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EVI1 mediated down regulation of *miR-449a* is essential for the survival of *EVI1* positive leukemic cells

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Running title: The role of miR-449a in *EVI1* pathogenesis

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SUMMARY

Chromosomal rearrangements involving the *Ecotropic Viral Integration site 1* (*EVI1)* locus are recurrent genetic events in myeloid leukemia and are associated with poor prognosis. In this study, we assessed the role of *EVI1* in the transcriptional regulation of microRNAs (miRNAs) involved in the leukemic phenotype. For this, we profiled expression of 366 miRNAs in 38 *EVI1* rearranged patient samples, normal bone marrow controls and *EVI1* knock down/re-expression models. Cross-comparison of these miRNA expression profiling data showed that *EVI1* rearranged leukemias are characterized by down regulation of *miR-449a*. Reconstitution of *miR-449a* expression in *EVI1* rearranged cell line models induced apoptosis resulting in a strong decrease in cell viability. These effects might be mediated in part by *miR-449a* regulation of *NOTCH1* and *BCL2* which are shown here to be *bona fide miR-449a* targets. Finally, we confirmed that *miR-449a* repression is mediated through direct promoter occupation of the *EVI1* transcriptional repressor.

In conclusion, this study reveals *miR-449a* as a crucial direct target of *EVI1* involved in the pathogenesis of *EVI1* rearranged leukemias and unravels *NOTCH1* and *BCL2* as important novel targets of *miR-449a*. This *EVI1-miR-449a-NOTCH1/BCL2* regulatory axis might open new possibilities for the development of therapeutic strategies in this poor prognostic leukemia subgroup.

KEYWORDS

EVI1; miR-449a; myeloid malignancies; NOTCH; BCL2

Introduction

Chromosomal rearrangements involving the Ecotropic Viral Integration site 1 (*EVI1*) gene on chromosome band 3q26.2 are a recurrent finding in malignant myeloid disorders (Nucifora 1997). *EVI1* gene rearrangements (translocations and inversions) account for approximately 5% of cytogenetic abnormalities in acute myeloid leukemias (AML), myelodysplastic syndromes (MDS) and chronic myeloid leukemias in blast crisis (CML-BC) (Buonamici, *et al* 2003). *EVI1* transcriptional activation has also been reported in approximately 8 – 15% of AML patients without chromosomal defects affecting the *EVI1* locus (Haas, *et al* 2008, Lugthart, *et al* 2008).

The *EVI1* gene encodes a zinc finger transcription factor that mainly acts as a repressor of gene expression, but relatively few data are available on the downstream targets and pathways involved in *EVI1* pathogenesis (Qiu, *et al* 2008, Spensberger and Delwel 2008, Takahashi and Licht 2002, Yatsula, *et al* 2005, Yuasa, *et al* 2005). As survival of patients with *EVI1* overexpressing leukemias is poor (Barjesteh van Waalwijk van Doorn-Khosrovani, *et al* 2003, Haas, *et al* 2008, Lugthart, *et al* 2008), new insights into the molecular pathogenesis of these leukemias are needed for rational development of targeted therapies.

MicroRNAs (MiRNAs) are 19-24 nucleotide long RNA molecules that can interfere with gene expression through degradation of the mRNA or inhibition of the translational machinery (Medina and Slack 2008). Recently, miRNA deregulation has been identified as a major contributor to cancer initiation and progression (Deng, *et al* 2008). In particular, miRNA genes have been shown to be directly regulated by activated oncogenes such as the *miR-17-92* cluster which is up regulated by *MYC/MYCN* (Bray, *et al* 2009, Mendell 2008). In view of these observations, we hypothesized that the malignant phenotype of *EVI1* overexpressing leukemic cells might be, at least in part, explained through deregulation of miRNAs controlling the expression of genes critically involved in oncogenesis. To test this hypothesis, we analysed the expression of 366 miRNAs in a large set of bone marrow samples from patients with *EVI1* rearrangements and crossed this data with miRNA profiling of two functional *EVI1* knockdown models.

METHODS

Patients and cell lines

Bone marrow samples from 27 AML patients, 6 MDS patients and 5 CML patients were collected in a multi-centre setting. Patients were included in the study after confirmation

of *EVI1* rearrangement by karyotyping and Fluorescence *In Situ* Hybridization (FISH) (RP11-362K14, RP11-82C9 and RP11-694D5) and confirmation of *EVI1* ectopic expression with reverse transcription quantitative PCR (RT-qPCR) (De Weer*, et al* 2008). Six normal bone marrow samples from patients without a hematological malignancy were also included in the study. The study was approved by the ethical committee of the Ghent University Hospital (2003/273).

