

Phosphorylation of the Leukemic Oncoprotein EVI1 on Serine 196 Modulates DNA Binding, Transcriptional Repression and Transforming Ability

Daniel J. White¹, Richard D. Unwin^{1#a}, Eric Bindels², Andrew Pierce¹, Hsiang-Ying Teng¹, Joanne Muter¹, Brigit Greystoke³, Tim D. Somerville³, John Griffiths^{1#b}, Simon Lovell⁴, Tim C. P. Somerville³, Ruud Delwel², Anthony D. Whetton¹, Stefan Meyer^{1,5*}

1 Stem Cell and Leukaemia Proteomics Laboratory, University of Manchester, Manchester Academic Health Science Centre, Manchester, United Kingdom, **2** Department of Hematology, Erasmus University, Rotterdam, The Netherlands, **3** Cancer Research UK Leukaemia Biology Laboratory, Paterson Institute of Cancer Research, Manchester, United Kingdom, **4** Faculty of Life Sciences, University of Manchester, Academic Health Science Centre, Manchester, United Kingdom, **5** Paediatric Haematology and Oncology, Royal Manchester Children's Hospital and The Christie NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, United Kingdom

Abstract

The *EVI1* (ecotropic viral integration site 1) gene at 3q26 codes for a transcriptional regulator with an essential role in haematopoiesis. Overexpression of *EVI1* in acute myeloid leukaemia (AML) is frequently associated with 3q26 rearrangements and confers extremely poor prognosis. *EVI1* mediates transcriptional regulation, signalling, and epigenetic modifications by interacting with DNA, proteins and protein complexes. To explore to what extent protein phosphorylation impacts on *EVI1* functions, we analysed endogenous *EVI1* protein from a high *EVI1* expressing Fanconi anaemia (FA) derived AML cell line. Mass spectrometric analysis of immunoprecipitated *EVI1* revealed phosphorylation at serine 196 (S196) in the sixth zinc finger of the N-terminal zinc finger domain. Mutated *EVI1* with an aspartate substitution at serine 196 (S196D), which mimics serine phosphorylation of this site, exhibited reduced DNA-binding and transcriptional repression from a gene promoter selectively targeted by the N-terminal zinc finger domain. Forced expression of the S196D mutant significantly reduced *EVI1* mediated transformation of Rat1 fibroblasts. While *EVI1*-mediated serial replating of murine haematopoietic progenitors was maintained by *EVI1*-S196D, this was associated with significantly higher *Evi1*-transcript levels compared with WT-*EVI1* or *EVI1*-S196A, mimicking S196 non-phosphorylated *EVI1*. These data suggest that *EVI1* function is modulated by phosphorylation of the first zinc finger domain.

Citation: White DJ, Unwin RD, Bindels E, Pierce A, Teng H-Y, et al. (2013) Phosphorylation of the Leukemic Oncoprotein *EVI1* on Serine 196 Modulates DNA Binding, Transcriptional Repression and Transforming Ability. PLoS ONE 8(6): e66510. doi:10.1371/journal.pone.0066510

Editor: Pierre-Antoine Defosse, Université Paris-Diderot, France

Received: January 26, 2012; **Accepted:** May 10, 2013; **Published:** June 12, 2013

Copyright: © 2013 White et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was funded by a Cancer Research UK (CRUK, <http://www.cancerresearchuk.org/>) Clinician Scientist Fellowship to SM (C1860/A5901) and LLR (UK) (<http://leukaemia.lymphomaresearch.org.uk/>). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: stefan.meyer@manchester.ac.uk

#a Current address: Centre for Advanced Discovery and Experimental Therapeutics (CADET), Central Manchester University Hospitals NHS Foundation Trust, Manchester, United Kingdom

#b Current address: The Paterson Institute for Cancer Research, The University of Manchester, Manchester, United Kingdom

Introduction

The *Ecotropic viral integration site-1* gene (*EVI1*), at the MECOM (MDS-*EVI1* complex) locus at 3q26.2 encodes a nuclear protein with an essential role in haematopoiesis [1]. Overexpression of *EVI1* in acute myeloid leukaemia (AML) is commonly a result of chromosomal rearrangements involving the 3q26.2 region and is associated with an extremely poor clinical outcome [2,3]. *EVI1* overexpression is in particular linked to leukemic transformation in individuals with Fanconi Anaemia (FA), which is an inherited chromosomal fragility disorder with predisposition to AML [4,5]. *EVI1* functions as a transcriptional regulator with two zinc finger domains that recognize specific genomic DNA target sequences [6,7] and mediates interaction of DNA with chromatin modifying proteins and protein complexes to regulate gene expression [8,9,10]. *EVI1* regulated genes have been reported to include *Pbx1* and *Fos* [11,12]. The oncogenic potential of overexpressed

EVI1 is reflected by the transformation of Rat1 fibroblasts to anchorage independent growth [13], as well as *EVI1* induced immortalization of primary bone marrow cells, and induction of myeloid dysplastic syndrome in mice [13,14,15]. *EVI1*-mediated transformation *in vitro* and *in vivo*, however, has been shown to be abrogated when interactions between *EVI1* and its co-regulatory protein complexes, or its target DNA sequences are disrupted [16,17]. Protein phosphorylation has been shown to be an important modulator of transcriptional regulators in development, haematopoiesis and differentiation [18,19,20]. Phosphorylation of *EVI1* was first identified by metabolic labelling studies more than 20 years ago [21], and more recently in large scale proteomic studies [22,23,24]. To test the hypothesis that *EVI1* phosphorylation plays a role in modulating *EVI1* protein function, we analysed endogenously expressed *EVI1* from the FA-derived AML

cell line SB1690CB by mass spectrometry and report here on the functional analysis of EVI1 phosphorylation on serine 196 (S196).

Results

EVI1 is phosphorylated on Serine 196

We detected high EVI1 protein expression in the FA-derived AML cell line SB1690CB, in which FA-associated 3q gains result in high *EVI1* transcript levels [5] (**Figure 1A**). This enabled immunoprecipitation of sufficient endogenous EVI1 protein to perform mass spectrometric analysis (**Figure 1B**). A multiple reaction monitoring-initiated detection and sequencing (MIDAS) scan for putative phosphopeptides detected a signal associated with potential phosphorylation of the peptide SYTQFSNLCR. Phosphorylation at serine 196 (S196) of EVI1 was confirmed by analysis of the MRM-triggered MS/MS spectrum from the putative phosphopeptide (**Figure 1C**). S196 is part of the evolutionarily conserved sixth zinc-finger motif within the N-terminal zinc-finger domain of EVI1 (**Figure 1D**), which specifically binds to the DNA sequence GA(T/C)AAGA(T/C)AAGATAA [6]. In addition, we confirmed previously described carboxy-terminal phosphorylation (S858 and S860) of EVI1 (data not shown) [22,23].

Impact of S196 phosphorylation on EVI1 protein structure

In order to determine the structural context of the phosphorylated serine 196, a comparative model of the sixth EVI1 zinc finger domain was generated using standard techniques with a model of the human C2H2 type zinc finger of protein 484 (pdb code 2EMH) as a template.

Serine 196 is on the surface of the zinc finger domain, which allows it to accommodate the phosphate group (**Figure 2A and B**). The impact of alanine and aspartate substitution at S196, used for functional analysis of S196 phosphorylation, was also modelled (**Figure 2C and D**). Given the solvent-exposed nature of this site, both aspartate and alanine can also be accommodated with no van der Waals overlaps. This suggests that the structural integrity of the proximal EVI1 zinc finger domain is maintained in the S196 phosphorylated, and alanine or aspartate substituted protein.

