SNAIL and miR-34a feed-forward regulation of ZNF281/ZBP99 promotes epithelial–mesenchymal transition

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Here, we show that expression of ZNF281/ZBP-99 is controlled by SNAIL and miR-34a/b/c in a coherent feed-forward loop: the epithelial–mesenchymal transition (EMT) inducing factor SNAIL directly induces ZNF281 transcription and represses miR-34a/b/c, thereby alleviating ZNF281 mRNA from direct down-regulation by miR-34. Furthermore, p53 activation resulted in a miR-34a-dependent repression of ZNF281. Ectopic ZNF281 expression in colorectal cancer (CRC) cells induced EMT by directly activating SNAIL, and was associated with increased migration/invasion and enhanced β -catenin activity. Furthermore, ZNF281 induced the stemness markers LGR5 and CD133, and increased sphere formation. Conversely, experimental down-regulation of ZNF281 resulted in mesenchymal–epithelial transition (MET) and inhibition of migration/invasion, sphere formation and lung metastases in mice. Ectopic c-MYC induced ZNF281 protein expression in a SNAIL-dependent manner. Experimental inactivation of ZNF281 prevented EMT induced by c-MYC or SNAIL. In primary CRC samples, expression of ZNF281 increased during tumour progression and correlated with recurrence. Taken together, these results identify ZNF281 as a component of EMT-regulating networks, which contribute to metastasis formation in CRC.

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Introduction

The ZNF281/ZBP-99 protein has characteristics of a transcription factor and contains four Krüppel-type zinc-finger domains (Law *et al*, 1999; Lisowsky *et al*, 1999). ZNF281 is

related to ZBP-89, which has been implicated in the regulation of cell proliferation (Bai and Merchant, 2001), apoptosis (Bai *et al*, 2004), differentiation (Li *et al*, 2006) and tumorigenesis (Law *et al*, 2006). ZNF281 mediates transcriptional repression and activation (Wang *et al*, 2008). For example, ZNF281 directly regulates the expression of *gastrin* and represses *ornithine decarboxylase (ODC)* by binding to GC-rich sequences in their promoters (Law *et al*, 1999; Lisowsky *et al*, 1999). Previously, we detected a direct interaction between ZNF281 and the c-MYC oncoprotein (Koch *et al*, 2007). Moreover, ZNF281 participates in the regulation and maintenance of pluripotency by interacting with transcription factors controlling stemness, such as Nanog, Oct4 and Sox2 (Wang *et al*, 2006, 2008). Furthermore, ZNF281 directly regulates *Nanog* expression and contributes to its auto-regulation by recruiting the NuRD complex in mouse embryonic stem cells (Fidalgo *et al*, 2011, 2012). The Sox4 transcription factor directly induces ZNF281 transcription (Scharer *et al*, 2009). Interestingly, Sox4 has been implicated in the regulation of differentiation, proliferation, epithelial–mesenchymal transition (EMT) and shows increased expression in many human cancers (Zhang *et al*, 2012). After DNA damage, the ZNF281 protein is phosphorylated by ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases (Matsuoka *et al*, 2007). However, other signals regulating ZNF281 activity and expression have remained elusive.

As a morphogenic programme, EMT is involved in the formation of tissue and organs during embryonic development and wound healing. During EMT, epithelial cells acquire mesenchymal features, such as decreased cell–cell contacts and loss of polarity, which promote increased motility and invasiveness. Thereby, EMT contributes to the progression of early-stage tumours to invasive malignancies (Thiery, 2002; Lee *et al*, 2006; Hugo *et al*, 2007). So far only a few transcription factors, such as SNAIL, SLUG, TWIST1/2 and ZEB1/2, are thought to constitute the central regulatory core of EMT (Peinado *et al*, 2007; Sanchez-Tillo *et al*, 2012). EMT has also been shown to promote stemness of cancer cells that may endow tumour initiating cells with traits necessary for metastasis formation, as shown for immortalized human mammary epithelial cells undergoing EMT (Brabletz *et al*, 2005b; Mani *et al*, 2008). Accordingly, SNAIL, TWIST and ZEB1 share the ability to induce both stemness and EMT (Mani *et al*, 2008; Wellner *et al*, 2009). Furthermore, tumour cells undergoing EMT show accumulation of active β -catenin in the nucleus (reviewed in Brabletz *et al*, 2005a).

Recently, microRNAs (miRNAs) have emerged as major regulators of EMT (Brabletz, 2012; Hermeking, 2012). For example, members of the miR-200 family and miR-205 promote MET by inhibiting EMT inducing factors like ZEB1 and ZEB2 (Gregory *et al*, 2008; Park *et al*, 2008), and

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miR-34a/b/c achieve the same effect by downregulating SNAIL expression (Kim *et al*, 2011; Siemens *et al*, 2011). Moreover, SNAIL directly represses *miR-34a/b/c* transcription (Siemens *et al*, 2011). The resulting double-negative feedback loop represents a bistable switch, which can be locked in the mesenchymal state by inactivation of *miR-34* genes by CpG methylation, which is often found in cancer cells (Hermeking, 2012; Siemens *et al*, 2013). Interestingly, the genes encoding the *miR-200* and *miR-34* families are direct p53 targets and their mediation of MET presumably contributes to tumour suppression by p53 (Hermeking, 2012).

Here, we show that ZNF281 expression is regulated by a feed-forward loop involving SNAIL and miR-34a. Taken together, we show that ZNF281 is an integral part of the EMT-regulating transcriptional network and controls processes relevant to colorectal cancer (CRC) progression, such as migration, invasion, stemness and metastasis.

Results

SNAIL regulates ZNF281 expression

We previously identified an interaction between c-MYC and ZNF281 proteins in a systematic analysis of c-MYC-associated protein complexes (Koch *et al*, 2007). ZNF281 was among the proteins represented by the highest number of mass-spectrometric sequence reads, indicating that it is associated with a large fraction of cellular c-MYC and presumably represents a significant regulator or effector of c-MYC. However, so far it is largely unknown how ZNF281 expression itself is regulated and whether it participates in regulatory pathways, which might be relevant for c-MYC function and tumour biology. In order to identify upstream regulators of ZNF281, we inspected the *ZNF281* promoter sequence for binding sites of transcription factors, which might hint towards cancer-relevant functions of ZNF281. Thereby, we identified several E-Box motifs (CACCTG) in the *ZNF281* promoter, which represent putative SNAIL binding sites (SBSs; Figure 1A). SNAIL is a known regulator of EMT (Mauhin *et al*, 1993; Batlle *et al*, 2000) and, similar to ZNF281, a zinc-finger-containing transcription factor (Nieto, 2002; Sanchez-Tillo *et al*, 2012; see also comparison in Supplementary Figure S1). Two of the SBSs were located ~500 and ~700 bp upstream of the transcription start site (TSS; Figure 1B). SBS2 and SBS4 are conserved between the human and mouse *ZNF281* promoters, indicating functional relevance (Figure 1B). When SNAIL was ectopically expressed in DLD-1 CRC cells using a Doxycycline (DOX) inducible episomal vector system an increase in the SNAIL

