental CarB xenografts (Cano *et al*, 2000) and the presence of blood vessels (Figure 9Aa and e). In contrast, tumors induced by CarB-LOXL2shRNA cells showed a less dense cellular pattern of growth with apparent absence of blood vessels and tumor cells had less spindle morphology (Figure 9Af and j). Interestingly, a significant increase in the number of apoptotic cells was clearly detectable in CarB-LOXL2shRNA xenografts (Figure 9B, right panel; 54 ± 7 TUNEL-positive cells/field) as compared with those induced by CarB-EGFPshRNA cells (Figure 9B, left panel; 18 ± 5 TUNEL-positive cells/field) and by parental CarB cells, which also show a higher mitotic index (data not shown). Immunofluorescence staining of tumors showed that the expression of fibronectin was lower in CarB-LOXL2shRNA xenografts (Figure 9A, compare panels b and g). Interestingly, the expression of vimentin (Figure 9Ac and h) and that of the invasive/angiogenic markers MMP-9 (Figure 9Ad and i) and CD-31 (Figure 9Ae and j) were also greatly decreased in the CarB-LOXL2shRNA xenografts. Significantly, E-cadherin re-expression at the protein level could be observed in 30–40% of the tumor cells of CarB-LOXL2shRNA xenografts grown for 35 days, and was detected in clusters of cells at apparent cell–cell contact regions (Figure 9C, right), in contrast to tumors induced by CarB-EGFPshRNA cells that do not express E-cadherin at any time point (Figure 9C, left). E-cadherin expressed in CarB-LOXL2shRNA-derived tumors at 35 days postinjection seems to form functional adhesion complexes, as supported by its colocalization with β-catenin at cell–cell contacts (data not shown), suggesting an initiation of tumor differentiation. Taken together, these results strongly support that LOXL2 is required for efficient growth and progression of tumors with invasive/angiogenic potential.

