Reactive oxygen species in health and disease
van der Wijst, Monique

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Prolonged re-expression of the hyper-methylated gene \textit{EPB41L3} using Artificial Transcription Factors and epigenetic drugs

Christian Huisman\textsuperscript{1}, Monique G.P. van der Wijst\textsuperscript{1}, Fahimeh Falahi\textsuperscript{1}, Juul Overkamp\textsuperscript{1}, Gellert Karsten\textsuperscript{1}, Martijn M. Terpstra\textsuperscript{3}, Klaas Kok\textsuperscript{3}, Ate G.J. van der Zee\textsuperscript{2}, Ed Schuuring\textsuperscript{1}, G. Bea A. Wisman\textsuperscript{2*} and Marianne G. Rots\textsuperscript{1*}

\textit{Departments of Pathology and Medical Biology}\textsuperscript{1}, \textit{Gynecological Oncology}\textsuperscript{2} and \textit{Genetics}\textsuperscript{3}, University of Groningen, University Medical Center Groningen (UMCG), Hanzeplein 1 9713 GZ Groningen, the Netherlands.

*Both authors contributed equally

Chapter 11

Abstract

Epigenetic silencing of tumor suppressor genes (TSGs) is considered a significant event in the progression of cancer. For example EPB41L3, a potential biomarker in cervical cancer, is often silenced by cancer-specific promoter methylation. Artificial Transcription Factors (ATFs) are unique tools to re-express such silenced TSGs to functional levels; however, the induced effects are considered transient. Here, we aimed to improve the efficiency and sustainability of gene re-expression using engineered zinc fingers fused to VP64 (ZF-ATFs) or DNA methylation modifiers (ZF-Tet2 or ZF-TDG) and/or by co-treatment with epigenetic drugs (5-aza-dC2'-deoxycytidine or Trichostatin A (TSA)). EPB41L3-ZF effectively bound its methylated endogenous locus, as also confirmed by ChIP-seq. ZF-ATFs reactivated the epigenetically silenced target gene EPB41L3 (~10 fold) in breast-, ovarian- and cervical cancer cell lines. Prolonged high levels of EPB41L3 (~150 fold) induction could be achieved by short-term co-treatment with epigenetic drugs. Interestingly, for otherwise ineffective ZF-Tet2 or ZF-TDG treatments, TSA facilitated re-expression of EPB41L3 by up to twofold. ATF-mediated re-expression demonstrated a tumor suppressive role for EPB41L3 in cervical cancer cell lines. In conclusion, epigenetic reprogramming provides a novel way to improve sustainability of re-expression of epigenetically silenced promoters.
**Introduction**

It has become increasingly clear that carcinogenesis is associated with epigenetic alterations, such as DNA hypermethylation of tumor suppressor genes (TSGs), causing aberrant gene expression (1, 2). Re-expression of silenced TSGs has emerged as potent anti-cancer tool (3) and epigenetic drugs are successfully exploited to reverse TSG silencing (4). However, these drugs act genome-wide and the disadvantages of these drugs include their lack of gene-specificity and low efficacy in solid tumors (5). A promising alternative is the gene-targeted re-activation of silenced genes using engineered DNA binding domains, including zinc finger proteins (ZFPs) and transcription activator–like effectors (TALEs), fused to gene activators (6). Such fusion proteins strongly exploit the reversibility of epigenetic silencing which, in contrast to genetic mutations, allow for functional re-expression of the target gene. Nevertheless, to induce long-lasting changes of cell fate in disease, sustained expressional changes are required. Artificial transcription factor (ATF)-mediated expressional changes however, have shown to be only transient, as targeted promoters are incompletely reprogrammed and genes tend to return to the ‘normal’ state after the ATF is removed (7, 8).

Recently, a number of TSGs were successfully reactivated using ZFPs linked to VP64, including maspin (7, 9, 10), CDKN2A (11) and C13ORF18 (12), and re-activation of these three TSGs was associated with site-specific DNA demethylation, while re-expression of the latter two was also shown to be accompanied with a decreased repressive histone methylation status. Despite these epigenetic changes, ATF-induced effects are transient, and more stable epigenetic reprogramming is required to prolong the effects (13). A possible strategy to achieve this is the co-treatment of ATFs with epigenetic drugs, such as the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) or the histone deacetylase inhibitor (HDACi) Trichostatin A (TSA). Although epigenetic drugs are often reported as transient actors, they have the potency to improve reprogramming in a number of applications. For example, 5-aza-dC and TSA have previously shown to improve epigenetic reprogramming of somatic cell nuclei (14), and also the reprogramming of somatic cells to become induced pluripotent stem cells (15) is increased in the presence of epigenetic drugs (16). Furthermore, TSA synergistically increases 5-aza-dC induced re-expression (17) and promoter demethylation (18), and has shown to increase the sustainability of 5-aza-dC induced re-expression (19). Moreover, epigenetic drugs facilitated ATF-mediated activation of epigenetic repressed genes (12, 20, 21). Such an approach of
combined treatment may thus prolong the transient effects of ATFs on gene expression.

An alternative strategy to induce long-lasting transcriptional changes may be through Epigenetic Editing (22), for example by targeting DNA demethylases to hypermethylated promoter regions, which can remove DNA methylation at the target site. Targeting members of the ten-eleven translocation (TET) protein family to inactive promoters indeed resulted in gene re-expression (23, 24). Also thymidine DNA glycosylase (TDG), an enzyme involved in the DNA repair pathway, induced similar effects upon targeting (25). Although the reported DNA demethylases re-activated gene expression, the observed effects are sometimes limited and efficiency as well as sustainability of re-expression can likely be increased. Methylation levels are quickly recovered by the presence of methyltransferases and other local factors enriched at methylated CpGs sites, resulting in remethylation and subsequent gene re-silencing (23). TSA, however, is also known to decrease the activity of DNA methyltransferase (26), and may thus inhibit the remethylation processes. Therefore, the effects of targeting DNA demethylases may be further improved in the presence of TSA.