occupancy of the *ZNF281* promoter was detected by chromatin immunoprecipitation (ChIP) analysis at SBS2 and SBS3, whereas SBS1, 4 and 5 did not display increased binding of SNAIL (Figure 1C). Also endogenous SNAIL protein selectively occupied SBS2 and SBS3 in SW620 CRC cells (Supplementary Figure S2A). Furthermore, ectopic SNAIL enhanced the expression of ZNF281 at the protein and the mRNA level in DLD-1 cells (Figure 1D and E). SNAIL also induced ZNF281 expression in SKBR3 breast cancer and MiaPaCa2 pancreatic cancer cells (Supplementary Figure S2B). Therefore, the induction of ZNF281 by SNAIL is not restricted to a specific cell type. In order to determine whether *ZNF281* is induced by SNAIL *via* SBS motifs, a region encompassing ~2 kbp upstream of the *ZNF281* transcriptional start site was subjected to a dual reporter assay (Figure 1F). Indeed, the wild-type reporter was induced by SNAIL, whereas mutation of SBS2 abolished and SBS3 mutation decreased the responsiveness to SNAIL. Also a reporter with combined mutation of SBS2 and SBS3 resulted in complete loss of responsiveness to SNAIL. A *CDH1* promoter reporter was repressed by SNAIL in an SBS-dependent manner in this assay. ZNF281 displayed the highest expression level in CRC cell lines with mesenchymal features, such as Colo320, SW480 and SW620, whereas HT29, DLD-1 and HCT-15 cells, which display an epithelial phenotype, showed comparatively low ZNF281 expression levels (Figure 1G). ZNF281 expression positively correlated with SNAIL and Vimentin and inversely with E-cadherin expression (Figure 1G). Moreover, analysis of publicly available mRNA expression profiles obtained from seven CRC cell lines (COLO205, HCC2998, HCT116, HCT15, HT29, KM12, SW620) within the NCI-60 panel (Shoemaker, 2006) confirmed a significant correlation between *ZNF281* and the mesenchymal markers *SNAIL*, *Vimentin* and *Fibronectin-1* (Supplementary Table S1). Taken together, these results suggested that the induction of ZNF281 by SNAIL may be an important component of the EMT programme induced by SNAIL. Indeed, when ZNF281 was downregulated using two different siRNAs the induction of EMT by SNAIL was prevented in DLD-1 cells (Figure 1H and I; Supplementary Figure S2C). Also the loss of E-cadherin from the outer membrane, which is typical for EMT, was prevented by simultaneous siRNA-mediated downregulation of ZNF281. Therefore, ZNF281 is required for SNAIL-induced EMT.

miR-34a directly regulates ZNF281 expression

The differences between the pronounced increase in ZNF281 protein levels and minor increase in mRNA levels after

Figure 1 ZNF281 is a direct SNAIL target required for SNAIL-induced EMT. For the following analyses (besides **F** and **G**) DLD-1 cells harbouring a pRTR-SNAIL-VSV vector were treated with DOX for the indicated periods to activate SNAIL-VSV expression. **(A)** Scheme of the *ZNF281* promoter and SNAIL binding sites (SBSs). Grey arrows indicate potential SNAIL binding sites; black rectangles exons and the bar a qChIP amplicon. TSS: transcription start site. **(B)** Sequence alignment of the indicated SBS in the indicated species. **(C)** ChIP analysis 24 h after addition of DOX or left untreated using anti-VSV and anti-rabbit-IgG antibodies for ChIP. Results are given as the mean $\pm$ s.d. ($n=3$). **(D)** Western blot detection of the indicated proteins at the indicated time points. **(E)** qPCR analysis with values representing the mean $\pm$ s.d. ($n=3$). **(F)** Luciferase assay in DLD-1 cells 48 h after transfection of pcDNA3-VSV (Ctrl.) or pcDNA3-SNAIL-VSV (SNAIL) vectors and the indicated pBV-ZNF281 promoter constructs or pXP2-E-cadherin/CDH1 vectors as controls (wt: wild type, mut: mutated). **(G)** Western blot analysis of the indicated proteins in CRC cell lines. 'epi.' = cells with epithelial, 'mes.' = cells with mesenchymal phenotype. **(H)** Cells were treated with DOX (+) or left untreated (–) for 96 h and simultaneously transfected with the indicated siRNAs. The indicated proteins were detected by western blot analysis. **(I)** Two upper panels: representative phase-contrast pictures (P/C) of the cells described in **(H)**. $\times 200$ magnification. Two lower panels: detection of E-cadherin by indirect immunofluorescence and confocal microscopy. Nuclear DNA was visualized by DAPI staining. $\times 200$ magnification. Scale bars represent 25 μ m. In **(D, H)**, detection of α -Tubulin and in **(G)**, detection of β -Actin served as a loading control. In **(D, H)**, relative densitometric quantifications are indicated. ZNF = ZNF281; E-cad = E-cadherin; α -Tub = α -Tubulin. In **(C, E** and **F)**, a Student's *t*-test was used. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. Source data for this figure is available on the online supplementary information page.

ectopic SNAIL expression suggested the possibility of an additional translational regulation mediated by miRNAs. Inspection of the *ZNF281* 3'-UTR using the TargetSCAN and Miranda algorithms (John *et al*, 2004; Grimson *et al*, 2007) revealed a conserved miR-34 seed-matching sequence (Figure 2A). Since we had previously shown that the *miR-34a* and *miR-34b/c* genes are directly repressed by SNAIL (Siemens *et al*, 2011), we hypothesized that at least part of the increase in *ZNF281* expression observed after SNAIL

induction might be due to a repression of *miR-34* genes. Indeed, ectopic *miR-34a* expression resulted in the downregulation of endogenous *ZNF281* expression at the protein and mRNA level in SW480 CRC cells (Figure 2B and C). This was also observed in MiaPaCa2 pancreatic cancer cells (Supplementary Figure S3A and B). Therefore, the regulation of *ZNF281* by miR-34a is not restricted to CRC cells. Furthermore, reporter constructs containing the complete 3'-UTR of *ZNF281* (720 bp) or a 77-bp fragment

Figure 2 Direct regulation of ZNF281 by miR-34a. (A) Schematic representation of the ZNF281 3'-UTR indicating seed-matching sequences (in red) and miR-34 seed sequences (blue letters) (adapted from www.targetscan.org). The black vertical bars indicate possible base pairing. (B) Western blot analysis of endogenous ZNF281 protein levels in SW480 cells harbouring a pRTR-pri-miR-34a vector after treatment with DOX for the indicated periods. Relative densitometric quantifications are indicated. ZNF = ZNF281; α -Tub = α -Tubulin. (C) Analysis of ZNF281 mRNA levels in cells corresponding to (B). (D) Mutagenesis of the ZNF281 3'-UTR. Black vertical bars indicate the remaining matches of the miR-34a seed (shaded black) with the miR-34 seed-matching sequence (shaded grey) in the ZNF281 3'-UTR sequence (wt: wild type, mut: mutated). (E) Dual luciferase reporter assay in SW480 cells 72 h after transfection with pre-miR-34a or control oligonucleotides and the empty pGL3 vector or pGL3 harbouring the indicated 3'-UTR-reporter constructs (fl: full length). A 3'-UTR reporter of the known miR-34a target TPD52 served as a positive control. (F) Western blot analysis of the indicated proteins in DLD-1 cells harbouring a pRTR-SNAIL-VSV vector transfected with the indicated oligonucleotides for 60 h and treated with DOX or left untreated for 36 h prior to cell lysis. In (B, F), detection of α -Tubulin served as a loading control. In (C, E), data represent the mean $\pm$ s.d. ($n = 3$). A Student's t -test was used. * $P < 0.05$ and *** $P < 0.001$. Source data for this figure is available on the online supplementary information page.