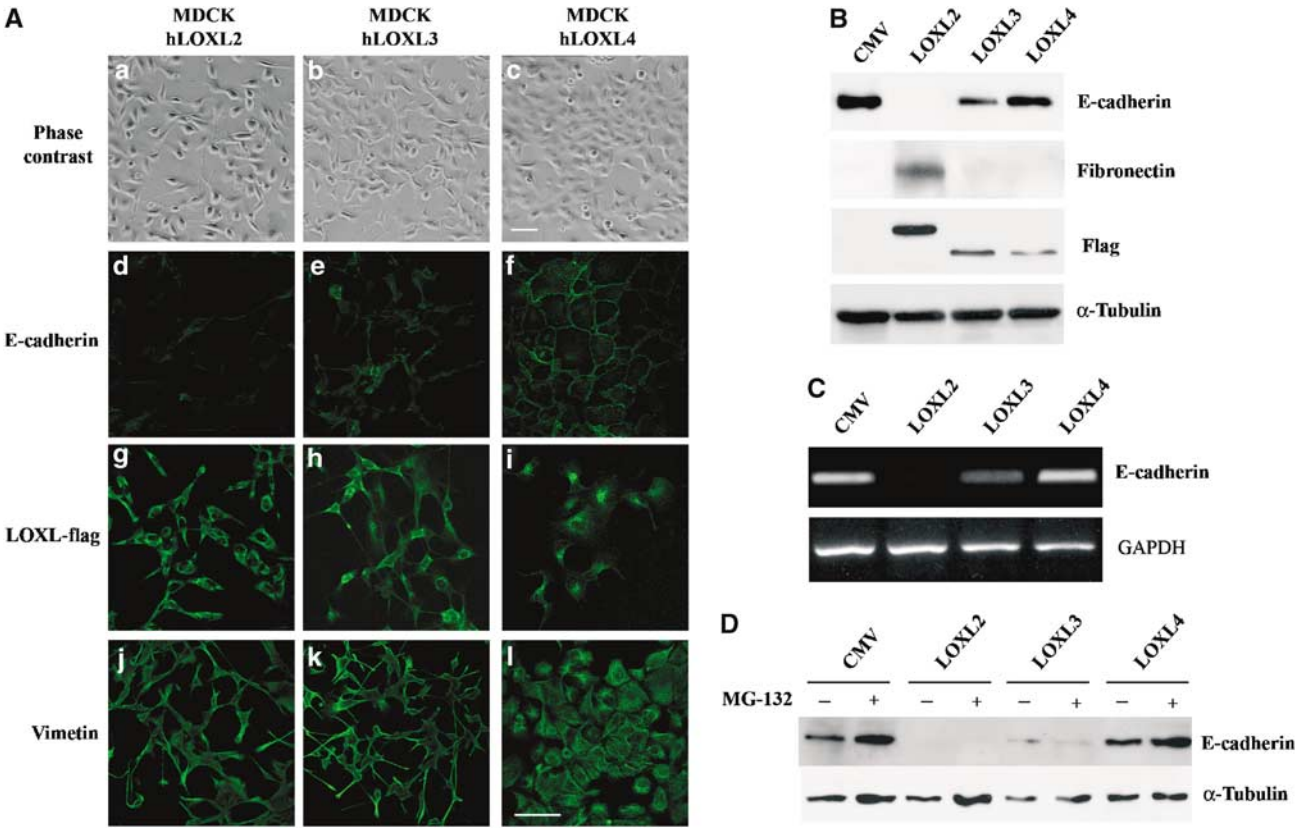


Figure 7 Stable expression of LOXL2 or LOXL3 in MDCK cells induces EMT. (**A**) Characterization of MDCK transfectants obtained after stable expression of hLOXL2 (left panels), hLOXL3 (middle panels) and LOXL4 (right panels) by phase contrast (a–c), and immunofluorescence for the expression of E-cadherin (d–f), ectopically expressed LOXL isoform (g–i) and vimentin (j–l) in the indicated cell clones. Bars, 40 μm. (**B**) Western blot analyses performed on whole cell extracts for the expression of E-cadherin, fibronectin and ectopically expressed LOXL isoform in the indicated MDCK-LOXL clones and in control mock-transfected MDCK cells (CMV). (**C**) RT–PCR analyses for detection of *E-cadherin* mRNA in the indicated MDCK-LOXL clones and control CMV cells. Levels of *GAPDH* transcript were used as a control of cDNA loading. (**D**) Western blot analyses performed on whole cell extracts obtained from the indicated cell clones treated for 5 h with or without 10 μM MG-132 to analyze the expression level of E-cadherin after proteasome inhibition. Detection of α-tubulin in panels B and D was used as a loading control.

Discussion

The local invasion process is thought to constitute the first event in the transition from premalignant to invasive malignant carcinomas. This step requires that tumor cells are able to disrupt the E-cadherin-mediated cell–cell adhesions and to acquire motility, and it is frequently associated with EMT (Thiery, 2002). In recent years, our understanding of the mechanism leading to the silencing of E-cadherin during tumor progression has been considerably expanded through the identification of several *E-cadherin* repressors, including zinc-finger factors (Snail, Slug, Zeb1 and Zeb2) and class I and II bHLH factors (E47 and Twist) (reviewed in Peinado *et al*, 2004c; Yang *et al*, 2004). It remains unsolved whether the different repressors may participate in silencing E-cadherin in different types of tumors or at defined stages of tumor