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SHORT COMMUNICATION

# Snail is a repressor of *RKIP* transcription in metastatic prostate cancer cells

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**Diminished expression of the metastasis suppressor protein RKIP was previously reported in a number of cancers. The underlying mechanism remains unknown. Here, we show that the expression of RKIP negatively correlates with that of Snail zinc-transcriptional repressor, a key modulator of normal and neoplastic epithelial–mesenchymal transition (EMT) program. With a combination of loss-of-function and gain-of-function approaches, we showed that Snail repressed the expression of RKIP in metastatic prostate cancer cell lines. The effect of Snail on RKIP was on the level of transcriptional initiation and mediated by a proximal E-box on the RKIP promoter. Our results therefore suggest that RKIP is a novel component of the Snail transcriptional regulatory network important for the progression and metastasis of cancer.**
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RKIP (Raf kinase inhibitor protein) is a member of an evolutionarily conserved group of proteins called PEBP (Phosphatidylethanolamine-binding protein). Recently, we and others have identified and characterized RKIP as a metastasis suppressor protein. On the molecular level, RKIP functions by inhibiting the proliferative and survival Raf-MEK-ERK and NF-κB signaling pathways (Chatterjee *et al*., 2004; Park *et al*., 2005). Consistent with its demonstrated inhibitory effect on Raf and NF-κB signaling, we and others have shown that the expression levels of RKIP are downregulated in a number of tumors, including highly metastatic prostate, breast and colon cancer, hepatocellular carcinoma, melanomas and insulinomas (Fu *et al*., 2003; Chatterjee *et al*., 2004; Schuierer *et al*., 2004, 2006; Zhang *et al*., 2004; Hegan *et al*., 2005; Al-Mulla *et al*., 2006; Lee *et al*., 2006). The importance of RKIP in metastases was highlighted by the finding that restoration of RKIP expression inhibits prostate cancer metastasis in a murine model (Fu *et al*., 2003, 2005). More recent studies have shown that RKIP is also a good prognostic marker of the pathogenesis of human prostate cancer (Fu *et al*., 2005) and a prognostic indicator for overall survival and disease-free survival in colorectal cancer (Al-Mulla *et al*., 2006). Collectively, these studies suggest that RKIP is a novel cancer metastasis suppressor and an effector of signal transduction pathways leading to apoptosis. In spite of the abundance of experimental evidence on the deleterious consequences of reduced RKIP expression in tumors, the mechanisms responsible for the downregulation of RKIP in cancer are not completely understood.

To set up a system to study the transcription regulation of RKIP, we examined RKIP expression levels in cancer cell lines with different metastatic capacity. In accordance with clinical tumor studies, we observed that expression levels of RKIP proteins progressively decreased in breast and prostate cancer cell lines of increasing metastatic potential. The expression of RKIP is low in invasive and metastatic breast (MB231, MB435 and 578T) and prostate (DU145 and PC3) cell lines and high in noninvasive cell lines like MCF7, BT20 and LNCaP (Figure 1a). Notably, RKIP protein levels correlated well with those of the intercellular adhesion protein E-cadherin (E-cad). E-cad is a well-documented tumor metastasis suppressor protein that is regulated by the Snail and closely related Slug transcription factors (Peinado *et al*., 2007). Quantitation of *RKIP* transcript levels in the different cancer cell lines by qRT-PCR demonstrated that they correlated with the levels of the protein, (Figure 1b), suggesting that *RKIP* expression is downregulated at the RNA level, via changes in mRNA stability or transcription initiation.