including the seed-matching sequence were repressed by co-transfection of pre-miR-34a, but not when the seed-matching sequence was mutated, demonstrating that it mediates repression by miR-34a (Figure 2D and E). The induction of ZNF281 by SNAIL was prevented by concomitant transfection of pre-miR-34a (Figure 2F). Therefore, the previously documented repression of the miR-34a gene by SNAIL (Siemens *et al*, 2011) is presumably necessary for the SNAIL-mediated increase in ZNF281 expression. In summary, these results

demonstrate that ZNF281 is directly regulated by miR-34a and that SNAIL induces ZNF281, at least in part, by repressing miR-34a.

p53 represses ZNF281 via miR-34a

Since the miR-34 genes represent direct p53 targets, we asked whether p53 represses ZNF281 expression *via* inducing miR-34a. Indeed, ectopic expression of p53 resulted in a decrease in ZNF281 at the protein and mRNA level

(Figure 3A and B). As expected, miR-34a/b/c levels were increased upon p53 activation, which is likely to mediate the decrease in ZNF281 protein expression (Figure 3C and D). Since miR-34b/c is expressed at least at 10-fold lower levels in CRC and CRC cell lines compared to miR-34a (Toyota *et al*, 2008; Siemens *et al*, 2013) we focussed on miR-34a in the further analyses. The recovery of *ZNF281* mRNA expression by 72 h of ectopic p53 expression is presumably due to the declining expression of ectopic p53 and therefore reduced *pri-miR-34* induction at this time point (Figure 3A, B and D). Nonetheless, ZNF281 protein was still downregulated 72 h after activation of p53 (Figure 3A). In order to determine whether downregulation of ZNF281 is a result of reduced SNAIL expression caused by direct interaction of SNAIL with p53 (Lim *et al*, 2010) or due to p53-induced *miR-34*, we directly interfered with miR-34a function using antagomirs. Indeed, *miR-34a*-specific antagomirs largely abolished the downregulation of ZNF281 after p53 induction, whereas a control antagomir did not affect the repression of ZNF281 by p53 (Figure 3E). The remaining minor repression of ZNF281 may be due to p53-induced *miR-34b* and *-c*, which are presumably not affected by the *miR-34a*-specific antagomir

used here. Additionally, we analysed the expression of ZNF281 in HCT116 *p53*^{+/+} cells and an isogenic clone with homozygous deletion of p53 resembling p53 inactivation in tumours. HCT116 *p53*^{+/+} cells expressed lower endogenous levels of ZNF281 protein and mRNA than p53-deficient cells (Figure 3F and G). As previously described (Siemens *et al*, 2011), the expression of the SNAIL protein was elevated in the HCT116 *p53*^{-/-} cells (Figure 3F). When SNAIL was downregulated using a *SNAIL*-specific siRNA, the expression of ZNF281 protein was only decreased to a minor extent in p53-deficient HCT116 cells (Figure 3H). Therefore, the increase in ZNF281 expression is presumably mainly due to the decrease in miR-34a expression in p53-deficient cells (Figure 3G). Taken together, these results show that miR-34a represents an important mediator for the repression of ZNF281 by p53.

ZNF281 induces EMT, migration and invasion

Since ZNF281 expression was induced by SNAIL and required for SNAIL-induced EMT, we determined whether ectopic expression of ZNF281 is sufficient to promote EMT. For this purpose, a pool of DLD-1 cells harbouring an episomal pRTR

Figure 3 p53 regulates the expression of ZNF281 via induction of miR-34a. In (A–E), SW480 cells harbouring a pRTR-p53-VSV vector treated with DOX for the indicated periods or left untreated were used. (A) Western blot analysis of endogenous ZNF281 expression at the indicated time points. (B) Ectopic p53 was expressed for the indicated periods before RNA was harvested and subjected to qPCR analysis. (C) Analysis of mature miR-34a/b/c expression levels 48 h after addition of DOX to induce p53 or left untreated. (D) Analysis of *pri-miR-34a* mRNA levels at the indicated time points. (E) Cells were transfected with the indicated oligonucleotides for 72 h in the presence or absence of DOX for the last 48 h prior to lysis of the cells. The indicated proteins were detected by western blot analysis. (F) Detection of the indicated proteins by western blot analysis in *p53*^{+/+} and *p53*^{-/-} HCT116 cells. (G) qPCR analysis of the ZNF281 mRNA expression in *p53*^{+/+} and *p53*^{-/-} HCT116 cells. (H) Western blot analysis of the indicated proteins in HCT116 *p53*^{-/-} cells 96 h after transfection of a *SNAIL*-specific siRNA. In (A, E), detection of β -Actin and in (F, H), detection of α -Tubulin served as a loading control and were used for relative densitometric quantifications in (E, H). ZNF = ZNF281; α -Tub = α -Tubulin. In (B–D and G), values represent the mean $\pm$ s.d. ($n = 3$). A Student's *t*-test was used. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Source data for this figure is available on the online supplementary information page.

construct that allows the DOX-inducible expression of ZNF281 was generated. After addition of DOX >90% of the cells were positive for eGFP, which is expressed from a bidirectional promoter also driving the expression of ZNF281 (Supplementary Figure S4A). After induction of ectopic ZNF281 expression DLD-1 cells changed from an epithelial morphology (dense islands of cobblestone-shaped cells) to a mesenchymal morphology with spindle-shaped cells forming protrusions and displaying a scattered growth pattern (Figure 4A). This was reminiscent of the effect of ectopic SNAIL expression observed in DLD-1 cells before (Siemens *et al*, 2011). Also molecular markers of EMT were regulated by the expression of ZNF281 (Figure 4B). The distinct membrane-bound expression of E-cadherin in DLD-1 cells was lost upon ZNF281 activation. Furthermore, ZNF281-expressing cells displayed an increased cytoplasmic expression of the mesenchymal marker Vimentin. In addition, F-actin, which forms stress fibres (Moreno-Bueno *et al*, 2009), was relocated from the membrane to the cytoplasm.

Subsequently, we determined whether ectopic ZNF281 expression influences cellular migration and invasion, since EMT has been previously linked to increased migration and invasion (reviewed in Christiansen and Rajasekaran, 2006). In a wound-healing assay, ectopic ZNF281 expression resulted in a minor, but reproducible increase in the closure of a scratch in a confluent layer of DLD-1 cells compared to controls (Figure 4C; Supplementary Figure S4H). When migration and invasion were examined in Boyden-chamber assays the effect of ectopic ZNF281 expression was more pronounced (Figure 4D and E). Furthermore, ectopic expression of ZNF281 significantly enhanced the ability of DLD-1 cells to form colonies in soft agar (Figure 4F). The addition of DOX to DLD-1 cells harbouring an empty vector control did not result in EMT-related morphological changes or significant effects in the above-mentioned assays (Supplementary Figure S4E–H). The effects of ectopic ZNF281 were not due to increased proliferation, since ZNF281 activation had a slight anti-proliferative effect (Supplementary Figure S5), which has also been described for other EMT-TFs, such as SNAIL (Peinado *et al*, 2007). Taken together, these results show that ectopic expression of ZNF281 is sufficient to mediate EMT and enhances migration, invasion and anchorage-independent growth.