Phosphorylation of S196 modulates DNA binding and transcriptional repression by EVI1

To investigate whether modulation of EVI1 S196 modifies the interaction with its target DNA sequences, we generated GST-fusion proteins with GST fused to the first zinc-finger domain of murine EVI1 [6], in which S196 was substituted with either aspartate (S196D) to mimic constitutively phosphorylated serine, or alanine (S196A), which mimics non-phosphorylated serine. We confirmed the strong interaction between the wtEVI1-GST fusion protein and the double stranded oligonucleotide containing the target DNA sequence GA(T/C)AAGA(T/C)AAGATAA recognized by the first zinc-finger domain of EVI1 by EMSA (**Figure 3A**) [6]. The S196A-substituted mutant EVI1-fusion protein mimicking non-phosphorylated EVI1 had equivalent binding to the target sequence as wtEVI1. However, the phosphomimetic EVI1-fusion protein showed decreased DNA binding (**Figure 3A**), suggesting that phosphorylation status of S196 modulates DNA binding of EVI1. We next assessed the impact of full length EVI1-S196 mutants on transcriptional repression using luciferase reporter constructs with *PLZF* and *Fos* promoters. *In vitro*, *PLZF* is regulated by the proximal Evil zinc finger domain [25], while the *Fos* promoter is regulated by the distal zinc-finger domain of EVI1 [11]. Wt-EVI1 repressed transcription from both reporter

constructs (**Figure 3 B and C**). The Evil-S196A mutant mimicking non-phosphorylated Evil repressed transcription from both reporter gene constructs as efficiently as wt-EVI1. In contrast, the phosphomimetic Evil-S196D mutant repressed transcription from the *Fos* promoter, but repression from the *PLZF* promoter was significantly abrogated, implying that S196 phosphorylation impacts selectively on transcriptional regulation by the first zinc finger domain of EVI1 (**Figure 3 B and C**). Western blot analysis confirmed similar protein levels of both mutant and wt-EVI1 in the above described reporter assays (**Figure 3D**).

Phosphorylation of S196 partially abrogates transforming ability of Evil

Transduction with EVI1 transforms Rat1 fibroblasts and enables anchorage independent growth in soft agar [13,26]. Rat1 fibroblasts transduced with and stably expressing S196A mutant EVI1 mimicking non-phosphorylated Evil had similar colony forming capacity in soft agar as Rat1 fibroblasts transduced with wt-Evil. However, Rat1 fibroblasts stably expressing the phosphomimetic EVI1 S196D mutation generated fewer ($p < 0.05$, t-test) and smaller colonies compared to wt-EVI1 (**Figure 4A+B**), despite similar Evil protein levels in all transduced Rat1 cells populations (**Figure 4C**). This implies that the ability of EVI1 to transform Rat1 fibroblasts is tightly regulated by the sixth zinc finger motif of the N-terminal zinc finger domain and is also modulated by S196 phosphorylation.

Phosphorylation of S196 modulates EVI1 mediated replating capacity in murine haematopoietic progenitor cells

To investigate EVI1 S196 phosphorylation in the context of haematopoiesis, wt-EVI1 and phosphorylation site EVI1-mutants were expressed in c-kit+ murine primary bone marrow progenitors (**Figure 5A**). In serial replating assays progenitor cells transduced with control vector only formed colonies for two rounds of replating only, whereas cells with forced expression of exogenous wt-EVI1 replated beyond four rounds as previously described [13] (**Figure 5B**). The forced expression of either the EVI1-S196A or EVI1-S196D mutants enhanced replating capacity to the same extent as wt-EVI1 (**Figure 5B**). Morphological analysis of May-Grünwald-Giemsa stained cells at the end of the third round of replating showed persistence of immature cell types in the wt-EVI1, EVI1-S196A and EVI1-S196D transduced populations, whereas vector control cells had almost completely differentiated (**Figures 5C and D**). The comparable replating efficiency of EVI1-S196D compared with wt-EVI1 and EVI1-S196A demonstrates that the phospho-mutant retains sufficient function to confer serial replating, despite the impaired function that was demonstrated in the Rat1 fibroblasts assay. Since EVI1-mediated serial replating of hematopoietic progenitors appears highly dependent on EVI1-transcript levels, and is most effective with intermediate levels of EVI1 expression [13], we hypothesized that for replating of EVI1-S196D transduced progenitors higher EVI1-S196D expression levels might compensate for the impaired function that we demonstrate using *in vitro* and Rat1 fibroblast assays. Cells harvested from colonies transduced with EVI1-S196D showed significantly higher Evil-expression after the first round of plating compared with either wt-EVI1 or S196A ($p < 0.01$) (**Figure 5E**) by qRT-PCR analysis of Evil transcript levels. Sequence analysis of Evil transcripts in the S196A and S196D transduced cells showed that the Evil transcripts were

