SHORT COMMUNICATION

EVI1 oncoprotein expression and CtBP1-association oscillate through the cell cycle

Roberto Paredes1,2 · Marion Schneider1,2 · Stella Pearson1,2 · Hsiang Yin Teng1,2 · James R. Kelly1,2 · Andrew Pierce1,2 · Tim C. P. Somervaille2,3 · Anthony D. Whetton1,2,4 · Stefan Meyer1,2,5,6

Received: 18 June 2020 / Accepted: 7 September 2020 / Published online: 26 September 2020
© The Author(s) 2020

Abstract
Aberrantly high expression of EVI1 in acute myeloid leukaemia (AML) is associated with poor prognosis. For targeted treatment of EVI1 overexpressing AML a more detailed understanding of aspects of spatiotemporal interaction dynamics of the EVI1 protein is important. EVI1 overexpressing SB1690CB AML cells were used for quantification and protein interaction studies of EVI1 and ΔEVI1. Cells were cell cycle-synchronised by mimosine and nocodazole treatment and expression of EVI1 and related proteins assessed by western blot, immunoprecipitation and immunofluorescence. EVI1 protein levels oscillate through the cell cycle, and EVI1 is degraded partly by the proteasome complex. Both EVI1 and ΔEVI1 interact with the co-repressor CtBP1 but dissociate from CtBP1 complexes during mitosis. Furthermore, a large fraction of EVI1, but not ΔEVI1 or CtBP1, resides in the nuclear matrix. In conclusion, EVI1- protein levels and EVI1-CtBP1 interaction dynamics vary through the cell cycle and differ between EVI1 and ΔEVI1. These data ad to the functional characterisation of the EVI1 protein in AML and will be important for the development of targeted therapeutic approaches for EVI1-driven AML.

Keywords EVI1 · CtBP1 · Cell cycle · AML

Introduction
Aberrantly high expression of EVI1 in acute myeloid leukaemia (AML) is commonly caused by chromosomal aberrations involving the MECOM (MDS-EVI1 complex) locus at 3q26 and associated with poor outcome [1, 2]. In AML, the overexpressed 1051 amino acid (aa) EVI1 protein can be co-expressed with the shorter ΔEVI1 isoform, which lacks a 324 aa sequence region (aa190-514), including the 6th and 7th zinc finger of the N-terminal zinc finger domain (Fig. 1a). The MDS-EVI1 isoform is usually not expressed at elevated levels [2]. DNA binding sites of the ΔEVI1 isoform largely overlap with those of EVI1, but it lacks in vivo transforming ability characteristic for EVI1 [3, 4]. While the reliance of EVI1 on interaction with other transcriptionally active proteins, e.g. CtBP1 [5], has been recognised and provides potential angles for therapeutic approaches, spatiotemporal dynamics of the EVI1 protein isoforms in AML are incompletely understood, but would be important for the development of EVI1-targeted therapeutic approaches. Here, we report on data that uncover cell cycle and isoform specific localisation and interaction dynamics of EVI1 and ΔEVI1.
Western blot, immunofluorescence and antibodies

Protein extracts from pelleted cells were resolved by protein electrophoresis (NuPAGE® Novex® 4–12% Bis-Tris Protein Gels, Invitrogen) and analysed by western blotting using standard methodologies.