Transcriptional regulation of EMT markers by ZNF281

Next, we determined whether ZNF281 also induces changes in the expression of genes previously implicated in the transcriptional programme of EMT. After activation of ectopic ZNF281 expression in DLD-1 cells, an upregulation of SNAIL was observed at the protein and mRNA level (Figure 5A and B). In addition, the mesenchymal markers *SLUG*, *ZEB1* and *Fibronectin-1* were induced after ectopic ZNF281 expression in DLD-1 cells (Figure 5B). In line with the indirect immunofluorescence results shown in Figure 4B, E-cadherin/CDH-1 was repressed at the protein level after induction of ZNF281 (Figure 5A), whereas expression of *CDH1* mRNA was not significantly affected by ZNF281 (Figure 5C). However, when ZNF281 was expressed in HT29 CRC cells *E-cadherin* was repressed at both the protein and mRNA level (Supplementary Figure S6A and C). Therefore, the regulation of EMT markers by ZNF281 is at least partially dependent on the cellular context. Other

epithelial markers, such as *OCN* and *CLDN-7*, were repressed at the mRNA level in DLD-1 and HT29 cells (Figure 5C; Supplementary Figure S6C), which is characteristic for EMT (Ikenouchi *et al*, 2003; Martinez-Estrada *et al*, 2006). Furthermore, ectopic ZNF281 expression resulted in the downregulation of a number of additional epithelial marker genes encoding components of tight junctions (*ZO-1/3*, *CLDN-1*) and adherens junctions (*CDH-3*), as well as desmosomes (*PKP2*, *DSP*) (Figure 5D), as previously shown for ZEB2 (Vandewalle *et al*, 2005).

Since SNAIL is a potent inducer of EMT, we determined whether the upregulation of SNAIL by ZNF281 is mediated by direct occupancy of the *SNAIL* promoter. ZNF281 is known to occupy GC-rich DNA sequences, as previously shown for the *ODC1* promoter (Law *et al*, 1999; Lisowsky *et al*, 1999). Similar GC-rich sequences are present in the *SNAIL* promoter (Figure 5E). When these regions were analysed by a ZNF281-specific ChIP, occupancy by ectopic and endogenous ZNF281 was detected in the vicinity of the *SNAIL* TSS (Figure 5F; Supplementary Figure S7A). The highest occupancy by ZNF281 protein was detected in a region encompassing the *SNAIL* promoter itself and ~600 bp upstream. A set of deletion constructs of the human *SNAIL* promoter (Barbera *et al*, 2004) was used to determine the regions mediating the regulation by ZNF281 in a reporter assay. The activation of the *SNAIL* promoter was most dominant for the –869/+59 reporter (Figure 5G), which was in line with the dominant binding of ectopic ZNF281 to a region ~600 bp upstream of the TSS in the ChIP assay (Figure 5F). Unexpectedly, the 1558/+92 construct resulted in a weaker response to ZNF281, which may be due to repressive elements or binding sites for other transcription- or co-factors in the region between –869 and –1558 bp. Also, the decreasing activity of further truncations indicates that the predominant region of ZNF281 binding is located between 514 and 869 bp upstream of the *SNAIL* TSS. Another, less effective binding region is presumably located closer to the TSS as also suggested by the ChIP results (Figure 5F; Supplementary Figure S7A). Occupancy by endogenous and ectopic ZNF281 was also detected in the promoter regions of *CDH1*, *OCN* and *CLDN-7* (Figure 5H; Supplementary Figure S7B).

When SNAIL was downregulated by RNA interference (Figure 5I and J) in DLD-1 cells ectopically expressing ZNF281 morphological changes associated with EMT were not observed (Figure 5J). Moreover, E-cadherin persisted at the cell membrane, whereas co-transfection of a control siRNA did not prevent its relocalization (Figure 5J). Therefore, ZNF281-induced EMT is mediated, at least in part, by SNAIL. In summary, these analyses show that ZNF281 directly regulates the expression of a subset of EMT regulators and effectors. The requirement of SNAIL for ZNF281-induced EMT suggests that at least some of these regulations are mediated and/or enhanced *via* the induction of SNAIL. Furthermore, ZNF281 directly induces SNAIL, which itself activates ZNF281 expression, thereby forming a positive feedback loop, which may enforce and stabilize the process of EMT.

ZNF281 regulates β -catenin localization and activity

Interestingly, ectopic expression of ZNF281 in DLD-1 cells resulted in the translocation of β -catenin from the cell membrane to the nucleus (Figure 6A), which is another

Figure 4 Ectopic ZNF281 induces EMT, migration and invasion in DLD-1 cells. DLD-1 cells harbouring a pRTR-ZNF281-VSV vector treated with DOX or left untreated were analysed. **(A)** Representative phase-contrast (P/C) pictures of the cells treated with DOX or left untreated for 96 h. $\times 200$ magnification. **(B)** Confocal laser-scanning microscopy of E-cadherin, Vimentin and F-actin proteins detected by indirect immunofluorescence 96 h after addition of DOX. Nuclear DNA was visualized by DAPI staining. $\times 200$ magnification. **(C)** Cells were treated with DOX or left untreated for 48 h before the scratch was applied. Upper panel: representative pictures of the wound areas at the indicated time points after scratching. $\times 100$ magnification. Lower panel: results represent the average (%) of wound closure determined by the final width of the scratch in three independent wells. Error bars represent $\pm$ s.d. ($n = 3$). Boyden-chamber assays of cellular migration **(D)** or invasion **(E)**. Cells were cultivated in the presence or absence of DOX for 72 h with serum starvation for the last 48 h. To analyse invasion, membranes were coated with Matrigel. After 48 h, cells were fixed and stained with DAPI. The average number of cells per well was counted in three different inserts. Relative invasion or migration is expressed as the value of treated cells to control cells with control set as one. **(F)** Cells were subjected to a soft-agar assay and treated with DOX or left untreated. Two weeks after seeding the resulting colonies were stained with crystal violet. Results represent the mean number of colonies in soft agar per well $\pm$ s.d. ($n = 3$). **(C–F)** A Student's *t*-test was used. $^{**}P < 0.01$ and $^{***}P < 0.001$. In **(A, B)**, scale bars represent 25 μ m.

characteristic of EMT (Brabletz *et al*, 2005b). Although, β -catenin mRNA and protein levels remained unchanged upon ZNF281 expression (Figure 6B and C), a significant increase in the transcriptional activity of β -catenin/TCF4 was observed after ZNF281 expression in DLD-1 and SW480 cells in a reporter assay (Figure 6D). Axin2 negatively regulates

the WNT/ β -catenin/TCF4 signalling pathway by promoting phosphorylation and degradation of β -catenin/TCF4 via a multi-protein complex including APC and GSK3 β (Lustig *et al*, 2002). Therefore, decreased levels of inhibitory Axin2 might result in the translocation of β -catenin. Indeed, Axin2 mRNA was repressed upon ectopic

expression of ZNF281 (Figure 6E). Furthermore, we detected increased binding of ZNF281 at the *Axin2* promoter, which harbours GC-rich regions representing potential binding sites for ZNF281 (Figure 6F). Therefore, it is conceivable that the direct repression of *Axin2* by ZNF281 contributes to the increased activity of TCF4/ β -catenin. In addition, the loss of E-cadherin from the cell membrane may contribute to the

activation of TCF4/ β -catenin after ZNF281 activation (see Discussion). In line with increased β -catenin/TCF4 activity *LGR5* and *CD133*, which are known β -catenin target genes (Katoh and Katoh, 2007; Fan *et al*, 2010; Glinka *et al*, 2011; Carmon *et al*, 2012), were induced after ectopic expression of ZNF281 in DLD-1 cells (Figure 6E). qChIP analysis revealed ZNF281 occupancy at the *LGR5* promoter, but not at the

CD133 promoter (Figure 6F). Since *LGR5* and *CD133* represent markers for cancer stem cells (Barker *et al*, 2007; Zhu *et al*, 2009; Munoz *et al*, 2012), the activation of ZNF281 may be accompanied by the acquisition of stem-cell traits. Indeed, ectopic ZNF281 expression significantly enhanced the formation of colono-spheres by non-adherent DLD-1 cells (Figure 6G and H). Taken together, ZNF281 enhances β -catenin activity and stemness of tumour cells. Both effects may contribute to metastasis formation.