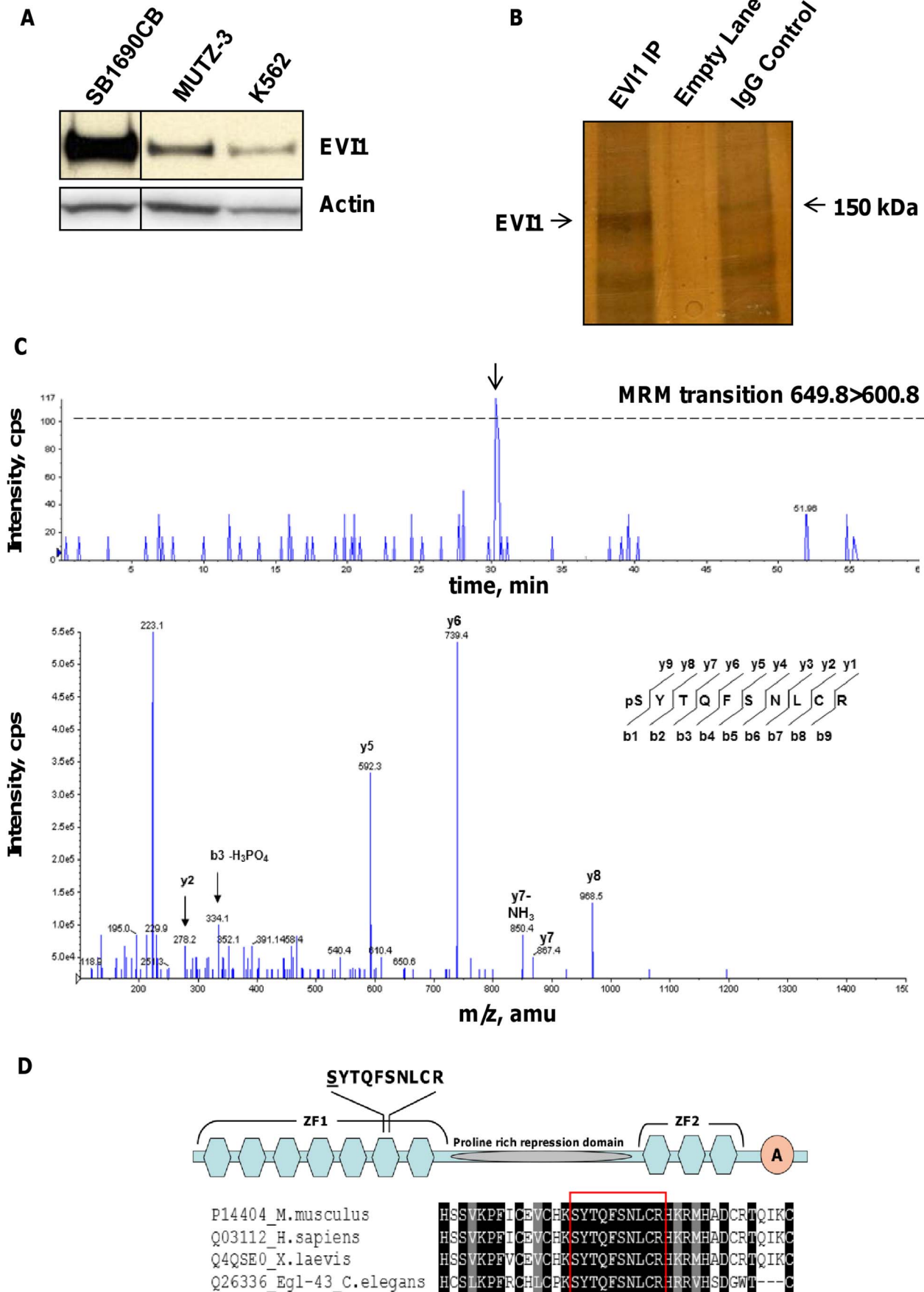


Figure 1. EVII is phosphorylated on Serine 196 in SB1690CB cells. (A) Western blot detection of EVII from whole cell lysate of leukemia cell lines SB1690CB, MUTZ-3 and K562. A vertical line has been inserted to indicate a repositioned gel lane. (B) Silver staining of immunoprecipitated EVII protein (arrow) from large scale SB1690CB cultures. Negative control lane showing immunoprecipitation with an irrelevant IgG antibody. (C) Extracted ion chromatograph showing signal intensity at time for the MRM transition $649.8 > 600.8$ designed to selectively detect the EVII peptide SYTQFSNLCR with a single serine or threonine phosphorylation. The threshold for triggering fragmentation and sequencing of the peptide was 100 counts per second (cps) and is indicated with a dashed line. A peak exceeding this threshold was eluted at 30.49 min and triggered fragmentation of the peptide (arrow). Lower panel: product ion spectrum generated by peptide fragmentation. Detected γ - or β -type fragment ions are indicated. Sequence of the EVII phosphopeptide with the β -ion and γ -ion series and position of the phosphorylated serine residue at the N-terminus of the peptide is shown. (D) Illustration showing the location of phosphorylated peptide within the EVII protein, with hexagons for zinc finger (ZF) motifs and acidic region, A-circle, at the carboxy terminal region (modified from [48]). Underlined is the serine residue at site of phosphorylation. Alignment was carried out using the t-coffee algorithm at www.t-coffee.org [49]. Shown is alignment to human EVII amino acids H182 to C219 with the phosphorylated peptide boxed in red. doi:10.1371/journal.pone.0066510.g001

expressed exclusively from the vector and not from the endogenous Mecom locus (Figure 5F)

Discussion

Overexpression of *EVII* is one of the most adverse prognostic markers in AML [2,3]. Insight into EVII function might therefore have therapeutic implications for *EVII* overexpressing AML. We hypothesised that the multiple functions of EVII might be partially modulated by protein phosphorylation. Therefore, we analysed endogenous EVII from an FA-derived AML cell line, which expresses high EVII transcript and protein levels as a result of FA-characteristic 3q gains [4,27,28]. This confirmed previously reported EVII phosphorylation sites (S858 and S860, data not shown), and detected a novel phosphorylation site at S196 within

the sixth zinc finger of the first zinc finger domain of EVII. Using the kinase substrate prediction software NetPhosK (<http://www.cbs.dtu.dk/services/NetPhosK/>) and NetworKin (<http://networkin.info>) several kinases were identified, which may be active on the EVII S196 site. These included: PKC, GSK3, CaM-II, CDC2, RSK, CKI, DNAPK, p38MAPK, PKA, PKG, ATM, CKII or NEK. As this phosphorylation site was detected in association with a defect in the FA-DNA damage response pathway, further investigations will focus on DNAPK, ATM and p38MAPK for this event, as these kinases in particular have been implicated in FA and the DNA damage response [29,30,31].

An *in silico* analysis of the structural impact of EVII S196 phosphorylation does not predict structural changes of this phosphorylation event on EVII structure. However, by functional analysis of the phosphomimetic mutant we demonstrated de-

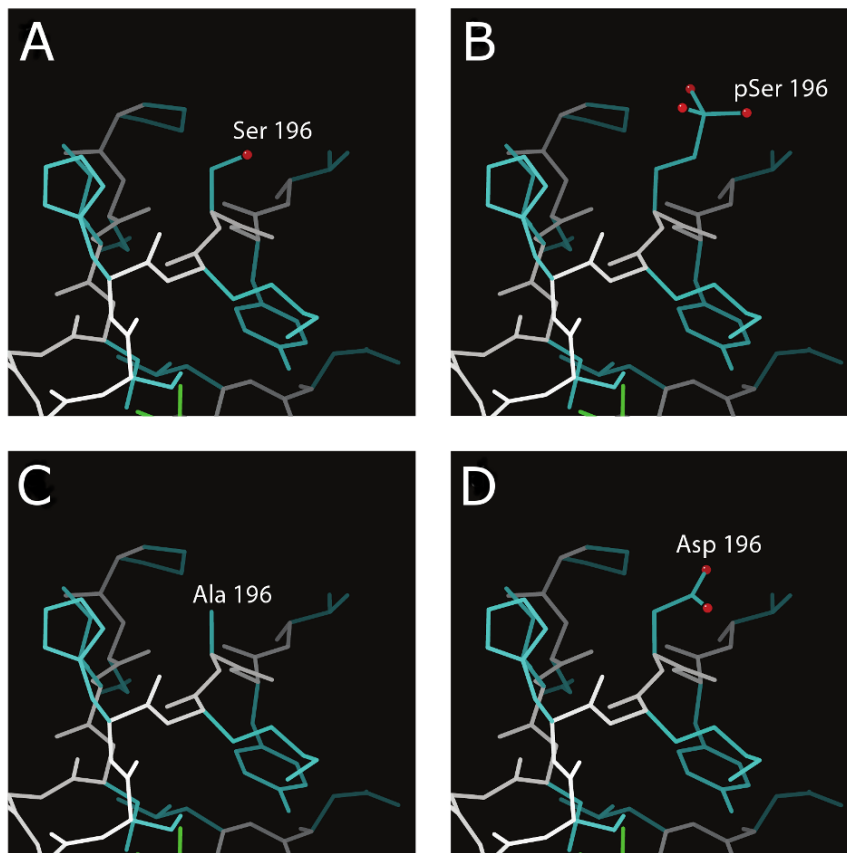


Figure 2. Structural context of phosphorylation and substitutions at EVII S196. Main chain is shown in white and side chains in blue. Oxygen atoms on residue 196 are indicated by red spheres. (A) The zinc finger domain with unmodified serine, (B) phosphorylated serine, (C) aspartate substitution and (D) alanine substitution. All residues can adopt a similar conformation and extend into the solvent. doi:10.1371/journal.pone.0066510.g002