progression (Rosivatz *et al*, 2002; Peinado *et al*, 2004c). In this report, we add a new member to the puzzling mechanism of *E-cadherin* gene repression during tumor progression; we describe the identification of LOXL2 and LOXL3, two members of the LOX gene family, as enzymes participating in the Snail-mediated silencing of *E-cadherin*. Our results show that both LOXL2/3 require the SNAG domain of Snail for interaction and collaboration with the transcription factor in *E-cadherin* repression. The involvement of the SNAG domain in LOXL2/3 interactions indicates additional regulatory functions of this domain apart from the recruitment of corepressor complexes (Peinado *et al*, 2004a). Furthermore, we have identified two Snail lysine residues (K98 and K137) as required for Snail-mediated repression of *E-cadherin* and functional collaboration with LOX2/3. Importantly, the effect of LOXL2/3 seems to be Snail-specific since it does not affect related members of the Snail superfamily such as Slug. This specificity unveils a functional difference in the mechanism of action of the two family members. Colocalization of LOXL2/3 with Snail in the perinuclear envelope and the observed collaboration in *E-cadherin* silencing suggest that the possible modification of Snail occurs before entry of the transcription factor into the nucleus and that it is required for Snail to achieve its full repressor activity. Previous studies have shown that Snail activity can be regulated by phosphorylation-dependent nuclear export (Dominguez *et al*, 2003) and, interestingly, the K98/K137 residues are flanking the reported NES domain of Snail. In addition, we have shown that K98 and K137 residues are involved in corepressor recruitment and, importantly, in the regulation of Snail stability by modulating its interaction with GSK3β, recently reported to phosphorylate Snail (Zhou *et al*, 2004; Yook *et al*, 2005). Furthermore, we have demonstrated that the K98/K138 residues are required for Snail repressor activity,