An important therapeutic target for epigenetic re-expression in cancer is \textit{EPB41L3}. This gene is part of the 4.1 family of proteins, which actions are implicated in cell adhesion, cell motility and cell growth (27-29). In many cancer types, \textit{EPB41L3} is found to be frequently methylated, including breast (30), ovarian (31) and lung cancer (32). As a potential marker for the early detection of (pre)malignant cervical cancer, we previously found that 68% of cervical scrapings of CIN3 are methylated for \textit{EPB41L3} versus 14% of normal cervices (33). This finding was confirmed by others who showed \textit{EPB41L3} as the best marker for detecting CIN2/3 (34). Such promoter methylation patterns indicate that \textit{EPB41L3} is silenced in cervical cancer. A functional consequence of EPB41L3 downregulation in cancer is a disruption of the organization in the cytoskeleton, leading to increased metastasis and invasion of cancer cells. In addition, EPB41L3 overexpression has been linked to strong tumor growth suppression in various cancer types (28, 31, 35, 36), in part through the induction of apoptosis. These properties make \textit{EPB41L3} an attractive therapeutic target for re-expression in cancer therapy.

In this study, we aimed to induce sustainable re-expression of \textit{EPB41L3} in cancer. For gene-specific re-expression of \textit{EPB41L3}, we engineered two ATFs (ZFPs +VP64) and confirmed their ability to re-express \textit{EPB41L3} in breast, ovarian and cervical cancer cell lines, displaying various degrees of \textit{EPB41L3} methylation. In
addition, we analyzed the genome-wide binding of an *EPB41L3*-targeting ZFP using ChIP-Seq. Furthermore, we tested whether the sustainability of *EPB41L3* re-expression by the ATFs could be enhanced by gene-targeting of DNA methylation modifiers (Tet2, TDG) (Epigenetic Editing) and/or by adding epigenetic drugs. Finally, we studied the functional effects of *EPB41L3* re-expression in cervical cancer cell lines, since a tumor suppressive role of this gene has not been studied in this malignancy.

**Results**

*EPB41L3* mRNA expression and epigenetic status

The promoter region for the two main splice variants of *EPB41L3* is shown in Fig. 1A. The CpG island at the 3′-side of the main transcription start site (TSS) suggests that *EPB41L3* expression might be controlled by DNA methylation. We analyzed the mRNA expression of *EPB41L3* in breast (MDA-MB-231, SKBR3), ovarian (SKOV3, A2780) and cervical (HeLa, CaSki, C33A, SiHa and CC-11) cancer cell lines (Fig. 1B): *EPB41L3* was silenced in most cell lines, except for SKBR3, C33A and SiHa cells (mRNA relative to GAPDH: 3.8x10⁻⁵ (SKBR3), 1.5x10⁻⁴ (C33A) and 7.6x10⁻⁴ (SiHa)). Examination of the DNA methylation status (region 2, Fig. 1A) showed that *EPB41L3* silencing was associated with extensive promoter hypermethylation in SKOV3 (75±9%), HeLa (88±1%) and CaSki (90±1%), but less in MDA-MB-231 (28±1%) and A2780 (58±14%) (Fig. 1C). The cell lines expressing *EPB41L3* showed lower degrees of promoter methylation (SKBR3 (6±3%) and C33A (19±5%)).

The cell line with the highest expression (SiHa) was highly methylated (96±4%). Sequencing of the *EPB41L3* mRNA of SiHa confirmed expression from the main TSS (isoform 3) (Fig. 1A), and no genetic mutations were observed. Expression of *EPB41L3* mRNA in SiHa was confirmed on protein level (Fig. 1B insert). Sequencing of another region in closer proximity to the TSS (region 1) also showed high DNA methylation levels for SiHa (~89%, Suppl. Fig. 1). Other cell lines showed comparable methylation levels for *EPB41L3* at this more upstream part of the promoter, except for SKOV3 (lower percentage of CpG methylation in this region). To quantify the reduction in methylation levels on single CpGs, we performed pyrosequencing for 15 CpGs in the promoter regions of *EPB41L3* (Fig. 1A, 1D, Suppl. Fig. 2). The methylation patterns showed high DNA methylation levels for the cervical cancer cell lines (except C33A), and intermediate or low levels of DNA methylation for the ovarian and breast cancer cell lines.
Chapter 11

A

EPB41L3 isoform 1

EPB41L3 isoform 3

bs seq. region 1

bs seq. region 2

ChIP region

pyrosequencing region

B

EPB41L3 mRNA
(relative to GAPDH)

CD

D

C33A: low DNA methylation

SKOV3: intermediate DNA methylation

CaSki: high DNA methylation

E

% of input

F

Fold induction of EPB41L3


is RNA expression


d_{aza-dC}

TSA
**Sustained re-expression of EPB41L3 by ATFs and epigenetic drugs**

**Figure 1. Epigenetic regulation of EPB41L3 (see previous page).** (A) Schematic representation of the EPB41L3 gene (two splice variants) and the EPB41L3 promoter, spanning bp -100 to +700. Shown are the TSS, CpGs (indicated as vertical bars), target sites ZFPs (21ab, 22ab) and bisulfite/ChIP regions. (B) EPB41L3 mRNA expression in a panel of breast- (MDA-MB-231, SKBR3), ovarian- (SKOV3, A2780) and cervical (HeLa, CaSki, C33A, SiHa and CC-11) cancer cell lines. Insert shows the protein expression of an expressing (SiHa) and non-expressing cell line (HeLa) visualized by western blotting using an EPB41L3 specific antibody (4.1B (sc-25965) Santa Cruz). (C) DNA methylation status of the EPB41L3 promoter for eight cell lines from B. Three clones per cell line were analyzed by bisulfite sequencing and each third of a circle represents a clone. Also shown are the predicted binding sites of EPB41L3 targeting ATFs (21ab-VP64 (△), 22ab-VP64(▼)) and the TSS. (D) The DNA methylation status of 15 CpGs in the EPB41L3 promoter of hypomethylated (C33A), intermediately methylated (SKOV3) and high methylated (CaSki) cells as quantified by pyrosequencing. Values represent the mean of at least two independent experiments ± SEM. (The other cell lines from C are shown in supplemental figure 2) (E) Quantitative ChIP for histone modifications associated with the gene promoter of EPB41L3 (n=3 or more, (H3K9Ac n=2)). (F) mRNA expression of EPB41L3 after treatment with epigenetic drugs (5-aza-dC (500 nM (+)), 5 mM (++)(three days) and/or TSA (400 nM) (one day)) relative to untreated cells (set as 1). Statistical significance was determined using a student’s t-test (*p<0.05).