DNA methylation is an important epigenetic mechanism for gene silencing, commonly used by cancer cells to inactivate tumor suppressor genes (Baylin and Herman, 2000; Herman and Baylin, 2000). Methylation usually occurs at small stretches of DNA containing the CpG dinucleotide or the CpG islands located in the proximal promoter region. Methylated CpG islands are docking

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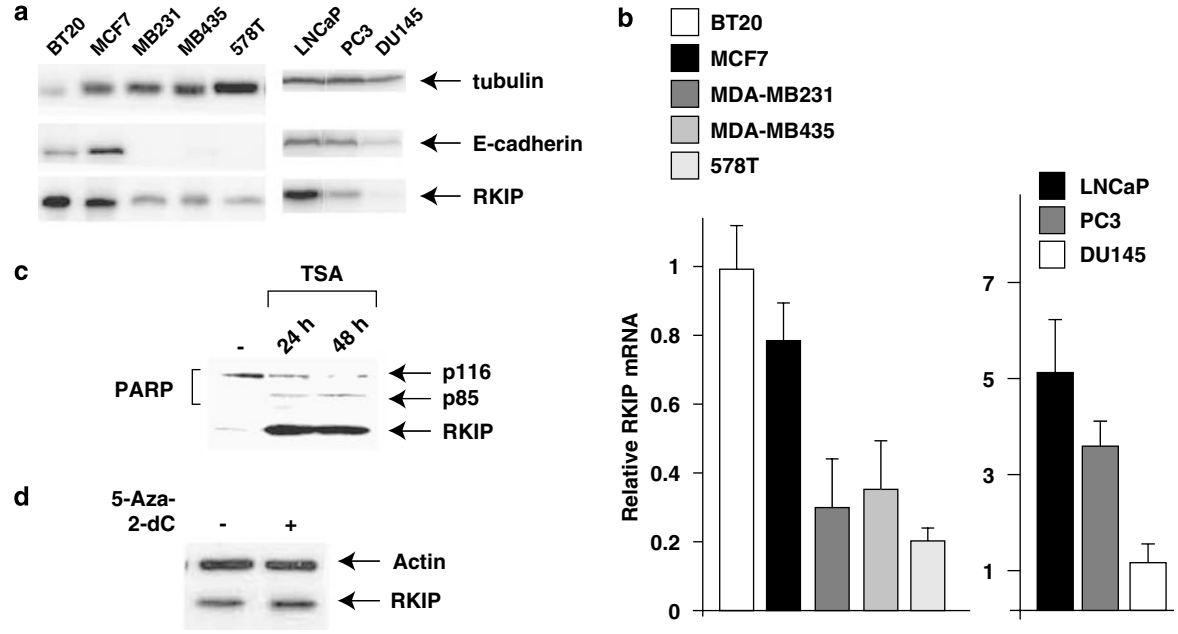


**Figure 1** The expression of RKIP is repressed in highly metastatic cancer cells. (**a**) Immunoblot analysis of extracts from prostate or breast cancer cells with specific antibodies (Abs). (**b**) The endogenous levels of *RKIP* mRNA in prostate cancer cells as measured by real-time quantitative PCR (qRT-PCR) and normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (left) or *mATP6*. Each bar represents the mean ± s.e.m. of the PCR reactions in triplicate. (**c**, **d**) Immunoblot analysis of extracts from prostate cancer cells DU145 treated with (**c**) trichostatin A (TSA) or (**d**) 5-Aza-2-dC for 24 or 48 h as indicated. Data shown are representative of at least three independent experiments.

sites for the recruitment of histone deacetylases, resulting in stable transcriptional repression. The repressed state of a methylated promoter can be reversed by the methylation inhibitor 5-Aza2dC or by the histone deacetylase inhibitor, trichostatin A (TSA). To determine whether RKIP is repressed by methylation in metastatic prostate cell lines, we examined the effect of TSA on RKIP expression. Immunoblotting against RKIP showed that TSA treatment caused a robust increase in RKIP expression in DU145 cells. Consistent with its role as an important apoptosis regulator, the increase in RKIP expression in TSA-treated cells was also accompanied by extensive apoptosis, as measured by cleavage of PARP, a common apoptosis marker (Figure 1c). To directly show that RKIP expression is repressed by methylation, DU145 cells were treated with 3 μM 5-Aza-2dC for 72 h. Expression of RKIP was monitored by immunoblot with RKIP-specific antibody. However, unlike TSA, 5-Aza-2dC had no effect on RKIP expression (Figure 1d). As reported (Soengas *et al*., 2001), treatment of melanoma cells with the same 5-Aza-2dC concentration caused a robust induction of the pro-apoptotic Apaf1 (not shown). These results using the demethylation agent 5-Aza-2dC clearly show that promoter hyper-methylation is not the cause of RKIP downregulation in DU145. Our finding that the expression of RKIP can be induced by TSA implies that RKIP expression may be actively repressed in cancer cells.