Requirement of ZNF281 for EMT, migration and invasion

Next, we thought to determine whether ZNF281 is not only sufficient for the induction of EMT but also necessary for the maintenance of an EMT-like state in the CRC cell line SW480, which displays mesenchymal features such as low E-cadherin and high SNAIL expression (Figure 1G), as well as enhanced migration, invasion and metastasis. In line with our previous findings, SW480 cells displayed high levels of endogenous ZNF281 when compared to the more epithelial cell lines DLD-1 and HT29 (Figure 1G). Therefore, we generated cell pools with DOX-inducible expression of a ZNF281-specific miRNA or the respective control driven by an episomal pRTS vector. These cell pools showed ectopic expression of an inducible, co-expressed mRFP marker in ~90% of the cells after addition of DOX (Supplementary Figures S8A, S9A and S11A). After induction of the ZNF281-specific miRNAs, endogenous ZNF281 was repressed at the mRNA and protein level (Figure 7A and B; Supplementary Figure S9B and D). Simultaneously, mesenchymal markers, such as SNAIL and Vimentin, were repressed, whereas the epithelial marker E-cadherin was induced at the protein level (Figure 7B; Supplementary Figure S9D). Furthermore, SW480 cells lost their mesenchymal morphology and gained epithelial phenotypes with an increase in cell-cell contacts (Figure 7C; Supplementary Figure S9C) and diminished expression of Vimentin (Figure 7B and C; Supplementary Figure S9D). Moreover, downregulation of ZNF281 in SW480 cells resulted in decreased migration in a scratch and a Boyden-chamber assay (Figure 7D and E; Supplementary Figure S8E and F), as well as diminished invasion in a Matrigel-transwell assay (Figure 7E; Supplementary Figure S9F). The downregulation of ZNF281 had no significant effects on cell proliferation, cell-cycle distribution and apoptosis

(Supplementary Figure S10). In addition, ZNF281 downregulation resulted in a decrease in colony formation in soft agar (Figure 7F) and a reduction in sphere formation (Figure 7G). Similar effects were observed after expression of the other ZNF281-specific miRNA (Supplementary Figure S9G and H), whereas a non-specific control miRNA did not result in significant effects in any of the assays described above (Supplementary Figure S11B–E). Taken together, downregulation of ZNF281 induces a MET of SW480 cells, which is associated with the loss of migratory and invasive capacities, as well as reduced stemness. Therefore, expression of ZNF281 is not only sufficient for induction of EMT but presumably also required to maintain a mesenchymal state in CRC cell lines. Furthermore, ZNF281 is not only sufficient to induce SNAIL, but also required for its continued expression.

Requirement of ZNF281 for c-MYC-induced EMT

We recently observed that ectopic c-MYC expression effectively induces EMT in DLD-1 cells, which was accompanied by an activation of SNAIL expression, mediated, to a large extent, by AP4 (Jackstadt *et al*, 2013). Since we had detected an interaction between c-MYC and ZNF281 before (Koch *et al*, 2007), we asked whether ZNF281 is required for c-MYC-induced EMT. Interestingly, c-MYC activation resulted in an induction of ZNF281 expression at the protein and mRNA level (Figure 8A and B). This was presumably indirect since MYC binding sites were not identified in the ZNF281 promoter region. Interestingly, ectopic c-MYC expression and concomitant transfection of a SNAIL-specific siRNA diminished the induction of ZNF281 (Figure 8C). When c-MYC was ectopically expressed in DLD-1 cells in the presence of siRNAs directed against ZNF281, the repression of E-cadherin and also the induction of SNAIL was less pronounced than with co-transfection of control siRNA (Figure 8D). In addition, siRNA-mediated downregulation of ZNF281 prevented the adoption of a mesenchymal phenotype after activation of c-MYC in DLD-1 cells (Figure 8E). After activation of ectopic c-MYC and concomitant transfection of ZNF281-specific siRNAs, E-cadherin remained at the membrane, whereas co-transfection of a control siRNA did not interfere with the c-MYC-induced loss of membranous E-cadherin (Figure 8E). Taken together, these results show that c-MYC-induced EMT is mediated by ZNF281.

Figure 5 ZNF281 activates a transcriptional EMT programme that includes activation of SNAIL. Unless mentioned otherwise, DLD-1 cells harbouring a pRTR-ZNF281-VSV vector treated with DOX or left untreated were analysed. (A) Western blot detection of the indicated proteins after ectopic expression of ZNF281 for the indicated periods. Relative densitometric quantifications are indicated. ZNF = ZNF281, E-cad = E-cadherin, α -Tub = α -Tubulin. (B) Expression of the indicated mRNAs was determined by qPCR analyses. Results represent the mean $\pm$ s.d. ($n = 3$). (C) Cells were induced with DOX for the indicated periods or left untreated and qPCR analyses were performed to determine the indicated mRNA expression levels. (D) Cells were treated with DOX for 96 h or left untreated and the indicated mRNAs were analysed by qPCR. In (C, D) results represent the mean $\pm$ s.d. ($n = 3$). (E) Schematic depiction of the SNAIL promoter. Amplicons (black bars) used for ChIP analysis, exons (black rectangles) and the TSS (transcription start site) are indicated. (F) ChIP assay of DLD-1/pRTR (Ctrl.) or DLD-1/pRTR-ZNF281-VSV (ZNF281) cells 24 h after addition of DOX using anti-VSV and anti-rabbit-IgG antibodies. The previously described ZNF281 occupancy at the *ODC1* promoter served as a positive control. Results represent the percentage of input chromatin of induced versus control cells $\pm$ s.e. ($n = 2$). (G) The indicated pGL3-SNAIL promoter plasmids were co-transfected with pcDNA3-VSV (Ctrl.) or pcDNA3-ZNF281-VSV (ZNF281) plasmids into Hek293T cells, which were subjected to a reporter assay after 48 h. (H) ChIP assay of DLD-1/pRTR (Ctrl.) or DLD-1/pRTR-ZNF281-VSV (ZNF281) cells 24 h after addition of DOX using anti-VSV and anti-rabbit-IgG antibodies. Results represent the mean $\pm$ s.d. ($n = 3$). (I) Cells were transfected with a SNAIL-specific siRNA or the respective control (Ctrl.) for 96 h and treated with DOX or left untreated for 72 h. Detection of the indicated proteins by western blot analysis. (J) Representative phase-contrast (P/C) images of cells analysed in (I) at $\times 200$ magnification. Lower two panels: detection of E-cadherin by indirect immunofluorescence 96 h after transfection of the indicated siRNAs and treatment of DOX or left untreated as in (I) using confocal laser-scanning microscopy. Nuclear DNA was visualized by DAPI staining. $\times 200$ magnification. Scale bars represent 25 μ m. In (A), detection of α -Tubulin and in (I) detection of β -Actin served as a loading control. In (B–D) and (G), a Student's *t*-test was used. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Source data for this figure is available on the online supplementary information page.