Analysis of the histone marks in the hypermethylated cervical cancer cell lines HeLa, CaSki and SiHa revealed a close association of DNA methylation with the repressive histone mark H3K9Me3 (Fig. 1E). Activating histone marks (H3Ac, H4Ac, H3K4me3 and H3K9Ac) were only weakly, and to a similar degree in all three cell lines, associated with the hypermethylated EPB41L3 promoter. Epigenetic drugs (5-aza-dC + TSA) increased re-expression efficacy for EPB41L3: ~83 fold in HeLa, ~32 fold in CaSki and ~58 fold in C33A. No further increase of EPB41L3 expression in SiHa was observed (Fig. 1F). Based on these findings, EPB41L3 seems a suitable target for gene-targeted interventions by ATFs.

**Targeted re-expression of EPB41L3**

Next, we investigated whether EPB41L3 induction from the endogenous locus can be achieved by engineering two ATFs (21ab-VP64 and 22ab-VP64) targeted at the EPB41L3 promoter (see Fig. 1A, 2A for the target sites). Efficient expression of EPB41L3-targeting ATFs was demonstrated by qRT-PCR and FACS (Suppl. Fig. 3). EPB41L3 could be significantly upregulated (Fig. 2B-D) by 21ab-VP64 compared to the effects of an empty vector (pMX) in MDA-MB-231 (11±2.7 fold), SKBR3 (3.6±0.6 fold), A2780 (8.5±2.5 fold), SKOV3 (13±3.3 fold), HeLa (14±4.3 fold), CaSki (13±3.1) (all p<0.01) and in C33A (26±8.0 fold: p<0.05). Also 22ab-VP64 significantly increased EPB41L3 expression in SKBR3 (11±1.4 fold (p<0.01)), CaSki (7.2±2.4 fold (p<0.05)) and C33A (6.5±1.1 fold (p<0.01)). In CC-11 cells, which are
more primary cervical cancer cells (37), \textit{EPB41L3} was not significantly upregulated by either ATF. Nevertheless, expressing both ATFs simultaneously resulted in significant gene-induction of \~32 fold \((p<0.05)\) (Fig. 2E). The overexpression of \textit{EPB41L3} cDNA in CC-11 cells yielded levels of \~8.1x10^3 fold \((p<0.05)\) compared to the pMX empty vector (Fig. 2E). In Caski cells, EPB41L3 re-expression was also confirmed on the protein level (Fig. 2D insert). Controls, consisting of zinc fingers lacking an effector domain (NoEf) showed comparable \textit{EPB41L3} expression as the empty vector control. C33A cells were transduced to express two irrelevant ATFs, which did not influence gene expression of \textit{EPB41L3} (data not shown).

**Targeted reversal of histone marks by \textit{EPB41L3}-ATFs, but moderate binding specificity**

Previously it was shown that ATFs indirectly affect local epigenetic features, such as histone modifications (11, 12). Therefore, we also analyzed if the \textit{EPB41L3}-targeting ATFs changed the histone marks after ATF-mediated re-expression. First, we confirmed successful association of the engineered ZFP 21ab with its target site in the \textit{EPB41L3} promoter. Indeed, an enrichment of 10% of input DNA was obtained for 21ab expressing CaSki cells (0.06% for pMX) using ChIP (Fig. 3A). Genome-wide sequencing revealed an enrichment of DNA fragments at the targeted site (area of 20 kb analyzed (Fig. 3B)), while empty vector had little or no reads at this location. Abundant off-targets were identified, but most of them with low coverage per peak (Fig. 3C). The analysis for all peaks with a coverage of 10 or more reads per peak showed that ZFP 21ab did not only bind to the \textit{EPB41L3} promoter, but also to other promoter regions (empty vector: 0.43% of all reads mapped to promoter sides, 21ab-NoEf: 7.73%) (Fig. 3D). The number of identified promoter binding sites was 1397 for the \textit{EPB41L3}-ZFP, while the number of background binding sites for virus only treated cells (pMX empty) was 204 (Fig. 3E).

To determine whether \textit{EPB41L3} re-expression by ATFs influenced the histone marks at the ATF target site, H3Ac and H3K9me3 levels were assessed both in methylated (CaSki) or unmethylated (C33A) cells (Fig. 3F) after treatment with a control (21ab-NoEf) and 21ab-VP64. In control cells, CaSki cells showed more association of the repressive H3K9me3 mark with the \textit{EPB41L3} promoter than C33A (CaSki 21ab-NoEf 2.4±0.2% of input, C33A 21ab-NoEf 0.5±0.1% of input \((p<0.01)\)), which is consistent with the more silenced/methylated state of \textit{EPB41L3} in CaSki. \textit{EPB41L3} re-expression by 21ab-VP64 significantly decreased the repressive mark H3K9me3 by 2.3±0.5 fold \((p<0.05)\) in CaSki cells and 3.4±0.6 fold
Sustained re-expression of \textit{EPB41L3} by ATFs and epigenetic drugs

(\(p=0.056\)) in C33A cells. Interestingly, increased \textit{EPB41L3} expression in unmethylated C33A cells was associated with a strong increase in the H3Ac mark (23±2.5 fold (\(p<0.01\))), while enrichment of H3Ac in the methylated CaSki cells could not be detected upon \textit{EPB41L3} re-expression.
Figure 2. Endogenous re-expression of EPB41L3 by ATFs (see previous page). (A) Graphical representation of the EPB41L3-targeting ZFPs. Shown are the 18-bp target sequence and the orientation of the VP64 transcriptional activator. EPB41L3 mRNA induction in breast- (B), ovarian- (C) and cervical (D, E) cancer cell lines by ATF 21ab-VP64 and/or 22ab-VP64 targeted at the EPB41L3 promoter. Quantification of mRNA was performed using qRT-PCR and induction levels were normalized to pMX empty vector (set as 1). ZFP without effector domains (NoEf) were used as extra controls. Each bar represents the mean of at least three independent ± SEM. Statistical significance was determined using a student’s t-test (*p<0.05 and **p<0.01). The insert in D show EPB41L3 re-expression in CaSki cells after expressing 21ab-VP64 or EPB41L3 cDNA as visualized by Western blotting. Spice variant 1 is estimated to be 120 kDa, while the overexpressed splice variant cloned from SiHa cells is estimated to be 100 kDa.
Figure 3. Targeted reversal of histone marks by EPB41L3-ATFs, but moderate binding specificity (see previous page). Association of 21ab-NoEf with its targeted site as analyzed by an HA tag ChIP (A) and ChIP-Seq (B) for the 21ab-ZFP and an empty vector in EPB41L3 methylated CaSki cells. For the ChIP-Seq, the local coverage is shown for a region of 20 kbp as visualized by R using the coverage distribution report obtained from NextGENe. Indicated is the ZFP target site (▲). (C) Representation of the number of identified ChIP-Seq peaks versus the average coverage per peak (shown for peaks with a coverage of four or more (maximum 100)) determined by the Peak Identification Report obtained from NextGENe. (D) Percentage of ChIP-Seq peaks (with a coverage of ten or more) bound to promoters, genes or other regions (such as noncoding DNA). (E) Graphical representation of peaks with a coverage of ten or more bound to promoter regions. (F) Change in histone marks after re-expression of EPB41L3 in methylated CaSki (left) and unmethylated C33A cells (right). A quantitative ChIP for H3Ac and H3K9me3 was performed after treatment with 21ab-VP64 and/or 21ab-NoEf. Values represent the mean percentage of input of three independent experiments ± SEM, (HA tag one experiment).