Gene-expression studies have identified the transcription repression of E-cad to be the key event in cancer metastasis. Among the transcription factors implicated in this process, Snail has been shown to be the strong repressor of *E-cad* transcription (Nieto, 2002; Thiery, 2002). In light of the downregulation of RKIP expression in metastatic cancer cell lines, as well as the coexpression of RKIP and E-cad in several cancer cell lines (Figure 1a), we hypothesized that Snail may act in the same pathway as RKIP. Consistent with this hypothesis, we observed a negative correlation of Snail protein expression with that of RKIP in prostate cancer cell lines (Figure 2a). To directly examine the causal role of Snail in RKIP expression, we introduced a Snail expression construct by retroviral infection into the non-metastatic cancer cell line LNCaP, which has relatively high RKIP levels. To circumvent the possible effect of the highly unstable Snail on RKIP expression, a mutated stable variant (Snail-6SA), which has all its six phosphorylable Ser in the consensus GSK-3β sites mutated to Ala (Zhou *et al*., 2004), was used along with the wild-type Snail. As expected, considerable amounts of Snail-6SA were detected in stably infected LNCaP cells (Figure 2b), although wild-type Snail was almost undetectable.

In accordance with previously published results, ectopic expression of Snail-S6A downregulated expression of E-cad in LNCaP cells. In addition, we observed a significant decrease in RKIP expression both at the protein and RNA level in the same cells, while infection with empty virus control had no effect (Figures 2b and c). The suppression of RKIP expression correlated with Snail expression levels, as the Snail variant S6A had a stronger effect relative to wild-type Snail (Figures 2b