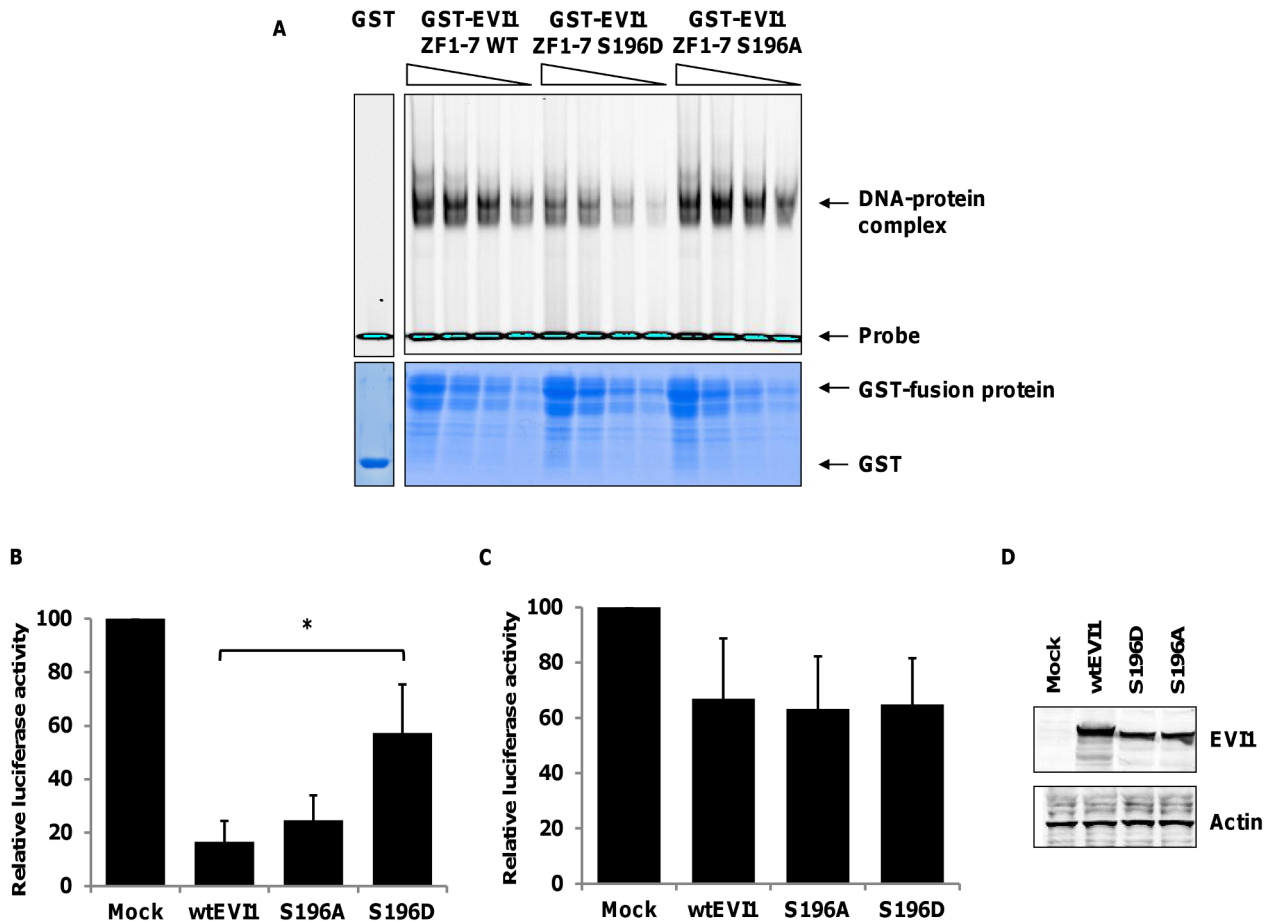


Figure 3. EVI1 S196 phosphorylation disrupts DNA binding and transcriptional repression. (A) Electro-mobility shift assay (EMSA) with GST alone or GST-Evi1 (ZF1-7) fusion proteins corresponding to WT or mutant Evi1 mimicking non-phosphorylated (S196A) or phosphorylated serine 196 (S196D) (upper panel). The DNA-protein complex and the free, labelled DNA probe are indicated. Lower panel: GST-fusion proteins separated on a 12.5% (w/v) SDS-PAGE gel and stained with colloidal coomassie blue. Specificity of protein/DNA interaction was confirmed by incubation with excess concentration of unlabelled random DNA substrate (data not shown). Graphs show mean (\pm s.d., n=4) luciferase activity relative to an empty vector control from reporter gene assays using either (B) a PLZF-luc reporter or (C) a Fos reporter co-transfected into HEK 293 cells with wild-type Evi1, S196 mutants or empty vector control. Luciferase activities were normalised against Renilla activity and calibrated to the empty vector control, *P<0.01, ns = non-significant (D) Western blotting of wild-type and mutant Evi1 protein levels in the reporter gene assays. doi:10.1371/journal.pone.0066510.g003

creased DNA binding and selectively abolished transcriptional repression from a promoter targeted by the first EVI1 zinc finger, as the mutant *Evi1-S196D* had no effect on transcriptional repression of the *Fos* promoter, which is regulated by the second zinc finger domain [11]. In the Rat1 fibroblast assay a mutation mimicking phosphorylated Evi1-S196 nearly entirely abrogated the transforming ability of Evi1. By EVI1-mediated serial replating of murine haematopoietic progenitor cells we show that the phosphomimetic S196D mutant retained EVI1 function, and conferred serial replating as we also demonstrated for wt-EVI1 and EVI1-S196A. However, replating with Evi1-S196D was associated with higher transgene expression compared to wt-EVI1 or EVI1-S196A. Since only a narrow range of *EVI1* expression appears compatible with transformation and replating [12,32] the significantly higher levels of S196D expression is consistent with impaired efficacy of the EVI1-S196D that we demonstrate *in vitro* and in Rat1 fibroblasts, which is compensated for by higher transcript levels.

In line with the observation that EVI1-mutations disrupting the first zinc-finger domain abolish the ability of EVI1 to induce myelodysplastic syndrome in mice [16], these data confirm a

central role for the first-zinc finger domain of EVI1 in transformation, which is modulated by phosphorylation. EVI1-S196 phosphorylation appears to modulate EVI1 function by reducing affinity of the N-terminal zinc finger domain to specific DNA target sequences, without evidence of significantly affecting functions of other domains with respect to transcriptional regulation.

Zinc finger domain phosphorylation has been detected on other transcriptional regulators [33,34], and phosphorylation mediated abrogation of DNA binding with effect on transforming ability has also been described for other leukemia associated transcription factors, including HOXA9, which intriguingly cooperates with EVI1 in leukaemogenesis [18,35]. Given that EVI1 phosphorylation at S196 was detected in cells derived from AML originally arising in an FA-patient, our findings might be in particular relevant in the context of leukaemia associated with FA. However, methylation patterns associated with EVI1 overexpression overlap largely between this FA-derived AML cell line and sporadic AML [8]. It has previously been reported that DNA binding and transcriptional regulation of EVI1 is also regulated by acetylation via the P/CAF acetyltransferase, which prevents EVI1 binding to