Sustained re-expression of EPB41L3.

To study sustainability of gene re-expression, we constructed 21ab-VP64 DOX-inducible cell lines (HeLa, SKOV3) and analyzed expression of 21ab-VP64 and EPB41L3 over time, also after removal of DOX. DOX treatment for two days resulted in ATF expression (Fig. 4A), which in turn, upregulated EPB41L3, with highest level reached one day after removal of DOX (HeLa 86±19 fold (Fig. 4A), SKOV3 6.5±1.5 fold (Fig. 4B)). Then, EPB41L3 levels decayed over time, with a delay of 24 hours relative to DOX-induced 21ab-VP64 expression levels (as shown in HeLa cells).

To explore possibilities to circumvent instable reactivation of the targeted gene, DOX induction was combined with epigenetic drug treatment (5-aza-dC or TSA) in SKOV3 21ab-VP64 stable transfectants (Fig. 4B). In the clinical setting, such “hit-and-run” approaches in which short-term treatment is sufficient to induce sustained effects would be preferred above a long-term drug treatment. Here, we could show that the combination of ATFs and suboptimal doses of epigenetic drugs acts synergistically, as also demonstrated by others (20). Interestingly, the improved effects were maintained over time and the reactivation of the targeted gene could be prolonged; Co-treatment strongly increased EPB41L3 re-expression compared to single treatment for 5-aza-dC (at day 2: 5.6±1.4 fold (p<0.05) and TSA (day 2: 2.9±0.8 fold (p=0.064)). Moreover, co-treatment of ATFs with 5-aza-dC or TSA resulted in a sustained increase of EPB41L3 expression levels after removal of DOX (day 5: 5-aza-dC 4.5±1.0 fold (p<0.05), TSA 7.0±1.7 fold (p<0.05)). Such
sustained re-expression was also observed for the 5-aza-dC only treated cells (although less prominent), but was not observed for this dose of TSA only.

![Graphs A and B]

**Figure 4. Kinetics of re-expression of EPB41L3 using ATFs and epigenetic drugs.** Re-expression of EPB41L3 (and 21ab-VP64) mRNA in HeLa (A) and SKOV3 (B) cells stably transduced with 21ab-VP64 after treatment with DOX. Expression of the ATF was confirmed on protein level after DOX treatment in HeLa cells as visualized with Western blotting (insert A). For SKOV3, DOX-treatment was also administered in the presence of 5-aza-dC (5 µM) or TSA (400 nM) (see treatment schedule at the bottom). Expression of EPB41L3 mRNA was measured during a period of five days. Quantification of mRNA was performed using qRT-PCR and induction levels were normalized to untreated cells. Each data point represents the mean of at least three independent experiments measured in triplicate ± SEM. Statistical differences (B) were determined between single treatment (5-aza-dC, TSA) and co-treatment with DOX (5-aza-dC + DOX, TSA + DOX) using a student’s t-test (*p<0.05 and **p<0.01).

**Targeting demethylating enzymes in the presence of HDACi TSA**

Next, we fused the catalytic domain of Tet2 or TDG to the EPB41L3-targeting ZFPs, delivered the construct into CaSki cells and measured the expression of EPB41L3 in the presence or absence of TSA. Without TSA, we failed to significantly re-activate the EPB41L3 promoter after expressing 21ab-Tet2, 22ab-Tet2, 21ab-TDG or 22ab-TDG (Suppl. Fig. 4). However, in the presence of TSA, EPB41L3 could be significantly re-activated (Fig. 5A). Co-treatment of 22ab-Tet2 with TSA reached the highest levels of EPB41L3 mRNA (pMX+TSA 1.0±0.1 fold vs 22ab-Tet2-CD+TSA 2.7±0.8 fold (p<0.05)). These re-expression levels were approximately similar to the EPB41L3 expression levels reached by the VP64-ATFs (without co-treatment (Fig. 2D)). To study if the TSA-induced effects were associated with downregulation of methyltransferases, DNMT3b levels were analyzed after expressing 21ab-Tet2, 22ab-Tet2, the combination of both or irrelevant ZFPs fused
to Tet2-CD (GFP expression of cells transduced to express Tet2 fusion proteins was approximately similar as for cells treated with empty vector (Fig. 5B) or VP64-ATFs expressing viruses (Suppl. Fig. 3D) as monitored by FACS). We found that TSA decreased DNMT3b expression (~32±7%, p=0.05) in control cells (Fig. 5C), which was consistent with a previous report (26). Interestingly, we also found that cells transduced to express the Tet-fusion proteins demonstrated a twofold increase in DNMT3b mRNA expression (p<0.05), while no increase in DNMT3b expression was observed in control cells expressing an empty vector (compared to untreated cells). Such increase of DNMT3b mRNA was diminished when Tet-expressing cells were co-treated with TSA.