For immunofluorescence SB1690CB cells were spun at 200 rpm for 2 min (Cytospin 2, Shandon) onto POLYSINE slides (VWR International) and fixed with methanol-free 4% formaldehyde (Thermo Scientific) for 10 min. Cells were washed in PBS and blocked with 5% goat normal serum (Cell Signaling Technology) and 0.3% Triton-X100 (Sigma) containing PBS. Primary antibodies (details listed in Supplementary Material) were used with secondary antibodies following standard procedures in a 0.1% BSA (Cell Signaling Technology) and 0.3% Triton-X100 containing PBS. Single confocal plane and sequential channel acquisitions were performed in a Fluoview1000 confocal system (Olympus), using a 60X UPLSAPO oil immersion lens. To determine levels of co-localisation of EVI1 and CtBP1, Pearson product-moment correlation coefficient (Pearson’s coefficient was used to measure the linear signal correlation (dependence) between the EVI1 and CtBP1 IF signals. Person’s coefficient ranged from 1 (total positive correlation) to −1 (total negative correlation). 200 + circular (r = 1.5 mm) Regions of interest (ROI) were analysed per condition with the co-localisation plug-in of the ImageJ software. To discard signal saturated ROIs, the images were analysed using HiLo (High-Low) intensity Look Up Table (LUT). Pearson coefficients were plotted either in a dispersion graph (cell distribution in a single experiment) or as average from at least three biological replicates. One-way Analysis Of The Variance (ANOVA) with the Tukey post-test statistical analysis was used to compare the means (GraphPad Prism). Alternatively, linear ROIs of 5 mm in length were analysed in terms of signal intensity and plotted as signal histograms. Automated foci detection and counting was performed by the FociPicker3D plugin for ImageJ [7]. Briefly, nuclear ROIs were created for Individual cells at independent microscope panels and foci were detected and counted under the follow criteria: foci > 0.35 µm in diameter and a MinISetting of 0.5 (Minimum intensity setting). For the antibodies used, please refer to the figure legends and Supplementary Table 1.
(Sigma P5726), 1X Phosphatase Inhibitor Cocktail 3 (Sigma P0044), supplemented with: (1) 150 µg/mL digitonin (Sigma D141) and glycerol (Fisher Scientific, BP-229-1) to extract cytosol proteins; (2) 0.5% Tween-20 (Sigma P1379) and glycerol (Fisher Scientific, BP-229-1) to extract mem-

brane proteins. The remnant pellet was extracted with 10 volumes of a high salt lysis buffer [420 mM NaCl, 20 mM CaCl2, 0.6% CHAPS (Sigma C9426), 1 mM Na3VO4, 1 mM PMSF, 1X Protease Inhibitor Cocktail (Sigma P8340), 1X Phosphatase Inhibitor Cocktail 2 (Sigma P5726), 1X Phosphatase Inhibitor Cocktail 3 (Sigma P0044) and 250 U/µL Pierce Universal Nuclease (Thermo Scientific 88,700)] to extract chromatin associated proteins. The residual pellet was extracted with 2X LDS buffer (Invitrogen) to dissolve the nucleoskeleton. Protein extracts were resolved by protein electrophoresis using the (NuPAGE® system, Invitrogen) and analysed by western blotting.

Results

EVI1 is degraded during mitosis

To investigate endogenously expressed EVII in AML we studied 3q26 rearranged SB1690CB AML cells, which express high levels of both EVII and ΔEVI1, but no MDS-EVI1 (Fig. 1a) [6]. Mimosine treatment-associated G1 arrest and release resulted in reduction of both EVII and ΔEVI1 levels (Fig. 1b), with EVII only starting to recover 7 h after release (Fig. 1b). In contrast, a nocodazole-induced G2/M arrest resulted in higher EVII levels compared with levels at G1 arrest (Fig. 1c), which suggests that degradation of over-expressed EVII occurs during or shortly after mitosis (iden-
tical findings with forced EVII expression also in another cell line model, see Supplementary Fig. S1). To further test this hypothesis, we induced a G2/M arrest and monitored EVII levels over 14 h post release. We observed gradual reduction in EVII and ΔEVI1 levels during mitotic progression with similar patterns as CYCLIN B1 (Fig. 1c), which is degraded by the anaphase-promoting complex (APC/Cyclosome) to exit mitosis [8]. To investigate whether EVII degradation is also proteasome dependent, we blocked de novo protein synthesis with cycloheximide alone, or in combination with the proteasome inhibitor MG-132 (Fig. 1d). Cycloheximide treatment alone resulted in a marked reduc-
tion of EVII levels, which was partly reversed by MG-132 treatment (Fig. 1d), with patterns resembling those of CYC-
LIN B1 (Fig. 1e). Intriguingly, degradation of ΔEVI1 was not reversible to the same extent by MG-132, implying additional and alternative degradation dynamics for ΔEVI1.

CtBP1 dissociates from EVII during mitosis

G1 block and long-term release over 26 hrs confirmed oscillation of EVII expression during cell cycle progression, exhibiting similar patterns to the MLL protein [9], which was used as a control (Fig. 2a). Both EVII and ΔEVI1 levels recovered at the transition between G1 and S (calibrated by the cell cycle markers CYCLIN B1, CYCLIN E2 and p-H3 (Ser10) (Fig. 2b). With respect to EVII interacting proteins, we observed for CtBP1 similar cell cycle dependent oscillation patterns, while the EVII interacting proteins BRG1 and HDAC1 [10, 11] displayed stable expression levels during cell cycle progression. Both EVII and ΔEVI1 interact with CtBP1 (Fig. 2c), and EVII co-localises with CtBP1 most strongly during telophase (Fig. 2d, e).