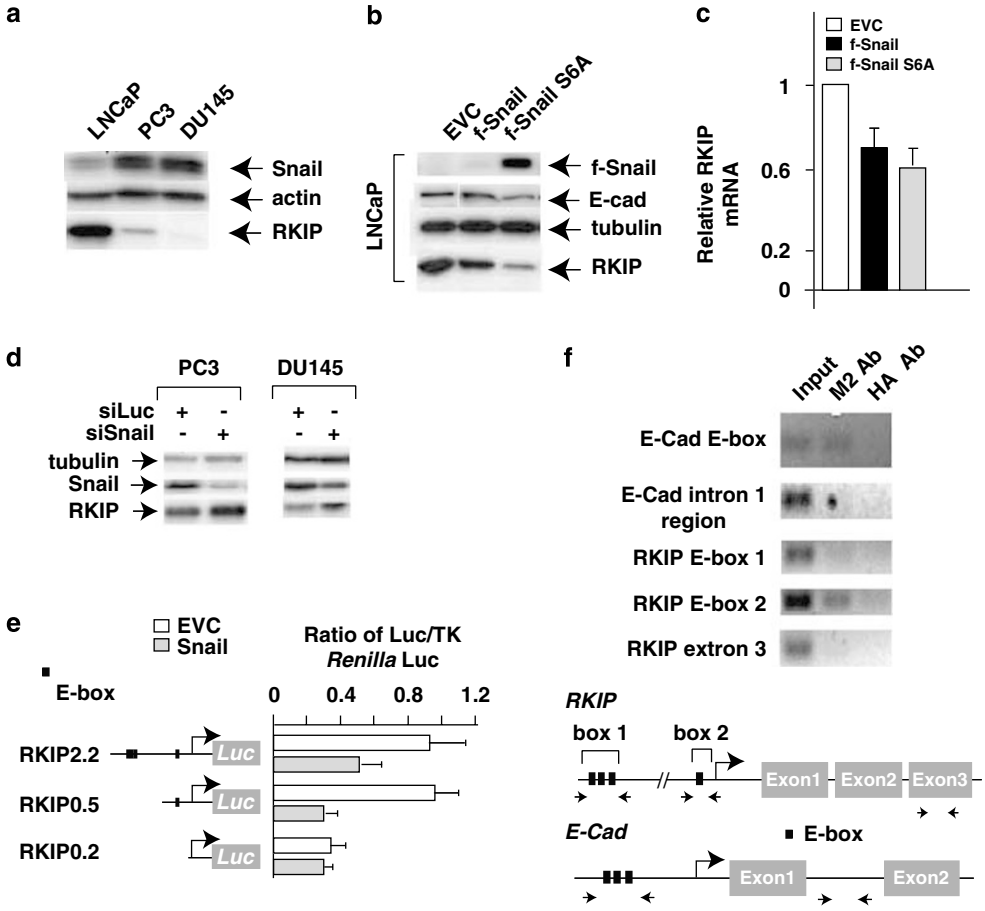


**Figure 2** Snail is a direct repressor of RKIP expression. (**a**) Comparison of RKIP and Snail expression in prostate cancer cell lines by immunoblot analysis. Triton X-100 extracted cell lysates (30 μg) were immunoblotted using polyclonal anti-RKIP or Snail antibodies. The same membrane was reblotted with an anti-actin serum as a loading control. (**b**, **c**) Ectopic expression of Snail represses RKIP expression. (**b**) Immunoblot analysis of extracts from cancer cells stably infected with the indicated retroviruses or EVC (empty vector control) with specific antibodies (Abs). (**c**) Comparison of *RKIP* mRNA levels in LNCaP cells stably infected with the indicated retroviruses. The endogenous levels of RKIP mRNA in infected cells were measured by qRT-PCR and normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each bar represents the mean ± s.e.m. of the PCR reactions in triplicate. (**d**) Downregulation of Snail in prostate cancer cells enhances *RKIP* expression. Cells were stably infected with the indicated retroviruses expressing short hairpin RNA (shRNA) for *luciferase* or *Snail*. The endogenous levels of RKIP, tubulin and Snail proteins in infected cells were measured by immunoblot analysis. (**e**) Snail represses *RKIP* promoter reporter in cancer cells. MCF7 cells were transfected with the indicated effector and reporter plasmids. Forty-eight hours after transfection, cells were harvested for luciferase assay. Schematic representation of the *RKIP*–LUC reporter containing the RKIP promoter region was shown on the left. Positions of the putative E-box are indicated. *TK* stands for thymidine kinase. (**f**) Snail associates with *RKIP* promoter. Chromatin immunoprecipitation (ChIP) assays were performed with anti-Flag or anti-HA Ab on LNCaP cells that stably express Flag-tagged Snail protein. DNAs that were immunoprecipitated down with the Abs were amplified with primer pair by PCR as indicated by arrows in the lower panel.

and c). Conversely, expression levels of RKIP were increased in metastatic PC3 and DU145 prostate cancer cells, when expression of Snail was knocked down by specific siRNA (Figure 2d), implying that Snail is a physiologically relevant repressor of RKIP.

Inspection of the *RKIP* promoter revealed the presence of at least four potential Snail-binding consensus sites (E-box: CANNTG)) clustered in two locations in the proximal *RKIP* promoter. To determine whether Snail regulates *RKIP* expression in an E-box-dependent manner, we examined the effect of overexpressing Snail on the activity of *RKIP* promoter-driven luciferase reporters in MCF7 breast cancer cells, which have a low level of native Snail expression and are comparatively easy to transfect. Three different *RKIP* promoter luciferase reporters containing all four, one or no E-box-binding sites were used. Consistent with the observation that ectopic expression of Snail downregulated RKIP, forced expression of Snail repressed *RKIP* luciferase reporters that contained one or all four E-box *cis*-elements, while the *RKIP* reporter lacking all E-box elements responded poorly to Snail repression (Figure 2e).