Figure 5. Gene-induction of EPB41L3 with demethylating reagents in the presence of TSA. (A) EPB41L3 mRNA expression after treatment with pMX, 21ab-Tet2-CD, 22ab-Tet2-CD, 21ab-TDG-CD or 22ab-TDG-CD (or combined) in CaSkI cells in the presence of TSA (400 nM). (B) Percentage GFP positive cells after retroviral transduction of pMX, 21ab-Tet2-CD, 22ab-Tet2-CD or 21ab/22ab-Tet2-CD. (C) DNMT3b mRNA expression after retroviral transduction of with pMX, 21ab-Tet2-CD, 22ab-Tet2-CD, 21ab/22ab-Tet2-CD or expression of control ZFPs carrying the Tet-CD. Quantification of mRNA was performed using qRT-PCR and induction levels were normalized to an empty vector. Each bar represents the mean of at least three independent ± SEM. Statistical significance was determined using a one-tailed t-test. A p-value of 0.05 or less was considered statistical significant.
EPB41L3 as tumor suppressor gene

Previously, EPB41L3 was identified as a TSG in, among others, breast and ovarian cancer, but in cervical cancer the role of EPB41L3 is currently unknown. To study if ATF-mediated re-activation of EPB41L3 affects cell growth in the EPB41L3 methylated cervical cancer cells, a five day MTT assay was performed for HeLa (Fig. 6A) and CaSki cells (Suppl. Fig. 5). The highest re-expressor of EPB41L3, 21ab-VP64, significantly decreased cell growth compared to controls at day five (pMX 100±15%, 21ab-NoEf +125±16%, 21ab-VP64 -41±10% (p<0.05), 22ab-VP64 -50±16% (ns)). For CaSki, growth was even further decreased at day five (~80%) compared to pMX (Suppl. Fig. 5).

Next, we studied if the ATF-mediated decrease in growth upon re-expression of EPB41L3 in methylated/silenced cell lines could be partially explained by the induction of apoptosis (Fig. 6B), as demonstrated by others for breast and ovarian cancer cells using EPB41L3 cDNA (31, 36). Indeed, apoptosis was induced by 21ab-VP64, the highest re-expressor of EPB41L3, in the methylated breast and ovarian cancer cells (MDA-MB-231 19±5%, SKOV3 11±3%, A2780 66±18%) compared to pMX (p<0.05). Interestingly, also for the methylated cervical cancer cells, significant apoptosis was induced by 21ab-VP64 (HeLa 13±0.1% (p<0.01); CaSki 21±2% (p<0.05)). ZFPs with no effector domain had similar background levels of apoptosis compared to pMX. The EPB41L3-targeting ATFs also strongly decreased the colony forming potential of the cells compared to NoEf as shown for HeLa cells (Fig. 6). For the more primary CC-11 cells, the re-activation of EPB41L3 by combining 21ab-VP64 and 22ab-VP64 resulted in significant cell death compared to pMX (Fig. 6D). The induced level of cell death was similar to a control condition were EPB41L3 cDNA (isoform 3) was overexpressed (pMX ~13%, ATFs ~31%, cDNA ~29%). As EPB41L3 is known to affect cell adhesion, we analyzed whether targeted re-expression of EPB41L3 affected cell adhesion; indeed, compared to pMX empty, a significant increase in adhesion was observed for 21ab-VP64 and 22ab-VP64, as shown in Fig. 6E.

To gain better understanding of how EPB41L3-targeting ATFs induce the effects on cell growth in cervical cancer, we analyzed the mRNA expression of the cell cycle regulators cyclin-dependent kinase inhibitor 1 (CDKN1A), cyclin-dependent kinase inhibitor 2A (CDKN2A) and tumor protein 53 (TP53) in CaSki cells (in which 21ab-VP64 induced the most pronounced apoptotic and growth effects) (Fig. 6F). All three proteins are deregulated in most cancers and play critical roles in cell cycle progression (38, 39). However, in cervical cancer, p53 is degraded by the human papillomavirus (HPV) E6 protein, and considered
Sustained re-expression of EPB41L3 by ATFs and epigenetic drugs functionally inactive (39). We observed that p21 expression levels were further upregulated by 21ab-VP64 compared to pMX (5.1±1.0 fold (p<0.05)), while CDKN2A levels were down-regulated (0.28±0.14 fold (p<0.05)). P53 expression levels were not changed, as could be expected based on its functional inactive states in cervical cancer. These results suggest that EPB41L3 is related with cell cycle regulators (CDKN2A, p21), which could be associated with the less malignant phenotype of the cells.
Figure 6. EPB41L3 decreases cell growth and induces apoptosis (see previous page). (A) Relative cell proliferation was measured with a MTT assay in HeLa cells after transduction with the EPB41L3-targeting constructs. Each data point represents the mean of five independent experiments ± SEM. (B) Percentage of apoptotic cells in methylated MDA-MB-231, A2780, SKOV3, HeLa and CaSkI after transduction with the 21ab-VP64 and controls (pMX and 21ab-NoEf) measured by a DiIC staining. All bars represent the mean of three independent experiments ± SEM. (C) Visualization of the number of colonies after transduction of the EPB41L3-inducing ATFs and controls in HeLa cells. Picture shows a representative of a triplicate measurement. (D) Percentage of cell death after treatment with EPB41L3 cDNA, 21ab-VP64 + 22ab-VP64 and pMX. (E) Relative cell adhesion of CaSkI cells after treatment with EPB41L3-inducing ATFs and a control (F) mRNA expression of the cell cycle regulating genes CDKN2A, p21 and p53 also after transduction of pMX and 21ab-VP64 in CaSkI cells. Quantification, statistics and representation is similar as in Fig.2.

Discussion
Here we describe an effective approach to increase sustainability of gene-targeted re-expression by combining effects of ATFs or epigenetic editors with epigenetic drugs. We showed that the DNA methylation inhibitor 5-aza-dC can increase the sustainability of transient ATF-induced re-expression. Furthermore, re-expression of the target gene EPB41L3 was obtained by targeting the demethylation inducers Tet2 or TDG when combined with epigenetic drug treatment (TSA). The combined actions of ATFs/epigenetic editors and epigenetic drugs may induce more complete promoter reprogramming than single treatment (19) as shown before for ATFs by others (20). As both epigenetic drugs and DNA targeting approaches are clinically explored, we thus provide a promising way to prolong sustainability of gene re-expression.