Differential subnuclear distribution of EVII and CtBP1 during interphase

We noticed different patterns of the nuclear signal distribution of EVII and CtBP1 in interphase: The EVII-signal in interphase has a speckled signal distribution (Fig. 3a) in the nuclei with distinctly separable foci formations (visualised in Fig. 3b, c), whereas the CtBP1-signal was more diffuse (Fig. 3a, b, c). Foci counts (Fig. 3d) per region of interest (ROI) were significantly higher for EVII foci than for CtBP1 (Fig. 3e). To further determine exact protein localisa-
tion within the nucleus, which might underly these findings, on cellular fractionation we found abundant CtBP1 in the nucleoplasmic fraction (Fig. 3f) (Supplementary Fig. S2). However, EVII, ΔEVI1, and only a fraction of CtBP1 and the EVII-interacting proteins BRG1 and HDAC1 co-elute in the nuclear chromatin fraction (Fig. 3f, Supplementary Fig. S2). Importantly, a fraction of EVII, but not ΔEVI1, is residing in the nucleoskeleton, evidenced by the presence of the nuclear envelop marker LAMIN A/C in that fraction (Fig. 3f, g), with similar staining patterns also during mitosis as the nucleoskeleton associated protein NuMA1 during interphase (Supplementary Fig. S3) [12].

Discussion

High EVII expression in one of the most aggressive onco-
genic events in leukaemia, and a similar role for aberrantly high EVII expression is emerging in some solid tumours [13, 14]. Understanding the functional interactions and spa-
tiotemporal associations of the different EVII isoforms is therefore important. We studied endogenously expressed EVII and ΔEVI1 in a robust AML cell line model with a
CtBP1 dissociates from EVI1 during mitosis. a SB1690CB AML cells were synchronised in G0/G1 with Mimosine treatment for 18 h and then released by replacement with fresh medium. Total protein extracts were produced at the time points as indicated after release from the G0/G1 block and expression levels of EVI1 and CtBP1 assessed by western blot. Levels of EVI1 interacting proteins BRG1 and HDAC1 were assessed as controls, Ser10-phospho H3 as a mitotic marker, CYCLIN B1 and CYCLIN E2 as cell cycle phase controls. MML180 was used as a marker which oscillates through the cell cycle and GAPDH as a loading control. b Quantitation of EVI1 isoform levels as in A from 3 independent experiments. c Co-immunoprecipitation of endogenously expressed EVI1 isoforms and CtBP1 from SB1690CB AML cells. d Dual colour EVI1 (green) and CtBP1 (magenta) immunofluorescence in SB1690CB AML cells. Single confocal planes acquired with a Fluoview 1000 system (Olympus). Cell cycle stage assessed by chromatin staining (DAPI, blue). Dashed lines denote cell boundaries. e Distribution of the Pearson Coefficient (P’sC) for the EVI1 and CtBP1 signal co-localization. 100 circular region of interests (ROI) were pooled from 5 different stains (3 µm in diameter). Statistical analysis: one-way ANOVA and Tukey post-test (n.s.=non-significant, ** p < 0.01, *** p < 0.001). (Color figure online)
3q26-aberration associated EVI1 overexpression [6]. Recent clinical data support the concept that all 3q-re-arranged AMLs constitute a uniform entity driven by EVI1 [2]; we therefore presume that our observations apply more generally to all EVI1-overexpressing AMLs. However, further confirmation of our findings in other cell lines and clinical samples would be important, also including EVI1-overexpressing leukaemia without 3q re-arrangements. Building on data showing that forced expression of EVI1 in hematopoietic progenitor cells inhibits normal cell cycle progression [15], here we illustrate the effect of cell cycle progression on EVI1. We can demonstrate a bimodal oscillation of EVI1 protein levels with maximum EVI1 levels at the end of S-phase, similar to that described for related transcription factors MLL and GATA2 [9, 16], and imply a role of the proteasome for EVI1-degradation, which could also provide...