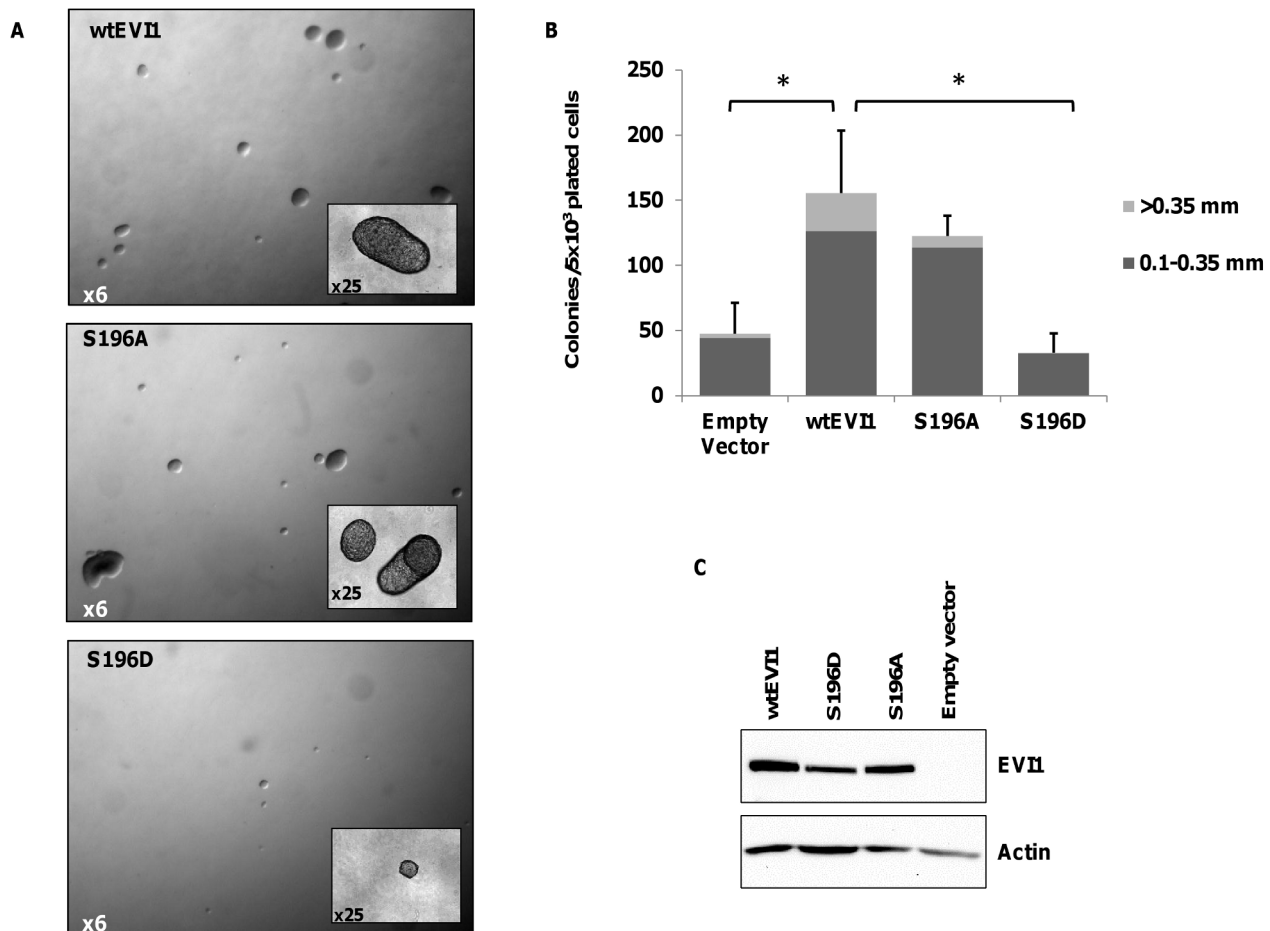


Figure 4. EVI1 S196 phosphorylation abrogates Rat1 transformation. (A) Representative photomicrographs of Rat1 fibroblast colonies transduced with wtEVI1 or S196 mutants (S196A and S196D) retroviral expression vectors. (B) Assays were scored after 24 days and colonies enumerated for number and size (mean \pm s.d., $n = 3$, $*P < 0.05$). (C) Detection of Evi1 protein in transduced Rat1 fibroblasts shown by western blot on whole cell lysates using the Evi1-SP137 antibody. doi:10.1371/journal.pone.0066510.g004

the promoter region of its target genes and modulates transcriptional activation [9,36]. This implies complex regulation of EVI1 function by a spectrum of post-translational modifications. We have addressed the functional consequences of S196 phosphorylation in biochemical assays, the Rat1 model and murine haematopoietic progenitor cells, which suggests a modulatory effect of S196 phosphorylation, which is likely to be further confounded by tissue specificity and cellular differentiation. Therapeutically, it will be important to understand how these post-translational modifications functionally interact and to identify mediators and dynamics of S196 phosphorylation. Investigations are underway to identify the cell signalling pathways controlling these processes.

Materials and Methods

Cell lines

The EVI1 overexpressing FA derived AML cell line SB1690CB was established from the leukaemia of an FA patient [5,27] and maintained under culture conditions as described previously [5,8,27]. Other cell lines were obtained from DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). The leukemia cell lines MUTZ-3 and K562 were maintained as described previously [5,27]. Rat1 cells were

cultured as described before [13,26,37,38]. GP-E86 cells lines were cultured in DMEM with 5% newborn calf serum, L-glutamine and penicillin/streptomycin. Plat-E and HEK-293T cells were cultured as previously described [39].

Western Blot and Immunoprecipitation

EVI1 western blot and immunoprecipitation was carried out using standard protocols. An EVI1 antibody, raised against the N-terminal EVI1 epitope MKSEDYPHETMAPDI (Eurogentec, Liege, Belgium), and anti-EVI1 #2265 (Cell Signalling Technology, Boston, MA, USA) were used. For mass spectrometric analysis EVI1 was immunoprecipitated from protein lysates of large scale cultures of approximately 6×10^8 SB1690CB cells. Evi1 detection in transformed Rat1 fibroblast was carried out with the anti-mouse Evi1-SP317 antibody (kind gift of Professor A. Perkins, Rochester, USA) [40].

Mass spectrometry

EVI1 was analyzed using multiple reaction monitoring-initiated detection and sequencing (MIDAS) [41,42]. MRM transitions were designed to detect EVI1 peptides (7 – 24 amino acids in length) with a single serine, threonine or tyrosine phosphorylation within a Q1 m/z range from 400 to 1300 and in both a double