Figure 6 ZNF281 mediates translocation of β -catenin to the nucleus and increases stemness properties. DLD-1 cells harbouring a pRTR-ZNF281-VSV vector were treated with DOX for the indicated periods to activate ZNF281-VSV expression. **(A)** Detection of β -catenin by indirect immunofluorescence and confocal microscopy 96 h after treatment with DOX. Nuclear DNA was visualized by DAPI staining. $\times 200$ magnification. The scale bar represents 25 μ m. **(B)** Western blot analysis of the indicated proteins. Detection of β -Actin served as a loading control. **(C)** qPCR analyses with results represented as mean values $\pm$ s.d. ($n = 3$). **(D)** Left: TOPflash reporter assay after addition of DOX for 48 h and concomitant transfection with TOP/FOP vectors. Right: TOPflash reporter assay of SW480 cells transfected with TOP/FOP vectors and control vector or pcDNA3-ZNF281-VSV vector (as indicated) for 48 h. Results represent the mean $\pm$ s.d. ($n = 3$). **(E)** qPCR analyses of DLD-1 pRTR-ZNF281-VSV cells with results depicted as the mean $\pm$ s.d. ($n = 3$). **(F)** qChIP assay 24 h after addition of DOX using anti-VSV and anti-rabbit-IgG antibodies for ChIP. qChIP values are represented as the percentage of input chromatin and qChIP amplicons are indicated. Error bars represent $\pm$ s.e. ($n = 2$). **(G)** Representative phase-contrast images of spheres grown on low adherence plates 7 days after addition of DOX. The scale bar represents 50 μ m. **(H)** Quantification of the sphere number per 1000 cells. Results represent the mean $\pm$ s.d. ($n = 3$). In **(C–E)** and **(H)**, a Student's *t*-test was used. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Source data for this figure is available on the online supplementary information page.

Role of ZNF281 in metastasis formation

Since EMT and the resulting cellular properties have been implicated in the metastatic process (Valastyan and Weinberg, 2011), we asked whether inactivation of ZNF281 in the highly metastatic CRC line SW620 would influence

metastasis formation in a xenograph mouse model. Therefore, we generated SW620 cells stably expressing luciferase2 to monitor the development of metastases over time in a non-invasive manner (Supplementary Figure S13). Transfection of SW620-Luc2 cells with two different ZNF281-

Figure 7 Requirement of ZNF281 for the mesenchymal phenotype of SW480 cells. SW480 cells harbouring a pRTS vector encoding a miRNA directed against endogenous *ZNF281* (*ZNF281*-specific-miRNA; *ZNF281*-spec.-miRNA#1) or pRTS non-specific control miRNA (non-spec.-miRNA) were analysed. **(A)** qPCR analysis of *ZNF281* mRNA expression 96 h after addition of DOX. Results represent the mean $\pm$ s.d. ($n = 3$). **(B)** Western blot analysis of the indicated proteins 96 h after addition of DOX. β -Actin served as a loading control. **(C)** Representative phase-contrast pictures 96 h after addition of DOX. $\times 200$ magnification. Right-hand side: confocal microscopy to detect Vimentin protein by indirect immunofluorescence staining. Nuclear DNA was visualized by DAPI staining. $\times 200$ magnification. Scale bars represent 25 μ m. **(D)** Wound-healing assay: SW480/pRTS-*ZNF281*-spec.-miRNA#1 cells were treated with DOX or left untreated 72 h prior to scratching. Results represent the mean average (%) of wound closure determined by the final width of the scratch in three independent wells $\pm$ s.d. ($n = 3$). **(E)** Boyden-chamber assay. Left panel: migration after treatment with DOX or left untreated for 72 h, serum starvation for the last 48 h and migration through the Boyden-chamber filter for 48 h. Right panel: invasion through a Matrigel-coated filter for 48 h. Results represent the relative change of cells detected in five fields in the Boyden chamber with untreated cells set as one $\pm$ s.d. ($n = 3$). **(F)** Cells were subjected to a soft-agar assay and treated with DOX or left untreated during the experiment. Three weeks after seeding the resulting colonies were stained with crystal violet. Results represent the mean number of colonies in soft agar per well $\pm$ s.d. ($n = 3$). **(G)** Quantification of colonies-spheres formed. The results are provided as the mean number of spheres formed per 1000 cells seeded $\pm$ s.d. ($n = 3$). A Student's *t*-test was used in **(A, D–G)**. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Source data for this figure is available on the online supplementary information page.

specific siRNAs resulted in a pronounced downregulation of ZNF281 expression, which was accompanied by a decrease in Vimentin and SNAIL protein (Figure 9A), in line with SNAIL being a target gene of ZNF281 (Figure 5A). Subsequently,

these cells were injected into the tail vein of NOD/SCID mice. Within 4 weeks, mice injected with control siRNA-transfected cells gave rise to luminescence signals in the lung indicating metastases, whereas mice injected with cells transfected with

Figure 8 Requirement of ZNF281 for c-MYC-induced EMT. DLD-1/pRTR-c-MYC-VSV cells were analysed. (A) Cells were treated with DOX for the indicated periods and subjected to western blot analysis of the indicated proteins. (B) qPCR analyses of *ZNF281* mRNA expression at the indicated time points. Results represent mean values $\pm$ s.d. ($n = 3$). A Student's *t*-test was used. *: $P < 0.05$. (C) Cells were transfected with the indicated siRNAs for 72 h and DOX was added for the last 24 h. Subsequently, the indicated proteins were detected by western blot analysis. (D) Cells were transfected with two different *ZNF281*-specific (*ZNF281* #1 and *ZNF281* #2) siRNAs or the respective control (ctrl.) for 96 h. For the last 24 h, DOX was added. Subsequently, the indicated proteins were detected by western blot analysis. Relative densitometric quantifications are indicated. E-cad = E-cadherin. siRNA controls had no effect on these cells in the absence of DOX (Supplementary Figure S12). (E) Representative phase-contrast (P/C) images of cells analysed in (D). $\times 200$ magnification. Detection of E-cadherin by indirect immunofluorescence using confocal laser-scanning microscopy. Nuclear DNA was visualized by DAPI staining. $\times 200$ magnification. Scale bars represent 25 μ m. In (A, C and D), detection of β -Actin served as a loading control. Source data for this figure is available on the online supplementary information page.

ZNF281-specific siRNAs did not show luminescence signals until 7–8 weeks after injection (Figure 9B and C). Nine weeks after injection luminescent metastases were easily detectable in mice, which had received control siRNA-treated cells, whereas cells treated with *ZNF281*-specific siRNAs only rarely gave rise to small metastases as evidenced by weak luminescence signals. At this time point, lungs displayed macroscopically visible metastases in the control group, whereas lungs from mice injected with cells transfected with *ZNF281*-specific siRNAs were devoid of macroscopically visible metastases (Figure 9C). Haematoxylin and eosin (H&E) staining revealed the presence of metastases in the lungs of the control siRNA group, whereas the knock-down of *ZNF281* largely prevented the colonization of SW620 cells in the lung (Figure 9C and D). Histological examination of the lungs revealed a significant decrease in the total number of metastatic nodules upon inhibition of *ZNF281* (Figure 9D). In conclusion, *ZNF281* is therefore necessary for metastatic colonization of CRC cells in this *in vivo* model.