The exact mechanism behind the re-inforcing effects between epigenetic drugs and ATFs/epigenetic editors is largely unknown. Several factors may contribute, such as increased binding of the ATF to its endogenous target site (12), acetylation of lysine residues within transcription factors (a mechanism by which cells can overcome gene repression (40) and enhanced expression of the transgenes (41). In addition, TSA-induced repression of DNA methyltransferases may slow down remethylation/de-reprogramming events (26). DNA methylation has a rapid turnover in human cells (42), and inhibition of the process of remethylation may therefore increase the reprogramming potential of ATFs. Interestingly, we observed that expression of the Tet2-fusion proteins was associated with increased expression of DNA methyltransferase DNMT3b. A possible explanation for this may be a feedback mechanism in which cells try to neutralize/antagonize decreased methylation levels induced by the demethylating reagents.
Previously, the relation between EPB41L3 promoter methylation and expression silencing has been demonstrated in many cancer types (29-32). In this study, we confirmed the association between promoter hypermethylation and EPB41L3 repression, as we found that, in general, the expressing cell lines showed lower degrees of promoter methylation. However, this relation was not observed for the highest expressing EPB41L3 cell line (SiHa) which turned out to be almost completely methylated in the analyzed part of the CpG island in the EPB41L3 promoter. This observation that TSS-region methylation allows expression is inconsistent with the bulk of literature, as such genes are typically silenced (43). However, exceptions to this general mechanism exist such as the DNA methylation-independent regulation of CDKN2A in epithelial cells during mouse mammary gland development (44). hTERT is another example of a gene with increased methylation in many cancer types while still being expressed (45).

In general, the re-expression of silenced genes by ATFs is considered an advantage compared to traditional cDNA overexpression, as all splice variants can be re-expressed in natural ratios, which may determine the functional outcome of the intervention (46). This may also explain why, despite lower EPB41L3 re-expression capacity, the combination of ATFs were equally efficient in inducing cell death compared to the cDNA overexpression of isoform 3 (EPB41L3 has 38 splice variants). The functional effects we observed, however, may also be partly caused by the aspecific binding of the EPB41L3-targeting ATFs, as also observed for other ZFPs (47). Such aspecific binding may result in modulation of off-target genes, although certain ZF-ATFs might regulate gene expression with single gene resolution (25, 48). A possible explanation for such observed gene specificity may be that most of the off-target promoters have an open chromatin structure which are easily accessible by the ATFs; however, the relative impact of ATFs on actively transcribed genes is small. In contrast, the impact of ATFs on repressed regions could be significant; however, as these regions are more difficult to be accessed by ATFs, such off-target events may thus be less frequent. For example, the EPB41L3-ZFP did not show enrichment at the epigenetically repressed C13orf18-promoter (12) in this cell line (data not shown).

The engineered VP64-ATFs in this study were effective re-activators of EPB41L3, capable of inducing EPB41L3 expression in breast, ovarian and cervical cancer cell lines, even in hypermethylated cell lines. Recently, TAL effectors and the CRISPR/Cas system have been presented as an alternative for gene targeting, promising higher success rate with regard to specificity (49, 50). The advantages of ZFPs may be that they are relative small proteins (6F-ZFP < 200 amino acids
(aa), TAL effectors > 800 aa), which may have more efficient access to epigenetically silenced regions, and thus might be better suited for gene re-expression. Indeed, the potency of TALEs to induce expression is sometimes hampered, possibly due to their large size (51). However, when comparing the ability to decrease DNA methylation levels at a certain target site when fused to large Tet1 domains, no differences were found between ZFP- or TALE-based fusions (23). Here, we showed that the two engineered VP64-based ATFs targeting a chosen TSS have indeed a high success rate when it comes to gene induction, but no relevant gene inductions were obtained when the ZFPs were fused to Tet domains. Co-treatment with TSA did allow gene induction by these larger constructs. An improvement in the field of TAL effectors was the targeting of combinations of various TAL effectors to a single gene, which resulted in an increase in gene activation compared to a single TAL effector (51). Such synergistic effects were also observed for ZFPs by simultaneously targeting several TDG constructs (25), DNA demethylases (Tet1) (23) or H3K9 methylases (52) to a single promoter. In addition, here we showed that simultaneously targeting two ZFP-based ATFs to the same promoter facilitates the ATF-effect, when single treatment did not result in gene re-expression.

For cervical cancer, this is the first study demonstrating that EPB41L3 can induce tumor suppressive effects like apoptosis and reduction of cell growth. As was already shown for ovarian and breast, we further validated the role of EPB41L3 as TSG (27-29, 31, 35, 36) by the ATFs. Based on its differential methylation profile in cervical cancer versus normal tissues (33) and the functional effects shown here, EPB41L3 might represent an interesting therapeutic target also for cervical cancer and its high-grade premalignant lesions. Several mechanisms have been described how EPB41L3 may mediate the inhibition of cell growth, for example by increasing activity of caspase-8 (36). Additionally, we found that ATF-mediated re-expression of EPB41L3 is related with expression of the cell cycle regulators CDKN2A (down) and p21 (up).

In the future, delivery methods need to be further optimized to reveal the in vivo effectiveness of re-expression of silenced tumor suppressor genes, as currently explored by us and others (7, 9, 10). Attempts are ongoing to make ZFPs, such as our EPB41L3-targeting platform, suitable for clinical use and an ongoing clinical trial (performed by Sangamo Bioscience) already showed promising results. In this trial, ZFPs fused to nucleases (designed to disrupt CDR5 on CD4 cells as "functional cure" for HIV/AIDS) were successfully delivered to target cells and decreased viral load in HIV patients (53). The recent approval for the
treatment of lipoprotein lipase deficiency by an adeno-associated viral vector engineered to express lipoprotein lipase (54), might facilitate the development of new therapeutic strategies based on gene targeting platforms for future clinical applications.

In conclusion, this study demonstrated that epigenetic drugs can increase the sustainability of selective gene re-expression by ATFs. Furthermore, we showed that the effect of epigenetic editors (Tet2, TDG) on gene-expression can be improved with the HDACi TSA. The synergistic effects of ATFs/epigenetic editors and epigenetic drugs exploit the gene targeted effects of the activators while limiting genome-wide side effects of the epigenetic drug which may be beneficial for future therapeutic applications. For cervical cancer, we showed for the first time the tumor suppressive role of EPB41L3, a gene recently identified as the best marker for detecting early stages of this malignancy (33, 34).

Materials and Methods

Cell lines
Human breast cancer cell lines (MDA-MB-231, SKBR3), human ovarian cancer cell lines (SKOV3, A2780) and human cervical cancer cell lines (HeLa, SiHa, CaSki and C33A) were obtained from ATCC (Manassas, VA) and cultured in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (BioWhittaker), 2mM L-glutamine and 50 μg/ml gentamycin. All cell lines were confirmed by STR profiling (BaseClear, Leiden, the Netherlands). CC-11 was derived from a cervical squamous cell carcinoma (37).