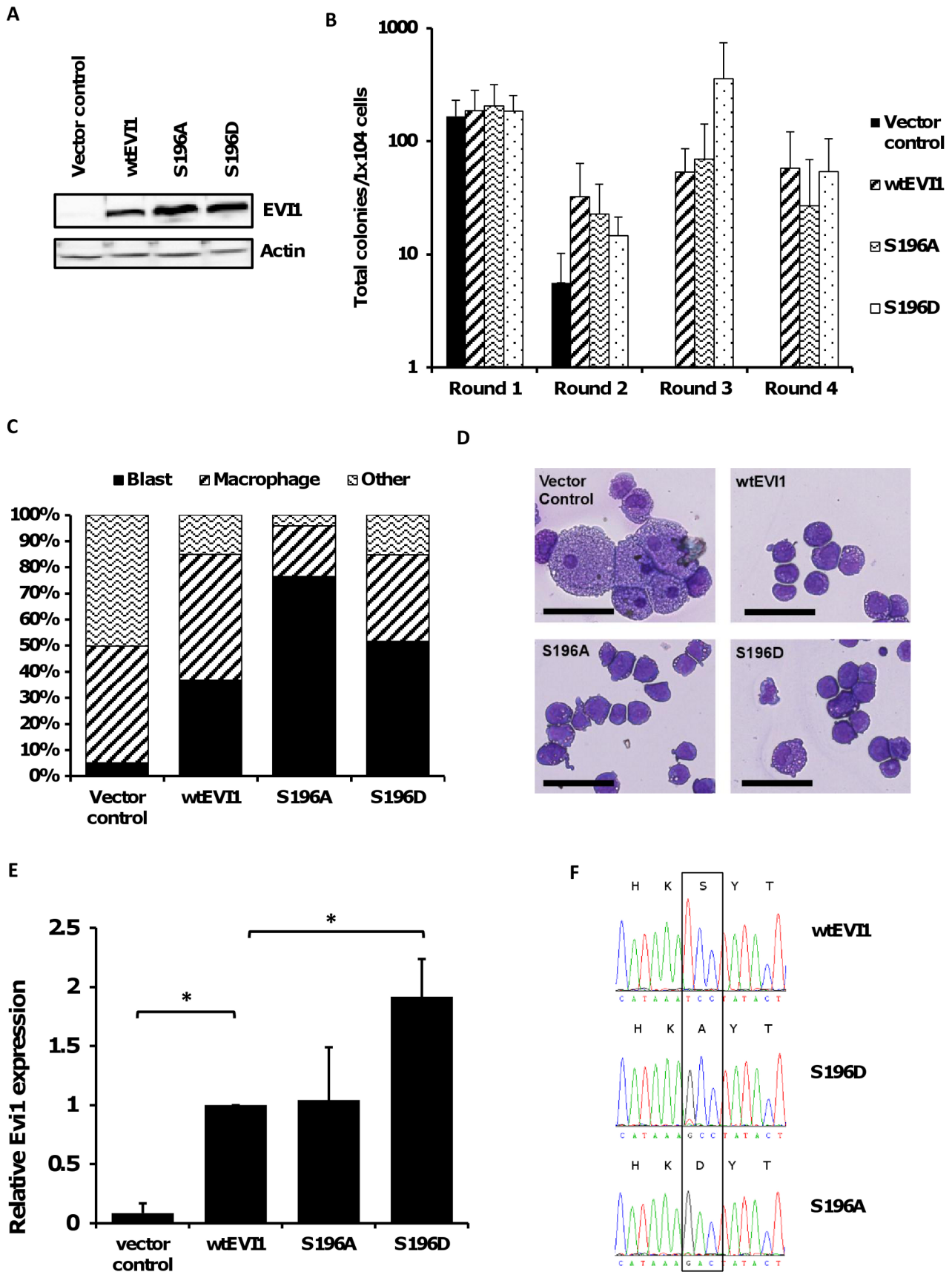


Figure 5. Serial replating of murine hematopoietic progenitor cells transduced with wtEvi1 and Evi1 phosphorylation site mutants. (A) Western blot analysis of Evi1 expression in PLAT-E cells two days after transfection with retroviral vectors encoding wtEVI1, Evi1-S196A, Evi1-S196D or empty vector control. (B) Total colony numbers (mean \pm s.d, n=5) formed in serial replating assays by c-kit positive cells transduced with wtEVI1, S196A, S196D or control retroviral expression vectors. (C) Percentage of cell types (blast, macrophage or other) enumerated by morphological analysis of May-Grünwald Giemsa stained cytopins after the third round of replating. Mean, n=4. Photomicrographs of May-Grünwald-Giemsa-stained cytopins after the third round of replating of transduced cells expressing WT or mutant EVI1 as shown. Scale bar indicates 50 μ m. (D) Photomicrographs of May-Grünwald-Giemsa-stained cytopins after the third round of replating of transduced cells expressing WT or mutant EVI1 as shown. Scale bar indicates 50 μ m. (E) Relative *Evi1* expression in c-kit+ cells after the first round of plating determined by qRT-PCR analysis. Mean \pm s.d, n=4. *P<0.01. (G) Sanger-Sequencing of *Evi1* transcripts from c-kit+ cells after the first round of plating. Electropherograms illustrating DNA-sequence around codon encoding S196. Nucleotide and amino acid sequence (single letter amino acid code) are shown. doi:10.1371/journal.pone.0066510.g005

and triple charge state. A Q3 mass of either 216.0 Da or Q1 minus 98 Da was used to identify tyrosine or serine/threonine phosphorylation, respectively. Following gel separation of immunoprecipitated EVI1, the EVI1 containing band was excised, and digested with trypsin. Peptides were separated by liquid chromatography prior to MIDAS using electrospray mass spectrometry on a 4000 Q-TRAP mass spectrometer (AB Sciex, Toronto, Canada).

Molecular modelling of the proximal EVI1 zinc finger domain

The phosphorylated serine is found in the first EVI1 zinc finger domain. No crystal structure is available for this domain. In order to assess the impact of phosphorylation or substitution of S196, a comparative model was generated using the human C2H2 type zinc finger of protein 484 (Database http://www.genome.jp/dbget-bin/www_bget?pdb:2EMH; pdb code 2EMH) as a template. For this, the sequences of EVI1 and the template structure were aligned using ClustalW [43] and 25 models built using Modeller [44]. The model with lowest discrete optimized protein energy (DOPE) score was selected. Models mimicking the specific mutations were built using KiNG (<http://kinemage.biochem.duke.edu>) [45]. In each case all low-energy rotamers [46] were considered and the rotamer with fewest van der Waals overlap used. For the phosphorylated serine standard bond lengths were used.

Plasmids

The retroviral *Evi1* expression vector p50FL-neo and the bacterial expression vector pGEX-Evi1-ZF1-7 encoding the first seven zinc-fingers of *Evi1* fused to GST have been described previously [6,37,38]. Mammalian expression constructs were generated by cloning *Evi1* excised from p50FL-neo (Wild-type, S196A, and S196D) into a pCMV backbone. Substitution of S196 in pGEX-EVI1-ZF1-7, p50FL-neo and pMIG-FLAG-Evi1 to alanine (A), or aspartic acid (D) was achieved by site directed mutagenesis using the QuikChange[®] II XL Kit (Agilent, Santa Clara, USA). The mutagenesis primer oligonucleotides used were as follows: S196A FW GCCCTTTATCTGTGAGGTCTGCCATAAAGCCTATACTCAG; S196A RV CTGAGTATAGGCTTTATGGCAGACCTCACAGATAAAGGGC; and S196D FW GCCCTTTATCTGTGAGGTCTGCCATAAAGCCTATACTCAG; S196D RV CTGAGTATAGTCTTTATGGCAGACCTCACAGATAAAGGGC. Mutations were confirmed by DNA sequencing. To generate pMIG-FLAG-Evi1 vectors a SalI FLAG-Evi1 fragment was excised from pCMV-FLAG-Evi1 (WT, S196A and S196D) and inserted into the XhoI site of pMIG. The reporter plasmids PLZF-luciferase, Fos-luciferase (a kind gift from Prof. Andrew Sharrocks, Manchester) and TK-Renilla luciferase have been previously described [25].

Electromobility shift assay (EMSA)

EMSA was carried out as described previously [6]. In brief, *E.coli* BL21 competent cells were transformed with pGEX-Evi1-ZF1-7 plasmids encoding for WT or S196-mutated GST-Evi1 fusion proteins. Fusion proteins were affinity purified using glutathione sepharose 4B and incubated with a double stranded DNA, sequence 5'ACCCATGGAGCTTTGAGCCCTGT-TATCTTGTGTCAGGGAAGTGTACCATGGA'3, labelled at the 5' end with IRDye. The reaction product was separated on a native 5% acrylamide gel and scanned on a Li-Cor Odyssey system (Li-Cor Biosciences, Lincoln NE, USA) to identify protein-DNA complexes.

Reporter gene assay

Reporter gene assays were carried out in HEK-293T cells. Cells were plated out in 6-well plates at 3.5×10^5 cells per well and co-transfected with 1 μ g luciferase reporter plasmid, 1 μ g effector plasmids (WT or mutants) and 5 ng TK-renilla luciferase using lipofectamine 2000 (Life Technologies, Carlsbad, CA,USA). After 48 hours cells were lysed and firefly and Renilla activity were assayed using the Dual-luciferase reporter assay (Promega, Madison, WI, USA) measured on a Victor³ plate reader (Perkin Elmer, Waltham,USA) or Flash'n Glow luminometer (Berthold Technologies, Bad Wildbad, Germany).