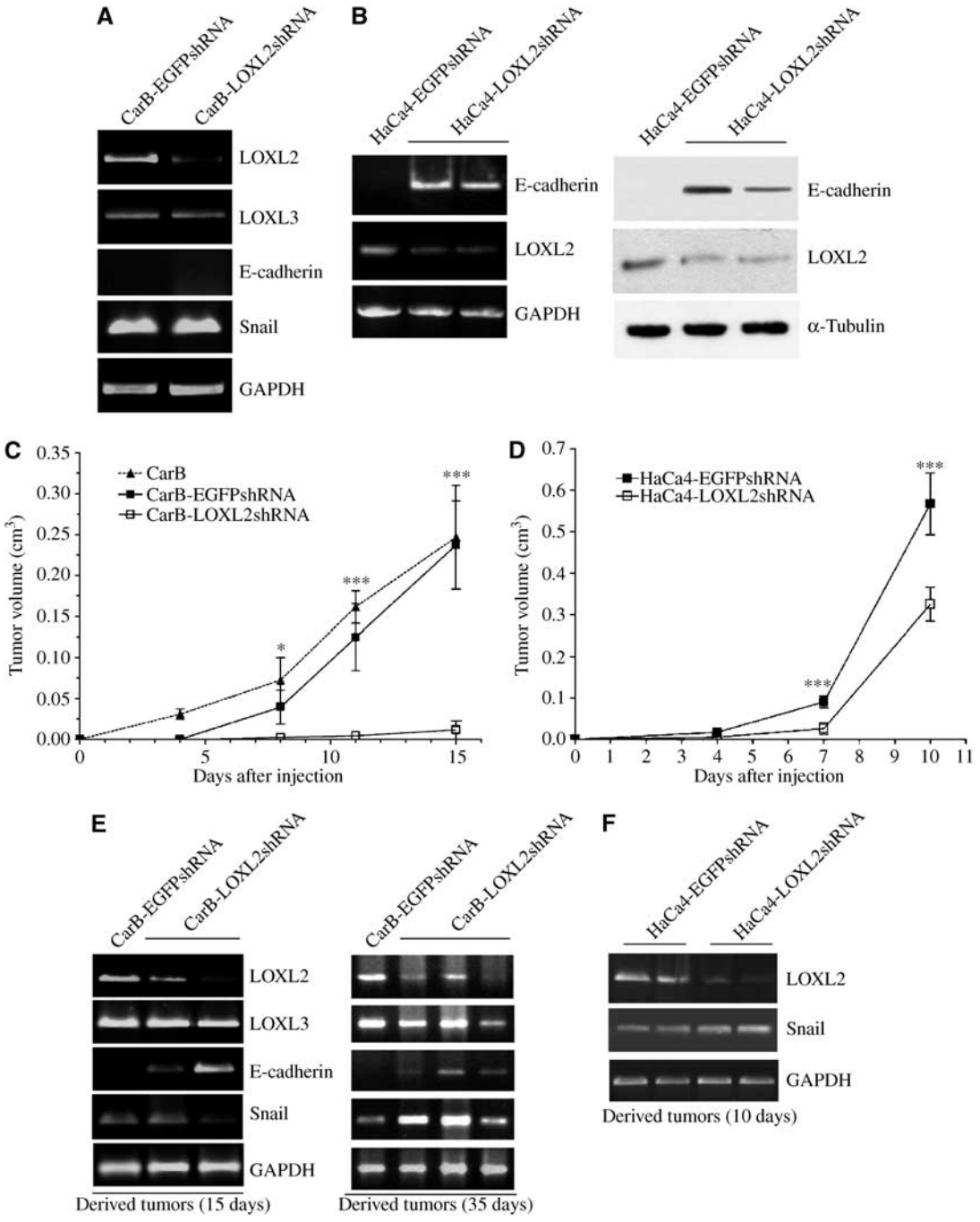


Figure 8 Silencing of LOXL2 strongly decreases the tumorigenic potential of CarB and HaCa4 cells. Highly tumorigenic and metastatic CarB or HaCa4 cells were stably transfected with pSuperior vector containing specific oligonucleotide sequences for mouse LOXL2 (LOXL2shRNA) or EGFP (EGFPshRNA) interference. (**A, B**) Levels of the indicated endogenous transcripts were determined by RT–PCR in CarB (A) or HaCa4 transfectant clones (B, left); Western blot analysis was performed in HaCa4 cells to detect E-cadherin and LOXL2 (B, right). Detection of *GAPDH* and α-tubulin was used as loading controls in RT–PCR and blot, respectively. (**C, D**) Tumorigenic potential of CarB- (C) and HaCa4-(D) derived clones after subcutaneous injection into nude mice. ANOVA analysis: $*P<0.05$; $**P<0.01$; $***P<0.001$. (**E, F**) RT–PCR analysis of the indicated genes, performed on RNA samples isolated from individual tumors generated by CarB (E) or HaCa4 (F) transfectants after the indicated days of injection. Note the reduced levels of *LOXL2* and increased expression of *E-cadherin* transcripts in tumors derived from LOXL2shRNA cells.

cooperation with LOXL2/3 and, importantly, for Snail-induced EMT. The increased degradation of mutant Snail K98R/K138R observed in the presence of LOXL2, in contrast to the wild-type form, also suggests that induction of specific modification/conformational changes involving those specific Lys residues are required for the Snail/LOXL2 collaboration. Therefore, our results add a new level in the regulation of Snail function and point to a potential collaboration between phosphorylation- and oxidation-dependent modifications of Snail. To our knowledge, this is the first report suggesting that LOX enzymes, besides extracellular matrix structural modification, could also play a role in regulating the functional activity of Snail transcription factor. This mechanism would also be in agreement with a recent report