ATF retroviral transduction/development of stable cell lines
Two target regions of ATFs, designated 21ab and 22ab, were selected based on proximity to the TSS and high affinity predictions (www.zincfingertools.org). (55) Double stranded DNA oligos (BIO BASIC, Markham, Canada) coding for the two 6-finger ZFPs predicted to bind the target sequences (21ab: GCAACAGGGGGCGGGGGG, 22ab: GGGGAGGAAGCCGCAGCC) were subcloned into the pMX-IRES-GFP containing either the gene activator VP64, no effector domain (NoEf) or the catalytic domain (CD) of Tet2 (24) or TDG (56) Tet2 and TDG DNA fragments were created by PCR (Phusion Hot Start II High-Fidelity DNA polymerase, Thermo Scientific) using construct-specific PCR primers flanked with MluI and PacI restriction sites on pcDNA3-Flag-TET2CD or TDG (57) and ligated into the pMX-IRES-GFP by sticky-end ligation with T4 ligase (Thermo Scientific).
ATF-21ab-VP64 (21ab-VP64) was subcloned in the Retro-X Tet-On advanced inducible expression system (Clontech, Mountain View, CA) according to the manufacturer's instructions. For overexpression of EPB41L3, the cDNA fragment of the gene was generated by PCR (Phusion Hot Start II High-Fidelity DNA polymerase) on SiHa cDNA using primers (Table 1) flanked with BamHI and EcoRI restriction sites and ligated into the pMX-IRES-GFP. Transduction of host cells with pMX-IRES-GFP or pRetroX-Tight-Pur was performed as previously described (12). For the expression of two ATFs simultaneously, viral supernatants were mixed (1:1).

To obtain stable RetroX-Tet-On double transfectants, cells transduced with pRetroX-Tet-On/ pRetroX-Tight-Pur (ratio 1:3) were placed under selection with G418 sulfate (InvitroGen, San Diego, CA) (600 µg/ml) and puromycin (InvitroGen) (1 µg/ml) for two weeks. To express 21ab-VP64 in double transfectants, cells were treated with doxycycline (DOX) (Clontech) (500 ng/ml) for 2 days. (DOX-treated) cells were also (co-treated with 5-aza-dC (Sigma, St Louis, MO) (5 μM) or TSA (Sigma) (400 nM).

Quantitative real-time PCR
RNA was extracted using the RNeasyPlus Mini Kit (Qiagen, Hilden, Germany) and converted into cDNA (Fermentas, Leon-Rot, Germany). 20 ng cDNA was used for qRT-PCR for the quantification of EPB41L3, ZFP-VP64 constructs, CDKN2A, p21, p53 and GAPDH, as previously described (12). Samples without amplification curves were assigned a Ct value of 40. Sequences of primers and probes are listed in Table 1. RNA levels were determined by the following formula \(2^{\Delta Ct}\) (relative to GAPDH) or \(2^{-\Delta\Delta Ct}\) (relative to empty vector).

Western blotting
Protein detection of EPB41L3, Actin and the ATFs was performed using standard Western blot techniques with antibodies detecting EPB41L3 (Santa Cruz, sc-100641 (1:250)), Actin (Millipore clone C4 (1:5000)) or HA tag (Abcam ChIP grade, Ab9110 (1:5000). Bands were visualized with the Pierce ECL chemoluminescence detection kit (Thermo Scientific, Rockford, USA). Protein mass was calculated using http://www.sciencegateway.org/tools/proteinmw.htm.
Table 1. Sequences mRNA primers.

<table>
<thead>
<tr>
<th>mRNA Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPB41L3 Fw</td>
<td>AGGAGGAGCAGCAGCAGGCC</td>
</tr>
<tr>
<td>EPB41L3 Rv</td>
<td>GCTGGTTTGAGCCCTGGCA</td>
</tr>
<tr>
<td>EPB41L3 probe</td>
<td>TGCAGAGGGAGTGACTGACAAG</td>
</tr>
<tr>
<td>GAPDH Fw</td>
<td>CCACATCGCTCAGACACCAT</td>
</tr>
<tr>
<td>GAPDH Rv</td>
<td>GCGCCCAATACGACCAAAT</td>
</tr>
<tr>
<td>GAPDH probe</td>
<td>GTTGAACCGACCTCACCTCCC</td>
</tr>
<tr>
<td>VP64 Fw</td>
<td>AAGCGACGCATTGGATGAC</td>
</tr>
<tr>
<td>VP64 Rv</td>
<td>GGAACGTCGTCGGGTAGTTAATT</td>
</tr>
<tr>
<td>VP64 probe</td>
<td>TCGGCTCCGATGCT</td>
</tr>
<tr>
<td>CDKN2A Fw</td>
<td>CACCCAATAGTTACGGTCCGA</td>
</tr>
<tr>
<td>CDKN2A Rv</td>
<td>GATGTAGAGCGGGCCTTCTGA</td>
</tr>
<tr>
<td>p21 Fw</td>
<td>GGCAGACCAGCATGACAGAT</td>
</tr>
<tr>
<td>p21 Rv</td>
<td>GATGTAGAGCGGCGCTTCTGA</td>
</tr>
<tr>
<td>p53 Fw</td>
<td>GTTGGCTCTGACTGTACCA</td>
</tr>
<tr>
<td>p53 Rv</td>
<td>CAAAGCTGGTCCGTCAGGTA</td>
</tr>
<tr>
<td>DNMT3b Fw</td>
<td>CGTGAAGCCAGGAGGGAAATA</td>
</tr>
<tr>
<td>DNMT3b Rv</td>
<td>TTCCGCAATACCAAGGTCGA</td>
</tr>
<tr>
<td>cDNA EPB41L3 Fw</td>
<td>GAGGGATCCGCGACCACATGAGCGAATCT</td>
</tr>
<tr>
<td>cDNA EPB41L3 Rv</td>
<td>TCTCAATGTTTCCGATCCTTCCCTCT</td>
</tr>
<tr>
<td>Bs seq. region 1 Fw</td>
<td>ATTATTTAAAGGGGAAATAGAAGGTTAA</td>
</tr>
<tr>
<td>Bs seq. region 1 Rv</td>
<td>CCCCCCTATTACAAAAACACC</td>
</tr>
<tr>
<td>Bs seq. region 2Fw</td>
<td>GTAATAGGGGGYGGGGGGAATAG61</td>
</tr>
<tr>
<td>Bs seq. region 2Rv</td>
<td>AACCCCCCTCGCAATCCCCCACCCTC</td>
</tr>
<tr>
<td>ChIP Fw</td>
<td>CCCGGGCTCTGATGACC</td>
</tr>
<tr>
<td>ChIP Rv</td>
<td>CCTCGGGCTCTTCTCCTCAGC</td>
</tr>
</tbody>
</table>

**Bisulfite-/pyrosequencing**

DNA of untreated cells was bisulfite converted (EZ DNA Methylation-GoldTM Kit, Zymo research, Irvine, CA) and amplified with primers (Table 1) specific for two regions in the EPB41L3 promoter (Fig. 1A). PCR products were cloned into the pCR2.1-TOPO Vector (Invitrogen, Carlsbad, CA) and sequenced subsequently.