Rat1 fibroblast transforming assay

Rat1 transduction with p50-M-X-neo, p50FL-neo, p50FL-196D-neo or p50FL-196A-neo retroviral constructs was carried out using the retroviral packaging cell line GP+E86, as described previously [13,26,37,38]. In brief, Rat1 cells were seeded into 100-mm tissue culture plates at 1×10^6 cells/plate and left to adhere. Medium was replaced with viral supernatant supplemented with polybrene (4 μ g/mL) until Geneticin (G418, 400 μ g/mL, Life Technologies) selection after 48 hours and maintained for 10 days to generate stable cell lines. Cells were seeded in soft agar and after 25 days quantified for colony number and size and documented using a standard inversion microscope.

Serial replating of murine haematopoietic progenitors

This was carried out essentially as previously described [47]. Briefly, bone marrow was harvested from the femurs and tibias of 8-10 week old C57/BL6 mice. C-kit+ cell were isolated using an Automacs pro system (Miltenyi Biotechnology, Bergisch Gladbach, Germany), and pre-stimulated overnight in RPMI 1640 supplemented with recombinant murine IL-3 (10 ng/mL), GM-CSF (10 ng/mL), SCF (20 ng/mL) and IL-6 (10 ng/mL) in 20% fetal bovine serum, prior to transduction. Viral supernatant was generated by transfecting Plat-E packaging cells with *WT-Evi1* and *Evi1-S196* mutant pMIG-FLAG-Evi1 or control pMIG retroviral vectors using Fugene HD transfection reagent. C-kit+ cells were infected by spinoculation (30 minutes at 1250 x g and 32°C) with 8 μ g/mL polybrene. Cells were cultured for an additional two

days in pre-stimulation medium prior to FACS selection of GFP+ cells. Mean transduction efficiency for *WT-Evi1* was, 11.9% GFP+ cells; *Evi1-196A*, 13.8%, *Evi1-196D*, 14.0%; and pMIG control, 35.1%.

For replating 1×10^4 GFP+ cells were plated out in Methocult M3231 medium (StemCell Technologies, Grenoble, France) supplemented with recombinant murine IL-3 (10 ng/mL), GM-CSF (10 ng/mL), SCF (20 ng/mL) and IL-6 (10 ng/mL). After 7 days colony number and type were enumerated. 1×10^4 cells were then replated and colonies scored for at least three subsequent rounds of replating. Cytospin preparations and May-Grünwald Giemsa stainings were carried out using standard methods.

EVI1 – transcript analysis

RNA was extracted from c-kit+ selected cells with an RNeasy Kit (Qiagen, Valencia, CA, USA). cDNA was generated using Omniscript reverse transcriptase (Qiagen) with random nonamers (Sigma, Dorset, UK) and quantitative RT-PCR reactions were carried out on an Applied Biosystems 7900 (Life Technologies). *Evi1* qRT-PCR reactions consisted of Taqman universal master-

mix (Life Technologies) and Taqman gene expression assay (Mm00514814_m1, Applied Biosystems). For sequence analysis an *Evi1* PCR fragment was amplified from cDNA using the following oligonucleotides: FW, TGTGAAAACCTGTGCCAAGGT; RV, ACTTAGATCCAGGGGCTGGT. Sanger sequencing of the purified PCR product was carried out using the standard techniques and the oligonucleotide primer AGGTAAGACCAG-CAGGATGC.

Acknowledgments

We thank Professor A Perkins, Rochester, for the *Evi1*SP317 antibody.

Author Contributions

Conceived and designed the experiments: SM ADW RD EB RDU AP DJW SL TCPS. Performed the experiments: DJW EB SM JM H-YT RDU JG TDS BG. Analyzed the data: RD SM ADW RDU JG DJW SL TCPS. Contributed reagents/materials/analysis tools: ADW SM RD TCPS. Wrote the paper: SM DJW ADW EB RD SL TCPS.