To determine whether Snail interacts with the *RKIP* promoter directly, we performed a chromatin immunoprecipitation experiment with purified cross-linked chromatin prepared from LNCaP cells stably expressing a flag-tagged Snail-S6A. In support of the regulatory role of Snail in RKIP expression, a detectable amount of

Snail was found associated with the *RKIP* promoter at E-box 2, but less at E-box 1 but not at exon 3 of the *RKIP* gene locus (Figure 2f). As expected, our positive control showed that Snail bound to its known direct target gene—the E-box in the *E-cad* gene promoter—but not to the intron region. These results confirmed the transient reporter assay, which showed that the proximal E-Box is sufficient for Snail-mediated repression of *RKIP* promoter.

We reasoned that if Snail is a physiologically relevant inhibitor of RKIP expression in prostate cancer, then we ought to observe a negative correlation between their expression patterns in cancer samples. We therefore interrogated publicly available DNA microarray expression data sets derived from human prostate cancers, including Oncomine (www.oncomine.org) and NCBI's Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gds), for both RKIP and Snail. We focused initially on data sets studying progressing prostate cancers and metastasis. Ten data sets were identified that included analyses of normal and/or benign prostatic hyperplasia, localized prostate cancer (PCA) and metastases of prostate cancer. One of them (Dhanasekaran *et al*., 2001), data set no. 1, showed a statistically significant increase of Snail expression from normal to localized to metastatic prostate cancer. As expected, the expression of E-cad, the proven direct target of Snail also decreased significantly between localized cancer samples and metastatic samples. Importantly, the same data set showed a significant decrease of RKIP expression as the prostate cancers progressed (Figure 3a). Samples were referenced against their own pool of normal adjacent prostate (NAP) tissue from prostate cancer patients.

To study the correlation between RKIP and Snail expression, we compared their expression in each prostate cancer sample in data set no. 1 (Figure 3b). We also directly quantified their expression relationship by plotting log-transformed expression units of Snail against RKIP and measured their expression similarity using Pearson's correlation coefficients $r$. As shown in Figure 3c, we observed a significant negative correlation between RKIP and Snail across all samples with $r=-0.58$ and $P$-value $=0.003$ (Figure 3e). As expected, expression of E-cad was also significantly negatively correlated with Snail across all samples ($r=-0.57$; $P$-value $=0.003$) (not shown). Similar results were obtained with another data set (data set no. 2), kindly provided by Dr Arul Chinnaiyan at the University of Michigan (not shown).

Since another member of the Snail superfamily, Slug, shares many physiological functions with Snail and is also implicated in promoting cancer invasiveness, it was of interest to determine whether its expression also negatively correlates with RKIP levels. Unexpectedly, not only did we not observe any increase in the expression levels of Slugs from normal, to benign prostatic hyperplasia, PCA and metastasis of prostate cancer samples, but in fact, there was a decrease in both data sets (Figure 3a). Although there was a small increase in Slug between PCA and metastasis of prostate cancer samples, the difference was not statistically significant.

RKIP was recently reported as being downregulated in metastatic prostate cancer (Fu *et al*., 2003, 2005). However, it is not known how RKIP is downregulated in cancer, or how it is transcriptionally regulated. We showed that by qRT-PCR that the steady-state RKIP mRNA was decreased in high metastatic prostate cancer cell lines PC3 and DU145. Studies with methylation inhibitor 5-Aza2dC and histone deacetylase inhibitor, TSA inferred that the transcription initiation of RKIP expression was actively repressed in prostate cancer metastases.