For pyrosequencing, bisulfite treated DNA of the EPB41L3 promoter was amplified with the Pyromark PCR kit (Qiagen, Hilden, Germany) using biotin-labeled primers (Table 1), and subsequently sequenced using the Pyromark Q24
MD pyrosequencer (Qiagen). Pyromark Q24 Software (Qiagen) was used to determine the methylation levels of single CpGs.

**Chromatin immunoprecipitation (ChIP)**
A ChIP for the detection of the ZFP (HA tag), acetylation of histone 3 (H3Ac) and trimethylation of Lys9 of histone H3 (H3K9me3) was performed 72 hour after transduction. ChIP was performed as previously described (12) with the following antibodies: normal rabbit IgG (ab46540) (Abcam, Cambridge, UK), HA tag (101P-200) (Covance, Uden, the Netherlands), H3Ac (06-599) and H3K9me3 (07-442) (Millipore, Billerica, MA). DNA specific for the EPB41L3 promoter (Fig. 1A) was amplified with primers listed in Table 1.

ChIP-Seq was performed as previously described (58) using the HA-tag antibody on aHiSeq2000 (Illumina). Reads were analyzed using NextGENe (SoftGenetics, LLC, USA). Alignment was done excluding reads mapping to multiple locations. Peak regions were identified by setting the coverage to four reads, gap to 110 basepairs and setting the noise to zero. The coverage per peak was calculated as the 75th percentile of the number of aligned basepairs across the region. The plot of the EPB41L3 region was made using the "coverage distribution rapport" obtained from NextGENe and plotted with R software and is shown as the amount and location of reads at the EPB41L3 locus.

**Apoptosis-, cell death-, cell growth- and colony forming assays**
To quantify the fraction of apoptotic cells, cells were incubated in 1,1’3,3,3’,3’- Hexamethylindodicarbocyanine iodide (DiIC) (Enzo Life Sciences, Farmingdale, NY) containing medium (50 nM, 20 min), and analyzed by flow cytometry using a FACS Calibur cytometer and CellQuest software (BD Biosciences, San Jose, CA). The fraction of living cells with decreased DiIC signal was considered apoptotic, as exemplified in Supplementary Fig. 6. A MTT assay (Sigma) was performed to examine metabolic activity representing cell growth of transduced cells as previously described (12) PI staining to measure late apoptosis was performed using standard PI staining protocol, as previously described (59). For colony forming assay, transduced cells were seeded at a density of 750 cells per well, incubated at 37°C for three weeks and subsequently stained with Coomassie brilliant blue.
**Adhesion assay**

To measure cell adhesion, transduced cells were seeded on laminin coated dishes and incubated for 1 hour at 37°C. Loose cells were detached by shaking the plate at 2000 rpm for 15 seconds. After washing, cells were fixed with paraformaldehyde and stained with crystal violet (0.05%) in distilled water for 30 minutes. After washing, the dye was dissolved in methanol and OD was measured at 540 nM.

**Acknowledgments**

This work was financially supported by the Netherlands Organization for Scientific Research (VIDI grant number 91786373 to MGR). We acknowledge Marcel Ruiters for helpful discussion.
References


Sustained re-expression of EPB41L3 by ATFs and epigenetic drugs


Chapter 11


Supplementary Data

Supplementary Figure 1. DNA methylation status of *EPB41L3*. DNA methylation status of the *EPB41L3* promoter (region 1, Fig. 1A) for SKOV3, SKBR3, SiHa, CaSki and HeLa cells. Each row represents an individual clone and the average methylation levels of the clones are shown. Methylation levels were analyzed by bisulfite sequencing.
Supplementary Figure 2. DNA methylation status of \textit{EPB41L3}. The DNA methylation status of 15 CpGs in the \textit{EPB41L3} promoter of untreated cells (MDA-MB-231, SKBR3, A2780, HeLa, SiHa and CC-11) as quantified by pyrosequencing. Values represent the mean of at least two independent experiments ± SEM (SKBR3 one experiment).
Sustained re-expression of EPB41L3 by ATFs and epigenetic drugs

Supplementary Figure 3. Expression of ATFs in cancer cell lines. Quantification of VP64 effector domain mRNA in MDA-MB-231, SKBR3 (A), SKOV3, A2780 (B), HeLa, CaSki and C33A (C) cells after retroviral delivery of EPB41L3 targeting ATFs and controls. Expression levels were quantified with qRT-PCR and the bars represent the mean of in general three independent experiments measured in triplicate± SEM. (D) Percentage of GFP positive CaSki and C33A cells after retroviral transduction of an empty vector (pMX), EPB41L3-targeting ATFs (21ab-VP64 and 22ab-VP64) and ZFPs without effector domains (21ab-NoEf and 22ab-NoEf) as measured by FACs.
Supplementary Figure 4. *EPB41L3* expression after expressing demethylating reagents (see previous page). Gene expression of *EPB41L3* after expressing demethylating reagents 21ab-Tet2-CD, 22ab-Tet2-CD, 21ab-TDG-CD or 22ab-TDG-CD or combinations in CaSki cells. Quantification of mRNA was performed using qRT-PCR and induction levels were normalized to cells expressing an empty vector (c). Each bar represents the mean of three independent ± SEM.

Supplementary Figure 5. *EPB41L3* decreases cell growth in CaSki cells. Relative cell proliferation was measured with a MTT assay in Caski cells after transduction with the *EPB41L3*-targeting constructs. Each data point represents the mean of two independent experiments ± SEM.

Supplementary Figure 6. Fraction of apoptotic cells determined with a DilC assay. From the living cell population (R1), the fraction of apoptotic cells was determined as the number of cells with a decreased DilC intensity (R2, R3).