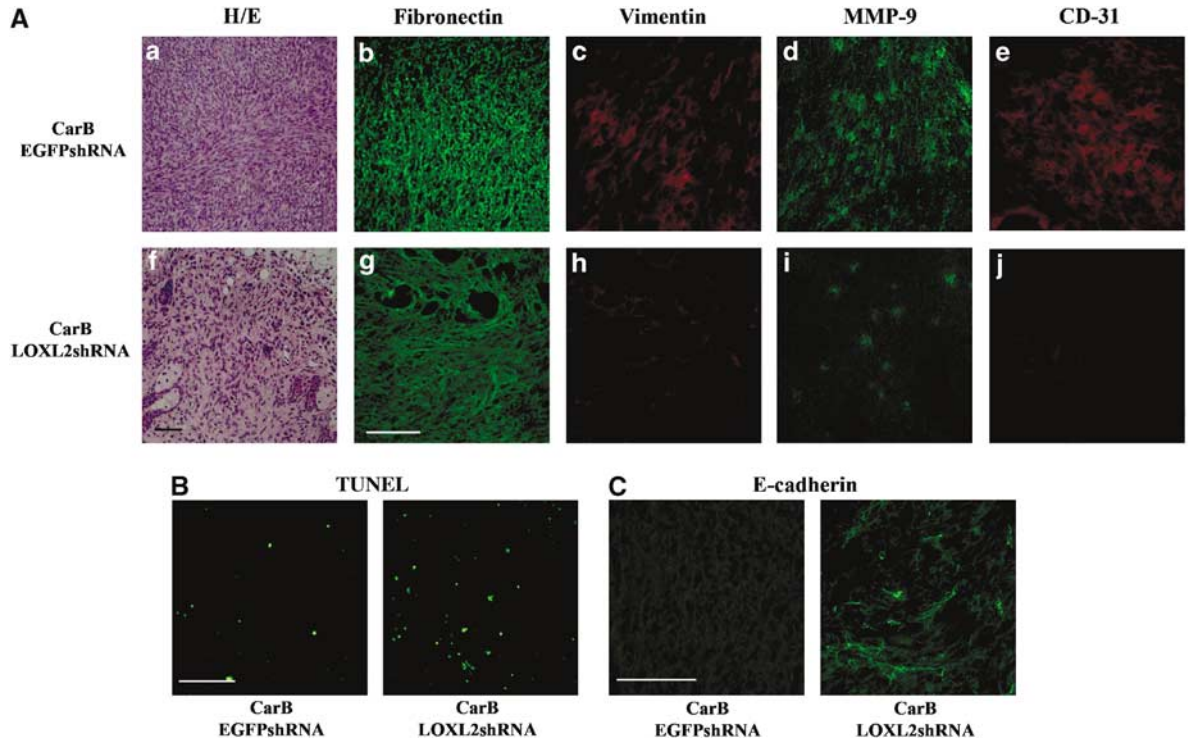


Figure 9 CarB-LOXL2shRNA xenografts show a less dedifferentiated and invasive/angiogenic phenotype. (**A**) Histological (a, f) and immunofluorescence analysis (b–e, g–j) of tumors induced by CarB-EGFPshRNA (a–e) and CarB-LOXL2shRNA (f–j) cells. Immunofluorescence of tumor sections shows detection of fibronectin (b, g), vimentin (c, h), MMP-9 (d, i) and CD-31 (e, j). Bar, 50 μm. (**B**) TUNEL assay on tumor sections obtained from CarB-EGFPshRNA (left) and CarB-LOXL2shRNA (right) xenografts. (**C**) Immunofluorescence analysis of E-cadherin in sections from 35 days CarB-EGFPshRNA (left) and CarB-LOXL2shRNA (right) xenografts. Bar, 50 μm. See the loss of the spindle phenotype and the presence of apoptotic cells in CarB-LOXL2shRNA-induced tumors, and decreased expression of vimentin and of invasive and angiogenic markers. Note also the expression of E-cadherin in late-stage tumors induced by CarB-LOXL2shRNA.

showing that nuclear export and, thus, functional activity of the yeast Yap1 transcription factor are regulated by its oxidation state (Wood *et al*, 2004).

Regarding the influence of LOX/LOXL enzymes in tumor progression, there is some controversy in the literature. LOX levels are reduced in several cancer cell lines as well as in Ras-transformed cell lines (Contente *et al*, 1999; Giampuzzi *et al*, 2001; Csiszar *et al*, 2002) and restitution of a nontransformed phenotype is associated with the re-expression of LOX (Krzyzosiak *et al*, 1992; Palamakumbura *et al*, 2004). However, other studies show overexpression of LOX in some types of carcinoma (Trivedy *et al*, 1999; Holtmeier *et al*, 2003). Moreover, LOX and LOXL2 are highly expressed in metastatic breast cancer-derived cell lines with a correlation between upregulation of LOX and LOXL2 and invasive/metastatic potential of breast tumors (Kirschmann *et al*, 2002; Akiri *et al*, 2003). Here, we also show a positive correlation between LOX2/3 and Snail expression and invasiveness, demonstrating that overexpression of LOXL2 in the prototypic MDCK epithelial cell line induces a complete EMT process. These data provide substantial new evidence that LOXL2 is involved in tumor progression, although a similar role for LOXL3 is also plausible. The importance of LOXL2 in tumor progression is strongly supported by knockdown expression studies. Silencing of LOXL2 in HaCa4 and CarB cells induces a strong reduction of tumor growth. However, in contrast to HaCa4, CarB LOXL2shRNA cells did not re-express E-cadherin in culture, suggesting that functional expression of other genes is necessary for this process. Indeed, CarB cells express at least two other repressors of *E-cadherin* and inducers of EMT, Slug and E47 (Perez-Moreno *et al*, 2001; Bolos *et al*, 2003), which may maintain E-cadherin silenced and the fibroblastoid phenotype of the cells in culture. Interestingly, the dramatic growth reduction of CarB-LOXL2shRNA xenografts was accompanied by increased apoptosis, upregulation of E-cadherin mRNA and protein and downregulation of invasive/angiogenic markers, suggesting that LOXL2 affects both the *in vivo* growth and the invasive and angiogenic properties of tumors. Interestingly, Snail has been recently involved in both cell survival and induction of angiogenesis (Peinado *et al*, 2004b; Vega *et al*, 2004). Therefore, the effects on tumor apoptosis and angiogenesis observed in the absence of LOXL2 might be related to the loss of Snail function, and support that in an *in vivo* context, blockade of Snail function can overcome the action of other *E-cadherin* repressors. Nevertheless, the action of LOXL2 on parallel, Snail-independent pathways cannot be presently discarded. Although further studies are necessary to fully elucidate the implication of LOXL2 in tumor progression and its potential collaborating genes, our present results indicate that LOXL2 activity might be a crucial modulator of Snail, providing an additional control mechanism of EMT and tumor progression; these results also raise the possibility of using LOXL2 expression as an additional predictive/prognostic marker for carcinoma progression.