References

- Goyama S, Yamamoto G, Shimabe M, Sato T, Ichikawa M, et al. (2008) Evi-1 is a critical regulator for hematopoietic stem cells and transformed leukemic cells. *Cell Stem Cell* 3: 207–220.
- Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, van Putten WL, Valk PJ, van der Poel-van de Luytgaarde S, et al. (2003) High *EVI1* expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. *Blood* 101: 837–845.
- Lugthart S, van Drunen E, van Norden Y, van Hoven A, Erpelinck CA, et al. (2008) High *EVI1* levels predict adverse outcome in acute myeloid leukemia: prevalence of *EVI1* overexpression and chromosome 3q26 abnormalities underestimated. *Blood* 111: 4329–4337.
- Meyer S, Bristow C, Wappett M, Pepper S, Whetton AD, et al. (2011) Fanconi anemia (FA)-associated 3q gains in leukemic transformation consistently target *EVI1*, but do not affect low *TERC* expression in FA. *Blood* 117: 6047–6050.
- Meyer S, Fergusson WD, Whetton AD, Moreira-Leite F, Pepper SD, et al. (2007) Amplification and translocation of 3q26 with overexpression of *EVI1* in Fanconi anemia-derived childhood acute myeloid leukemia with biallelic *FANCD1/BRCA2* disruption. *Genes Chromosomes Cancer* 46: 359–372.
- Delwel R, Funabiki T, Kreider BL, Morishita K, Ihle JN (1993) Four of the seven zinc fingers of the *Evi-1* myeloid-transforming gene are required for sequence-specific binding to GA(C/T)AAGA(T/C)AAGATAA. *Mol Cell Biol* 13: 4291–4300.
- Funabiki T, Kreider BL, Ihle JN (1994) The carboxyl domain of zinc fingers of the *Evi-1* myeloid transforming gene binds a consensus sequence of GAAGATGAG. *Oncogene* 9: 1575–1581.
- Lugthart S, Figueroa ME, Bindels E, Skrabanek L, Valk PJ, et al. (2011) Aberrant DNA hypermethylation signature in acute myeloid leukemia directed by *EVI1*. *Blood* 117: 234–241.
- Shimahara A, Yamakawa N, Nishikata I, Morishita K (2010) Acetylation of lysine 564 adjacent to the C-terminal binding protein-binding motif in *EVI1* is crucial for transcriptional activation of *GATA2*. *J Biol Chem* 285: 16967–16977.
- Yoshimi A, Goyama S, Watanabe-Okochi N, Yoshiki Y, Nannya Y, et al. (2011) *Evi1* represses *PTEN* expression and activates *PI3K/AKT/mTOR* via interactions with polycomb proteins. *Blood* 117: 3617–3628.
- Bard-Chapeau EA, Jayakani J, Kok CH, Muller J, Chua BQ, et al. (2012) Ecotopic viral integration site 1 (*EV1*) regulates multiple cellular processes important for cancer and is a synergistic partner for *FOS* protein in invasive tumors. *Proc Natl Acad Sci U S A* 109: 2168–2173.
- Shimabe M, Goyama S, Watanabe-Okochi N, Yoshimi A, Ichikawa M, et al. (2009) *Pbx1* is a downstream target of *Evi-1* in hematopoietic stem/progenitors and leukemic cells. *Oncogene* 28: 4364–4374.
- Bartholomew C, Kilbey A, Clark AM, Walker M (1997) The *Evi-1* proto-oncogene encodes a transcriptional repressor activity associated with transformation. *Oncogene* 14: 569–577.
- Buonamici S, Li D, Chi Y, Zhao R, Wang X, et al. (2004) *EVI1* induces myelodysplastic syndrome in mice. *J Clin Invest* 114: 713–719.
- Du Y, Jenkins NA, Copeland NG (2005) Insertional mutagenesis identifies genes that promote the immortalization of primary bone marrow progenitor cells. *Blood* 106: 3932–3939.
- Dickstein J, Senyuk V, Premanand K, Laricchia-Robbio L, Xu P, et al. (2010) Methylation and silencing of *miRNA-124* by *EVI1* and self-renewal exhaustion of hematopoietic stem cells in murine myelodysplastic syndrome. *Proc Natl Acad Sci U S A* 107: 9783–9788.
- Palmer S, Brouillet JP, Kilbey A, Fulton R, Walker M, et al. (2001) *Evi-1* transforming and repressor activities are mediated by CtBP co-repressor proteins. *J Biol Chem* 276: 25834–25840.
- Vijapurkar U, Fischbach N, Shen W, Brandts C, Stokoe D, et al. (2004) Protein kinase C-mediated phosphorylation of the leukemia-associated *HOXA9* protein impairs its DNA binding ability and induces myeloid differentiation. *Mol Cell Biol* 24: 3827–3837.
- Buitenhuis M, Verhagen LP, van Deutekom HW, Castor A, Verploegen S, et al. (2008) Protein kinase B (c-akt) regulates hematopoietic lineage choice decisions during myelopoiesis. *Blood* 111: 112–121.
- Costoya JA, Hobbs RM, Pandolfi PP (2008) Cyclin-dependent kinase antagonizes promyelocytic leukemia zinc-finger through phosphorylation. *Oncogene* 27: 3789–3796.
- Matsugi T, Morishita K, Ihle JN (1990) Identification, nuclear localization, and DNA-binding activity of the zinc finger protein encoded by the *Evi-1* myeloid transforming gene. *Mol Cell Biol* 10: 1259–1264.
- Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, 3rd, Hurov KE, et al. (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 316: 1160–1166.
- Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, et al. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 127: 635–648.
- Olsen JV, Vermeulen M, Santamaria A, Kumar C, Miller ML, et al. (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci Signal* 3: ra3.
- Takahashi S, Licht JD (2002) The human promyelocytic leukemia zinc finger gene is regulated by the *Evi-1* oncoprotein and a novel guanine-rich site binding protein. *Leukemia* 16: 1755–1762.
- Kurokawa M, Ogawa S, Tanaka T, Mitani K, Yazaki Y, et al. (1995) The *AML1/Evi-1* fusion protein in the t(3;21) translocation exhibits transforming activity on *Rat1* fibroblasts with dependence on the *Evi-1* sequence. *Oncogene* 11: 833–840.
- Meyer S, Fergusson WD, Oostra AB, Medhurst AL, Waisfisz Q, et al. (2005) A cross-linker-sensitive myeloid leukemia cell line from a 2-year-old boy with severe Fanconi anemia and biallelic *FANCD1/BRCA2* mutations. *Genes Chromosomes Cancer* 42: 404–415.
- Tonnies H, Huber S, Kuhl JS, Gerlach A, Ebell W, et al. (2003) Clonal chromosomal aberrations in bone marrow cells of Fanconi anemia patients: gains of the chromosomal segment 3q26q29 as an adverse risk factor. *Blood* 101: 3872–3874.
- Sasaki MS, Ejima Y, Tachibana A, Yamada T, Ishizaki K, et al. (2002) DNA damage response pathway in radioadaptive response. *Mutat Res* 504: 101–118.
- Castillo P, Bogliolo M, Surrallés J (2011) Coordinated action of the Fanconi anemia and ataxia telangiectasia pathways in response to oxidative damage. *DNA Repair (Amst)* 10: 518–525.
- Zhang S, Yajima H, Huynh H, Zheng J, Callen E, et al. (2011) Congenital bone marrow failure in DNA-PKcs mutant mice associated with deficiencies in DNA repair. *J Cell Biol* 193: 295–305.
- Kustikova OS, Schwarzer A, Stahlhut M, Brugman MH, Neumann T, et al. (2012) Activation of *Evi1* inhibits cell cycle progression and differentiation of hematopoietic progenitor cells. *Leukemia*.
- Black AR, Jensen D, Lin SY, Azizkhan JC (1999) Growth/cell cycle regulation of Sp1 phosphorylation. *J Biol Chem* 274: 1207–1215.

34. Sakamoto Y, Yoshida M, Semba K, Hunter T (1997) Inhibition of the DNA-binding and transcriptional repression activity of the Wilms' tumor gene product, WT1, by cAMP-dependent protein kinase-mediated phosphorylation of Ser-365 and Ser-393 in the zinc finger domain. *Oncogene* 15: 2001–2012.
35. Jin G, Yamazaki Y, Takuwa M, Takahara T, Kaneko K, et al. (2007) Trib1 and Evi1 cooperate with Hoxa and Meis1 in myeloid leukemogenesis. *Blood* 109: 3998–4005.
36. Pradhan AK, Mohapatra AD, Nayak KB, Chakraborty S (2011) Acetylation of the proto-oncogene EVI1 abrogates Bel-xL promoter binding and induces apoptosis. *PLoS One* 6: e25370.
37. Kilbey A, Bartholomew C (1998) Evi-1 ZF1 DNA binding activity and a second distinct transcriptional repressor region are both required for optimal transformation of Rat1 fibroblasts. *Oncogene* 16: 2287–2291.
38. Kilbey A, Stephens V, Bartholomew C (1999) Loss of cell cycle control by deregulation of cyclin-dependent kinase 2 kinase activity in Evi-1 transformed fibroblasts. *Cell Growth Differ* 10: 601–610.
39. Morita S, Kojima T, Kitamura T (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 7: 1063–1066.
40. Perkins AS, Fishel R, Jenkins NA, Copeland NG (1991) Evi-1, a murine zinc finger proto-oncogene, encodes a sequence-specific DNA-binding protein. *Mol Cell Biol* 11: 2665–2674.
41. Unwin RD, Griffiths JR, Leverenz MK, Grallert A, Hagan IM, et al. (2005) Multiple reaction monitoring to identify sites of protein phosphorylation with high sensitivity. *Mol Cell Proteomics* 4: 1134–1144.
42. Unwin RD, Griffiths JR, Whetton AD (2009) A sensitive mass spectrometric method for hypothesis-driven detection of peptide post-translational modifications: multiple reaction monitoring-initiated detection and sequencing (MIDAS). *Nat Protoc* 4: 870–877.
43. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947–2948.
44. Sali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* 234: 779–815.
45. Chen VB, Davis IW, Richardson DC (2009) KING (Kinemage, Next Generation): a versatile interactive molecular and scientific visualization program. *Protein Sci* 18: 2403–2409.
46. Lovell SC, Word JM, Richardson JS, Richardson DC (2000) The penultimate rotamer library. *Proteins* 40: 389–408.
47. Somerville TC, Matheny CJ, Spencer GJ, Iwasaki M, Rinn JL, et al. (2009) Hierarchical maintenance of MLL myeloid leukemia stem cells employs a transcriptional program shared with embryonic rather than adult stem cells. *Cell Stem Cell* 4: 129–140.
48. Metais JY, Dunbar CE (2008) The MDS1-EVI1 gene complex as a retrovirus integration site: impact on behavior of hematopoietic cells and implications for gene therapy. *Mol Ther* 16: 439–449.
49. Notredame C, Higgins DG, Heringa J (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol* 302: 205–217.