***ZNF281* is upregulated in human colon and breast cancer**

In order to evaluate whether the pro-metastatic functions of *ZNF281* are reflected in enhanced expression of *ZNF281* during progression of CRC and other carcinomas, we analysed the Oncomine database (Rhodes *et al*, 2004). In 11 out of 12 tumour entities *ZNF281* expression was found to be up-regulated in tumour versus normal tissue (Supplementary Figure S14A). In primary tumour samples of two colorectal and two breast cancer cohorts, cancer-specific upregulation of *ZNF281* was consistently found (Supplementary Figure S14B–E). In addition, an increased *ZNF281* expression in the primary tumour of CRC patients was associated with recurrence, and therefore presumably metastasis, 3 years

after removal of the primary tumour (Supplementary Figure 14F).

Discussion

Here, we could show that *ZNF281* is an integral part of the regulatory network that controls the transition between epithelial and mesenchymal states in CRC cells (see schematic model in Figure 9E). The results imply that *ZNF281* expression is induced by a coherent feed-forward loop consisting of SNAIL and miR-34a. Furthermore, *ZNF281* expression is sufficient to elicit an EMT and necessary to maintain a mesenchymal state in CRC cell lines. This effect was mediated by the direct activation of SNAIL and repression of epithelial marker genes and effectors.

As shown here, *ZNF281* is regulated in a coherent feed-forward loop, involving SNAIL, which directly binds to the *ZNF281* promoter and induces its transcription. Besides being mainly a transcriptional repressor, direct induction of target genes by SNAIL has been shown before (Guaita *et al*, 2002; De Craene *et al*, 2005; Vetter *et al*, 2010). The regulatory loop identified here involves miR-34a, which is repressed by SNAIL in a negative feedback loop (Kim *et al*, 2011; Siemens *et al*, 2011) and itself targets *ZNF281*. We recently demonstrated that increased SNAIL expression inversely correlates with *miR-34a* expression in a cohort of 94 colon cancer patients and was associated with liver metastasis (Siemens *et al*, 2013). Therefore, the increased expression of *ZNF281* in colorectal and other tumour types identified in public data sets may be caused by miR-34a down-regulation due to cancer-specific CpG methylation of *miR-34a* and/or p53 inactivation.

Ectopic *ZNF281* directly induced SNAIL transcription. However, this resulted in varying degrees of EMT effector

Figure 9 Requirement of ZNF281 for metastasis formation. (A) SW620 cells stably expressing a pLXSN-Luc2-tdTomato vector were transfected with the respective siRNAs. After 72 h, western blot analysis was performed to detect the indicated proteins. Detection of β-Actin served as a loading control. (B) Cells characterized in (A) were injected into the tail vein of 6- to 8-week-old immuno-compromised NOD/SCID mice and bioluminescence signals presented as 'total flux' per mouse were recorded at the indicated time points. Data are represented as mean $\pm$ s.d. (n = 5–6). (C) Left panel: representative images show bioluminescence signals 30 min and 3, 6 and 9 weeks after the intravenous injection of the siRNA-transfected SW620 cells. Middle panel: representative examples of the resected lungs per group. The arrow indicates the macroscopically visible metastatic tumour nodule in the control group. Right panel: representative example of the haematoxylin and eosin (H&E) staining of the resected lungs 9 weeks after tail vein injection of SW620 cells transfected with the respective siRNAs. Scale bars represent 200 μ m. (D) Quantification of detectable metastatic tumour nodules in the lung per mouse 9 weeks after the intravenous injection of SW620 cells transfected with the indicated siRNA. Data are represented as mean $\pm$ s.d. (n = 6). (E) ZNF281 as a regulator of EMT and stemness: schematic model integrating the results of this study with previous findings. Green rectangles represent factors that promote and red rectangles represent factors that inhibit EMT and stemness. In combination with increased cancer cell stemness, EMT promotes the formation of metastases. In (B) and (D) a Student's *t*-test was used with: **P < 0.01 and ***P < 0.001. Source data for this figure is available on the online supplementary information page.

regulations in the two different CRC cell lines DLD-1 and HT29. Nonetheless, ZNF281 expression consistently resulted in the loss of intercellular adhesions and enhanced migration and invasion in those two CRC cell lines. The subtle differences in the transcriptional response of different CRC cell lines to ZNF281 activation may be due to variations in related signalling pathways and therefore varying degrees of permissiveness for EMT or plasticity of the respective cells. We also provided evidence that ZNF281 is induced by SNAIL in other

types of carcinomas, such as pancreatic and breast carcinomas. Furthermore, miR-34a repressed ZNF281 in a pancreatic cancer cell line. Therefore, the regulations described here may also be important for the regulation of EMT and metastasis in other carcinomas besides CRC.

Our results demonstrate that ZNF281 represents a new EMT-promoting transcription factor. Notably, ZNF281 is structurally related to the zinc-finger containing transcription factors SNAIL, SLUG and ZEB1/2 (as indicated in

Supplementary Figure S1). The current literature suggests an extensive crosstalk between EMT-inducing transcription factors and miRNAs during the establishment and maintenance of the mesenchymal phenotype of cells (reviewed in Sanchez-Tillo *et al*, 2012 and De Craene and Berx, 2013). Our results further extend this network by adding reciprocal connections between SNAIL, ZNF281 and miR-34a.

We have previously shown that the oncoprotein c-MYC binds to ZNF281 (Koch *et al*, 2007). Here, we found that ectopic expression of c-MYC increased the ZNF281 expression. The absence of c-MYC binding sites in the promoter region of *ZNF281* implies the existence of alternative regulatory mechanisms. For example, c-MYC has been shown to directly downregulate *miR-34a* in B-cell lymphoma (Chang *et al*, 2008). Furthermore, c-MYC-induced SNAIL could repress *miR-34a*, which would contribute to increased ZNF281 expression. Alternatively, the association of c-MYC protein with ZNF281 may inhibit the turnover of ZNF281. Our results further imply that ZNF281 represents a necessary mediator of SNAIL- and c-MYC-induced EMT. Therefore, ZNF281 might be an important mediator of tumour progression in tumour entities with deregulation of c-MYC. It has been shown before that the EMT process yields cells with properties of stem cells (Polyak and Weinberg, 2009; Valastyan and Weinberg, 2011). For example, activation of the EMT inducers SNAIL and ZEB1 promotes the formation of tumour initiating cells with stem-cell properties (Mani *et al*, 2008; Wellner *et al*, 2009; Dang *et al*, 2011; Hwang *et al*, 2011). The ZNF281-induced sphere formation may also involve the previously reported links between ZNF281 and the stemness regulating transcription factors Nanog, Oct4 and Sox2 (Wang *et al*, 2006, 2008). Recently, it was shown that *miR-34a* and its target Notch1 form a bimodal switch which determines whether CRC stem cells (CCSCs) divide symmetrically or asymmetrically and thereby give rise to differentiated cells (Bu *et al*, 2013). The latter cells showed increased *miR-34a* and decreased Notch1 expression, whereas CCSC displayed the reverse expression patterns. It is conceivable that ZNF281 as another *miR-34a* target related to stemness may also contribute to fate determination by *miR-34a*. The enhancement of β -catenin activity by ZNF281 may contribute to ZNF281-induced stemness, since WNT signalling is necessary to maintain stem cells in the intestinal crypts (Pinto and Clevers, 2005; Brabletz *et al*, 2005b; Scoville *et al*, 2008). Interestingly, ZNF281 directly binds to the β -catenin promoter in human multipotent stem cells (hMSCs) (Seo *et al*, 2013). In addition, we found that ZNF281 directly represses *Axin2*, a negative regulator of the WNT pathway (Lustig *et al*, 2002). Thereby, ZNF281 presumably interrupts the negative feedback regulation of β -catenin by *Axin2* and allows β -catenin to accumulate in the nucleus and activate target genes. However, ZNF281 may also promote β -catenin/TCF4 activity by mediating the loss of E-cadherin expression, which is known to inhibit nuclear localization and activity of β -catenin by recruiting it to the cell membrane (Sadot *et al*, 1998; Orsulic *et al*, 1999). Furthermore, our results are consistent with the reported correlation of ZNF281 and β -catenin expression in hMSCs (Seo *et al*, 2013). We found that ZNF281 induces expression of the prognostic stem-cell markers CD133 and LGR5. Colon