Fig. 3 Subnuclear distribution of EVI1 and CtBP1 during interphase. a Dual colour EVI1 (green) and CtBP1 (magenta) immunofluorescence in AML cells. Single confocal planes acquired with a Fluoview 1000 system (Olympus) and presented individually in grey scale. Nucleus visualised by chromatin staining (DAPI). Signal intensity measured over 5 µm length (yellow lines numbered 1 and 2) linear ROIs for both, EVI1 and CtBP1 stains. b, c Histogram panels illustrating nuclear signal intensity distribution of EVI1 (green) and CtBP1 (purple). d Western blotting of AML cell fractionations and e Quantitation of EVI1 isoform levels in selected fraction (as in D) from 3 independent experiments. Statistical analysis for each isoform was performed by one-way ANOVA test and Tukey post-test (n.s. non-significant, **p < 0.01, ****p < 0.0001). (Color figure online)
therapeutic options for EVII-overexpressing leukaemia. Our data further implies that the interaction with the co-repressor CtBP1, which has been shown to be essential for some EVII functions [5], is likely to be mainly occurring through inter-phase and is located in the chromatin fraction and, while a large proportion of EVII is located at the nuclear matrix, where we did not see CtBP1. As the repressor protein CtBP1 is considered as a therapeutic target in various cancer types [17] and might have a role specifically for EVII overexpressing malignancies, these observations need to be considered when targeting CtBP1 interactions therapeutically. Reported differences of functional interactions of the EVII isoforms with respect to transcription and protein association [4, 17] may be partly explained by their dynamic sub-nuclear localisation. Our study reports on the EVII-CtBP1 interaction, but many more proteins have been described to interact with EVII [17, 18]. The detailed mechanistic understanding and the functional implications of transcription levels and protein turnover, which could be mediated by ubiquitination, sumoylation or other posttranslational modifications, and dynamic spatiotemporal interactions of EVII will be a critical consideration for targeted therapeutic approaches in EVII overexpressing leukaemia.

Acknowledgements
Not applicable.

Author contributions Conceptualisation and experimental design: RP, SM, ADW, AP, TCPS; Experimental work: RP, JK, AP, HYT, MS, SP; preparation of manuscript: RP, AP, ADW, TCPS, SM.

Funding Bloodwise (Grant No. 10037, 150380 and 19007); Cancer Research UK (C5759/A20971 and C18601/A5901); The Kay Kendall Leukaemia Fund (KKL 792), Children with Cancer, UK; The Elimination of Leukaemia Fund, UK; The UK Children’s Cancer and Leukaemia Group (CCLG, Toti Worboys Leukaemia Project Grant to JK), The Medical Research Council, UK.

Data availability All data generated or analysed during this study are included in this published article [and its supplementary information files].