The Snail superfamily of zinc-finger transcription factors is essential for the induction of epithelial–mesenchymal transition (EMT) during embryogenesis. Abnormalities in the EMT program have been shown to drive cancer cell invasion and metastasis (Thiery, 2002). The process of EMT involves a dramatic phenotypic change, including the loss of epithelial markers, the gain of mesenchymal markers and changes in cell shape. One of the downstream effector targets of Snail leading to EMT is *E-cad*. Expression of E-cad is central in maintaining the cell–cell adhesion of epithelial cells. Snail directly binds the *E-cad* promoter to strongly repress transcription (Batlle *et al*., 2000; Cano *et al*., 2000). An inverse correlation between the expressions of E-cad and Snail has been observed in various cancers including prostate cancer (Poser *et al*., 2001; Yokoyama *et al*., 2001; Blanco *et al*., 2002; Jiao *et al*., 2002). In this study, we presented compelling evidence implicating that RKIP is another transcriptional target of Snail in advanced prostate cancer. We observed a statistically significant negative-correlation between the RKIP expression levels with that of transcription repressor Snail in metastatic prostate cancer samples. We observed that RKIP was highly expressed in the low metastatic cell line LNCaP, but reduced in the DU145 and PC3 cell lines. RKIP expression in these cell lines was reduced in a similar fashion to E-cad expression, and inversely related to Snail expression. We found that overexpressing or knocking down Snail could modulate RKIP expression, and that Snail could repress RKIP promoter activity *in vitro*.

The transcription factor Snail is characterized as having a conserved C-terminal region containing four to six C2H2-type zinc-finger repeats (Knight and Shimeld, 2001) and an amino Snail/Gfi (SNAG) domain. While the fingers function as sequence-specific DNA-binding motifs, the SNAG domain is the effector domain that enhances repressor activity in mammalian cells (Grimes *et al*., 1996; Nakayama *et al*., 1998). It has been shown that Snail represses transcription initiation by binding to the E-box *cis*-elements and recruiting chromatin remodeling mSin3A and histone deacetylases containing repressor complexes. By chromatin immunoprecipitation assay, we showed that Snail was physically associated with the putative E-box 2, and to a lesser extent with E-box 1, in RKIP promoter. We also showed that the presence of E-box 2 correlated with the repression of RKIP promoter by Snail in a transient reporter assay. However, at present, we do not know whether the E-box 2 is required for the observed