tumours were shown to contain a subpopulation of CD133-positive cells with the ability to initiate tumour growth (Horst *et al*, 2008). Furthermore, high CD133 expression was associated with poor survival of CRC patients. Moreover, the combination of CD133 and the nuclear localization of β -catenin identified cases of low-stage CRC with a high risk for tumour progression (Horst *et al*, 2009). Since downregulation of ZNF281 prevented the formation of lung metastasis of a CRC cell line in a xenograft mouse model, it seems likely that enhancement of EMT and/or stemness by ZNF281 are important functions of ZNF281 during CRC progression. We also observed inhibition of metastasis formation after experimental downregulation of SNAIL in a similar assay (Jackstadt *et al*, 2013). Therefore, the effect of ZNF281 downregulation might be mediated, at least in part, by decreased expression of SNAIL and the concomitant loss of mesenchymal properties. Furthermore, the downregulation of ZNF281 by p53 via *miR-34a* indicates that limiting ZNF281 function is critical for tumour suppression by p53. The increased expression of *ZNF281* mRNA in primary colorectal and breast carcinomas also points towards cancer promoting effects of ZNF281. Furthermore, enhanced ZNF281 expression correlated with recurrence 3 years after removal of the primary colorectal tumour, suggesting that detection of elevated ZNF281 expression in primary tumours may have a prognostic value.

Materials and methods

Cell culture

The cell lines HCT-15, HEK293T, HT29, MiaPaCa2, SKBR3, SW480 and SW620, as well as human diploid fibroblasts (HDFs) were kept in DMEM. DLD-1, HCT116 p53^{-/-} and HCT116 p53^{+/+} cells were cultured in McCoy's medium and Colo320 in RPMI medium. Media were supplemented with 10% FCS (Invitrogen) and 1% Penicillin/Streptavidin (Invitrogen) and cells were kept at 5% CO₂ and 37 °C. For Hek293T cells, 5% FCS was used. All oligonucleotides (Ambion—Applied Biosystems) were transfected using HiPerfect (Qiagen).

Wound-healing assay

DLD-1 and SW480 cells harbouring a DOX-inducible *ZNF281* allele or respective miRNAs were cultured for the indicated periods in the presence of DOX (100 ng/ml for pRTR vectors and 1 μ g/ml for pRTS vector systems) or left untreated before applying the wound. Mitomycin C (10 ng/ml) was applied 2 h before scratching using a Culture-Insert (IBIDI). To remove Mitomycin C and detached cells, cells were washed twice with HBSS and medium containing DOX (100 ng/ml or 1 μ g/ml) was added where indicated. Cells were allowed to close the wound for the indicated periods and images were captured on an Axiovert Observer Z.1 microscope connected to an AxioCam MRm camera using the Axiovision software (Zeiss) at the respective time points.

Migration and invasion analysis in Boyden chambers

DLD-1 and SW480 cells harbouring DOX-inducible pRTR or pRTS vectors were cultured for the indicated periods in the presence or absence of DOX (pRTR: 100 ng/ml; pRTS: 1 μ g/ml). Cells were deprived of serum (0.1%) for 48 h before the analysis. To analyse migration, 5 $\times$ 10⁴ cells were seeded in the upper chamber (8.0 μ m pore size; Corning) in serum-free medium. To analyse invasion, membranes were coated with Matrigel (BD Bioscience) at a dilution of 3.3 mg/ml in medium without serum. After coating 7 $\times$ 10⁴ cells were seeded on Matrigel in the upper chamber. As a chemo-attractant, 10% FCS was used in the lower chamber. Cultures were maintained for 48 h, then non-motile cells at the top of the filter were removed and the cells in the lower chamber were fixed with methanol and stained with DAPI. Either the number of cells

per well or five different fields per condition were counted by microscopy. Relative invasion/migration was calculated in relation to the control.

Sphere formation assay

Cells were separated by treatment with trypsin after addition of DOX for 48 h. For each triplicate, 1×10^5 cells were seeded into a well of a 6-well plate coated with attachment preventing poly(2-hydroxyethyl-metacrylate) (PolyHEMA, Sigma) in 5 ml sphere medium (Yu *et al*, 2007). After 7 days, the resulting spheres were documented by phase-contrast microscopy at $\times 100$ magnification and spheres were dissociated into single cells using a 0.05% trypsin-EDTA solution. For quantification, 1×10^4 cells/well were seeded in Yu medium containing 1% methyl cellulose (Sigma) into PolyHEMA coated 96-well plates in the presence or absence of DOX ($n = 6$). The number of colonies larger than 50 μm in diameter were counted after 7 days.

In vivo lung metastasis assay

72 h after siRNA transfection 4×10^6 SW620-Luc2 cells were resuspended in PBS in a total volume of 0.2 ml and injected into the lateral tail vein of a 6- to 8-week-old age-matched male NOD/SCID mouse using a 25-gauge needle. For monitoring of the injected cells, mice were injected intraperitoneally with D-luciferin (150 mg/kg) and imaged under anaesthesia with the IVIS Illumina System (Caliper Life Sciences) 30 min after tail vein injection in order to have a reference point. The acquisition time was set to 2 min and imaging was repeated once a week to monitor the seeding and outgrowth of the cells. After 9 weeks complete lungs were resected and photographed. For H&E stainings, lungs were fixed with 4% paraformaldehyde and 5 μm sections were stained with hematoxylin and eosin. The number of metastases was determined microscopically. Mice were kept under IVC conditions and experiments were performed with permission of the Bavarian state (file number: 55.2-1-54-2532.8-188-11).

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Statistical analysis

Unless noted otherwise, each experiment was carried out in triplicates. A Student's *t*-test (unpaired, two-tailed) was used for calculation of significant differences between two groups of samples, with $P < 0.05$ considered as significant. Asterisks generally indicate: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. For correlation analyses, the SPSS software package 19 (SPSS Inc.) was used. Spearman rank correlation test was applied to the NCI-60 data in order to correlate mRNA expression with the expression of other mRNAs.

Additional detailed Materials and methods are available online. Oligonucleotides used for ChIP analysis and for cloning and mutagenesis are listed in Supplementary Tables S2 and S3, respectively. Expression plasmids are listed in Supplementary Table S4, primers used for qPCR in Supplementary Table S5, and antibodies used for IF, ChIP and WB analysis are listed in Supplementary Table S6.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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