Compliance with ethical standards
Conflict of interest The authors declare that they have no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References
1. Groschel S, Lugath S, Schlenk RF, Valk PJ, Eiwen K, Goudsward C, van Patten WJ, Kayser S, Verdonck LF, Lubbert M, Ossenkoppele GI, Germain U, Schmidt-Wolf I, Schlegelberger B, Krauter J, Ganss A, Dohner H, Lowenberg B, Dohner K, Delwel R (2010) High EVII expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. J Clin Oncol 28:2101–2107 1. Ottoma S, Mulet-Lazo R, Beverloo HB, Erpelinck C, van Herk S, van der Helm R, Havermans M, Grob T, Valk PJM, Bindels E, Haferlach T, Haferlach C, Smeek L, Delwel R (2020) Atypical 3q26/MECOM rearrangements genocopy inv(3)(t;3;3) in acute myeloid leukemia. Blood 136:224–234 3. Hoyt PR, Bartholomew C, Davis AJ, Yutzey K, Gamer LW, Potter SS, Ihle JN, Mucenski ML (1997) The Evii proto-oncogene is required at midgestation for neural, heart, and paraxial mesenchyme development. Mech Dev 65:55–70 4. Sayadi A, Jeyakani J, Seet SH, Wei CL, Bourgue G, Bard FA, Jenkins NA, Copeland NG, Bard-Chapeau EA (2016) Functional features of EVII and EVII Delta32 isoforms of MECOM gene in genome-wide transcription regulation and oncogenicity. Oncogene 35:2311–2321 5. Palmer S, Brouillet J-P, Kilbey A, Fulton R, Walker M, Crossley M, Bartholomew C (2001) Evii-1 transforming and repressor activities are mediated by CtBP co-repressor proteins. J Biol Chem 276:25834–25840 6. Meyer S, Fergusson WD, Whetton AD, Moreira-Leite F, Pepper SD, Miller C, Saunders EK, White DJ, Will AM, Eden T, Ikeda H, Ullmann R, Tuerkmen S, Gerlach A, Klopccki E, Tonnies H (2007) Amplification and translocation of 3q26 with overexpression of EVII in Fanconi anemia-derived childhood acute myeloid leukemia with biallelic FANCD1/BRC2a2 disruption. Genes Chromosomes Cancer 46:359–372 7. Du G, Drexlser GA, Friedland W, Greubel C, Hable V, Krucken R, Kugler A, Tonelli L, Friedl AA, Dollinger G (2011) Spatial dynamics of DNA damage response protein foci along the ion trajectory of high-LET particles. Radiat Res 176:706–715 8. Hershko A (1999) Mechanisms and regulation of the degradation of cyclin B. Philos Trans R Soc Lond B Biol Sci 354:1571–1576 9. Liu H, Cheng EH, Hsieh JJ (2007) Bimodal regulatory circuit lost in leukemogenic MLL fusions. Genes Dev 21:2385–2398 10. Chi Y, Senyuk V, Chakraborty S, Nuicifora G (2003) EVII promotes cell proliferation by interacting with BRG1 and blocking the repression of BRG1 on EZF1 activity. J Biol Chem 278:49806–49811 11. Bard-Chapeau EA, Gunaratne J, Kumar P, Chua BQ, Muller J, Bard FA, Blackstock W, Copeland NG, Jenkins NA (2013) EVII oncoprotein interacts with a large and complex network of proteins and integrates signals through protein phosphorylation. Proc Nat Acad Sci 110:E2885–E2894 12. Gueth-Hallonet C, Wang J, Harborth J, Weber K, Osborn M (1998) Induction of a regular nuclear lattice by overexpression of NuMA. Exp Cell Res 243:434–452 13. Koos B, Bender S, Witt H, Mertsch S, Felsberg J, Beschorner R, Korshunov A, Riesmeier B, Pfister S, Paulus W, Hasselblatt M (2011) The transcription factor evi-i is overexpressed, promotes proliferation, and is prognostically unfavorable in infratentorial ependymomas. Clin Cancer Res 17:3631–3637 14. Wang H, Schaefer T, Konantz M, Braun M, Varga Z, Paczulla AM, Reich S, Jacob F, Perner S, Moch H, Fehm TN, Kanz L, Schulze-Osthoff K, Lengerke C (2017) Prominent oncogenic roles of EVII in breast carcinoma. Cancer Res 77:2148–2160
15. Kustikova OS, Schwarzer A, Stahlhut M, Brugman MH, Neumann T, Yang M, Li Z, Schambach A, Heinz N, Gerdes S, Roeder I, Ha TC, Steinemann D, Schlegelberger B, Baum C (2013) Activation of Evi1 inhibits cell cycle progression and differentiation of hematopoietic progenitor cells. Leukemia 27:1127–1138
16. Koga S, Yamaguchi N, Abe T, Minegishi M, Tsuchiya S, Yamamoto M, Minegishi N (2007) Cell-cycle-dependent oscillation of GATA2 expression in hematopoietic cells. Blood 109:4200–4208
17. Ivanochko D, Halabelian L, Henderson E, Savitsky P, Jain H, Marcon E, Duan S, Hutchinson A, Seitoa A, Barsyte-Lovejoy D, Filippakopoulos P, Greenblatt J, Lima-Fernandes E, Arrowsmith CH (2019) Direct interaction between the PRDM3 and PRDM16 tumor suppressors and the NuRD chromatin remodeling complex. Nucleic Acids Res 47:1225–1238
18. Bard-Chapeau EA, Gunaratne J, Kumar P, Chua BQ, Muller J, Bard FA, Blackstock W, Copeland NG, Jenkins NA (2013) EVI1 oncoprotein interacts with a large and complex network of proteins and integrates signals through protein phosphorylation. Proc Natl Acad Sci USA 110:E2885–E2894

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.