Materials and methods

Yeast two-hybrid screen

The Matchmaker system 3 (Clontech) was used for the yeast two-hybrid screen. The bait protein consisted of the N-terminus of mouse Snail (residues 1–150). A fibroblast NIH3T3 library in pACT2 vector (Clontech) was used. Positive colonies were isolated based on their capacity to express the markers *ADE2*, *HIS3* and *LacZ*.

Generation of plasmids, expression vectors and stable cell lines

hLOXL2/3/4 and mLOXL2/3/4 constructions were generated using human and mouse cDNA, respectively, as template and specific primers for each isoform (Supplementary data S3). Point mutations within the sequence of Snail were performed by one-step PCR, using the Quick-change II site-directed mutagenesis kit (Stratagene). Fragments subjected to site-directed mutagenesis were sequenced completely to discard undesirable mutations. Snail deletion mutants were generated by RT–PCR using specific primers (Supplementary data S3). The different cDNAs were cloned in pcDNA3, pcDNA3-HA or pcDNA3-flag vectors.

Cell culture and stable transfections

MDCK-II, HEK293T and mouse spindle CarB cells were grown in DMEM medium and mouse MCA3D and HaCa4 cells were grown in Ham's F12 medium. The culture conditions, origin, tumorigenic properties, E-cadherin and Snail expression levels of all the mouse and human cell lines used have been previously described (Cano *et al*, 2000). MDCK stable transfectants were obtained by transfection of 3 μg of either pcDNA3-hLOXL2/3/4-flag or control pcDNA3 vector using Lipofectamine reagent (Invitrogen) and selection with G418 (400 μg/ml) for 3–4 weeks. Six independent clones from each transfection were analyzed and results representative of one single clone are shown in the figures. MDCK Snail wild-type and mutant stable transfectants were obtained as above using pcDNA3-Snail-HA, pcDNA3-SnailK98K137-HA or pcDNA3-HA vectors. A total of 20 clones from each transfection were characterized, and two representative clones are shown in the figures. MDCK stable transfectants expressing SnailshRNA or EGFPshRNA were obtained using pSuperior-shRNA against Snail (Jordá *et al,* 2005) or EGFP (Caplen *et al*, 2001) and puromycin selection (1 μg/ml) for 3 weeks. CarB- or HaCa4-LOXL2shRNA cells were generated by stable transfection using the pSuperior vector containing a specific sequence to interfere with mLOXL2 (CAACTGTCACGTAGGTGGA); as a control, CarB or HaCa4 cells were transfected with EGFPshRNA. Stable transfectants were obtained after selection with 1 μg/ml of puromycin for 2–3 weeks. Four independent clones from each transfection were characterized; one representative clone from each cell line is shown in the figures.

E-cadherin promoter analysis

The mouse *E-cadherin* promoter (−178 to +92) fused to *Luciferase* was used to determine the activity of the *E-cadherin* promoter as described previously (Peinado *et al*, 2004a). Cotransfections were carried out in the presence of the indicated amounts of Snail, Slug, or the indicated Snail mutants and/or LOXL2/3 cDNAs, all of them cloned in the pcDNA3 vector. The amount of total DNA was normalized with empty pcDNA3 vector. Luciferase and *Renilla* activities were measured using the dual-luciferase reporter assay kit (Promega) and normalized to the wild-type promoter activity detected in mock- (pcDNA3) transfected cells.