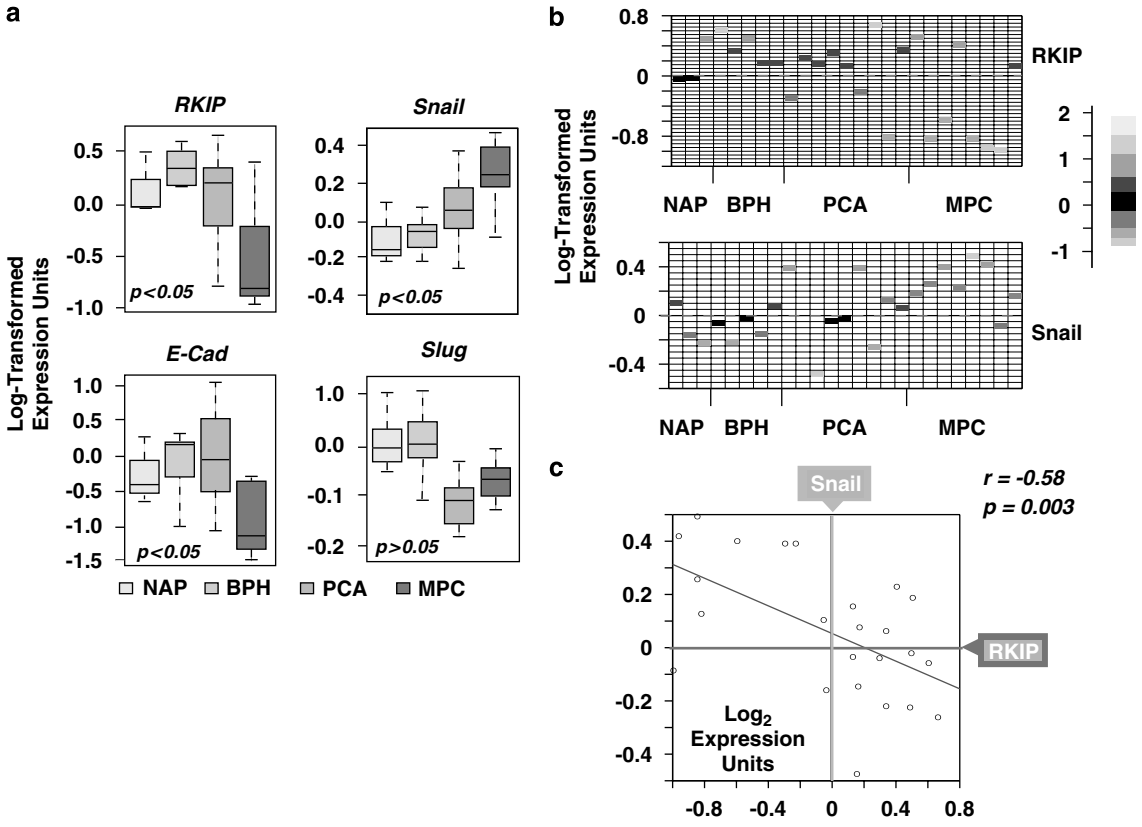


**Figure 3** The expression of *RKIP* negatively correlates with *Snail* in prostate cancer samples. (**a**) Box plots of *RKIP* and *Snail* levels in prostate cancer microarray data sets. The box represents the s.d. of the distribution, and the line through that box represents the mean of that distribution. The horizontal lines above and below the box represent the extreme values of the distribution. Ratios of prostate cancer samples referenced against a pool of normal adjacent prostate (NAP) samples from patients were log-transformed and plotted using the statistical program R. NAP, $N=3$; benign prostatic hyperplasia (BPH), $N=5$; localized prostate cancer (PCA), $N=10$; and metastases (MET), $N=7$. (**b**) The log transformed expression units of *RKIP* or *Snail* were plotted for each sample. Samples were grouped as NAP, BPH, PCA and metastasis of prostate cancer (MPC) as in (**a**). (**c**) Scatterplots of *Snail* versus *RKIP* of all samples displayed in (**a**).

repression mediated by Snail. Nor do we know if the zinc-fingers or the SNAG domain are essential for repression of RKIP expression.

In addition to *E-cad*, Snail has many other downstream effector targets. It has been shown that Snail could downregulate the expression of tight-junction components claudins and occludin, and epithelial marker mucin-1. It also increased the expression of the mesenchymal markers vimentin and fibronectin, proteins involved in cancer invasion such as metalloproteinase-2 and -9, and transcriptional factors ZEB-1 and LEF-1 (Nieto, 2002; De Craene *et al*., 2005). The molecular mechanism of how Snail negatively regulates gene expression has been partially delineated and involves the direct binding of Snail to targeted gene promoter. In contrast, very little is known about how Snail activates gene expression. It appears that Snail activates by indirect mechanisms involving another mediators. For instance, it has been shown that the induced expression of MMP-9 by Snail is dependent on Raf-MEK-Erk and PI3K signaling pathways (Jorda *et al*., 2005). An increase in the binding of NF-κB to the MMP-9 promoter was also observed in Snail-expressing cells. In light of inhibitory functions of RKIP on Raf and NF-κB signaling pathways, it is possible that the observed activation of MMP-9 or other target genes by Snail is caused by the repression of RKIP. In addition to the regulation of genes encoding proteins important for cell–cell and cell–matrix interaction, Snail also plays an important role in promoting resistance to apoptosis and regulating cell cycle progression (Kajita *et al*., 2004; Vega *et al*., 2004). The involved repertoire of Snail target genes has not yet been completely elucidated. Because of its demonstrated negative role in cell survival and proliferation, RKIP may represent a novel downstream effector of the Snail transcriptional axis important for the progression and metastasis of cancer.

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