Transient transfections, immunoprecipitations and Western blots

HEK293T (6×10^5) cells grown in P60 dishes were transiently transfected using Lipofectamine reagent (Invitrogen) with 2 μg of indicated plasmids. Cell extracts were obtained 36 h post-transfection and co-immunoprecipitation experiments were performed as described (Peinado *et al*, 2004a). Blots were incubated with rat anti-HA (Roche) (1:400), mouse anti-flag (Sigma Chemical Co.) (1:3000) or anti-GSK3β (Transduction Lab.) (1:1000), rabbit anti-ubiquitin (Sigma) (1:100) or anti-mSin3A (Santa Cruz Biotechnology) (1:200). The secondary antibodies used were HRP-coupled sheep anti-mouse (1:1000), goat anti-rabbit (1:4000) (Amersham) or anti-rat (1:10 000) (Pierce). For characterization studies, whole cell extracts were fractionated in SDS–PAGE gels and subjected to Western blot analyses with anti-E-cadherin, anti-fibronectin, anti-flag and anti-α-tubulin as described (Cano *et al*, 2000); rabbit anti-LOXL2 and anti-LOXL3 sera have been recently generated (Fong *et al*, unpublished). Proteasome inhibition was performed with MG-132 treatment (Calbiochem).

Protein stability

Snail-HA or variant K98R/K137R Snail-HA constructs were transiently transfected in HEK293T cells and after 24 h, cells were treated with 20 μM cycloheximide (Sigma) for the indicated time intervals; whole cell extracts were analyzed by Western blotting with anti-HA as above.

Immunofluorescence

MDCK cells grown on coverslips and transiently cotransfected as above were fixed in methanol (−20°C) for 1 min and stained with anti-HA (1:100) and anti-flag (1:300). The secondary antibodies used were anti-rat Alexa594 and anti-mouse Alexa488 (1:800) (Molecular Probes). Preparations were viewed in a Leica confocal TCSSP2 microscope. To analyze MDCK transfectants, cells were fixed as above and stained for E-cadherin, flag and vimentin and visualized in a Zeiss Axiophot microscope.

RT–PCR analysis

Total RNA from cell lines or tumors was extracted with Trizol reagent (Invitrogen) and then subjected to DNAseI treatment. A 2 μg portion of RNA was used in RT–PCR experiments using specific primers for transcript detection of E-cadherin, Snail and GAPDH using TaqExpand High Fidelity (Roche) as described (Perez-Moreno *et al*, 2001; Bolos *et al*, 2003); primers used for amplification of mouse and human LOX/LOXL isoforms are detailed in Supplementary data S3.

Induction of tumors

CarB/HaCa4 and derived cell lines were subcutaneously injected into the flanks of 8-week-old female Balb/C nude mice (Charles River) as described before (Cano *et al*, 2000). Tumors were excised in three regions and immediately frozen in liquid nitrogen and optimal cutting temperature (OCT)-embedded for immunofluorescence, directly frozen in liquid nitrogen for RT–PCRs or fixed in 10% formaldehyde for histology. Tumors used for RT–PCR were carefully dissected to eliminate all surrounding skin before freezing. All animal experiments were approved and performed according to Institutional guidelines for animal care. A total of eight tumors from each cell line were generated and, at least, four different tumors derived from each cell line were analyzed by histology, immunostaining and RT–PCR.

Immunofluorescence analysis of tumors

Sections (10 μm) of the OCT-embedded xenografted tumors were fixed and stained as described previously (Peinado *et al*, 2004b) for E-cadherin, vimentin, CD-31, fibronectin (1:200) and anti-MMP-9 (1:200, Chemicon) and appropriate secondary antibodies.

TUNEL assays

For detection of DNA fragmentation, paraformaldehyde-fixed cryostat sections were analyzed by the TUNEL method using the *in situ* Cell Death Detection kit (Roche) according to the manufacturer's protocol.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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