Two *EVI1* rearranged cell lines (Kasumi-3 and UCSD-AML1) were used to develop the knockdown models and cultured as previously described (Asou*, et al* 1996, Oval*, et al* 1990). The Kasumi-3 cell line was purchased from ATCC (Middlesex, UK; ATCC#CRL-2725) and the UCSD-AML1 was a generous gift from dr. Bernard O. (Paris, France). For modulation of miRNA expression, two additional AML cell lines, ME-1 (Yanagisawa*, et al* 1991) and NB-4 (Lanotte*, et al* 1991), without *EVI1* rearrangement or *EVI1* overexpression were included. Furthermore, the two AML cell lines TF-1 (*EVI1* overexpressing) and MOLM-13 (*EVI1* negative) were used for chromatin immunoprecipitation (ChIP) experiments and were maintained in RPMI-1640, supplemented with 1% penicillin-streptomycin, and 20% FBS (GIBCO-BRL, Grand Island, NY, USA) and additionally 5ng/ml GM-CSF (Promocell, UK) for TF-1. The HEK293T cell line was maintained in RPMI-1640 supplemented with 10% fetal bovine serum, Glutamine (2 mM) and penicillin (100 U/ml)-streptomycin (100 µg/ml). Patient and *EVI1* rearranged cell line characteristics and karyotypes are listed in Supplementary Table 1.

In order to validate the differentially expressed miRNAs, a tetracycline regulable U937 model system (U937T EVI1-HA) was used and propagated in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.5 µg/ml puromycin, 1 µg/ml tetracycline, and 500 µg/ml hygromycin (Konrad*, et al* 2009).

Knock down models

An *EVI1* knockdown model was developed in two *EVI1* rearranged cell lines, Kasumi-3 and UCSD-AML1, by electroporation of two *EVI1* targeting small interfering RNAs (siRNA) (Invitrogen, Belgium). To evaluate the role of *NOTCH1* and *BCL2* in *EVI1* pathogenesis, knockdown models of each gene were created in Kasumi-3 and UCSD-AML1 using a combination of four *NOTCH1* specific siRNAs (Dharmacon, USA) or four *BCL2* specific siRNAs (Dharmacon, USA), respectively. The siRNA sequences are listed in Supplementary Table 2.

The Kasumi-3 cells were electroporated at 250 V, 1000 µF and the UCSD-AML1 cells at 300 V, 1000 µF (exponential decay pulse) (Genepulser II, Bio-Rad, USA), respectively, using 100 nM of the *EVI1*, NOTCH1 or BCL2 siRNAs.

The cell lines were also electroporated with a scrambled control siRNA duplex (Invitrogen, Belgium or Dharmacon, USA) in order to account for any effect of the electroporation itself and for possible off-target effects. The cells were subsequently evaluated for knockdown at the mRNA level by RT-qPCR as previously described (Poppe*, et al* 2006, Vandesompele*, et al* 2002). The reference genes *RPL13A* and *YWHAZ* were used for normalization after selection using the geNorm software (Vandesompele*, et al* 2002). The qbasePLUS software version 1.2 (Biogazelle, Belgium) was used for the calculations of the RT-qPCR results (Hellemans*, et al* 2007). The primer sequences are listed in Supplementary Table 3.

Knock down of EVI1, NOTCH1 and BCL2 on the protein level was evaluated by Western blotting. Protein isolation was performed with the Nuclear protein Extraction Kit (Pierce, Belgium). Protein concentrations were determined using the Bradford reagent (Bio-Rad, Belgium). For Western blotting, 20 µg of protein was loaded onto a 7.5% (EVI1) or a 10% (NOTCH1 and BCL2) pre-cast gel (Bio-Rad, Belgium) and the Western blot procedure was performed according to the manufacturer's descriptions using a monoclonal EVI1 antibody (1/2000, #2265, Cell Signalling Technologies, The Netherlands), polyclonal NOTCH1 or BCL2 antibodies (1/1000, 2421S and 2876; Cell Signalling Technologies, The Netherlands) and an α-tubulin antibody (1/5000, Sigma-Aldrich, Belgium) (Girish and Vijayalakshmi 2004).

MicroRNA expression profiling

The Trizol reagent (Invitrogen, Belgium) was used to extract total RNA from total bone marrow samples or bone marrow leukocytes for all patients and normal bone marrow samples. All RNA fractions from cell lines were isolated with the miRNeasy mini kit (Qiagen, Belgium). MiRNA expression profiling was performed using high-throughput stem-loop RT-qPCR as previously described (Mestdagh*, et al* 2008, Mestdagh*, et al* 2009).

To identify differentially expressed miRNAs between the *EVI1* siRNA treated fraction and controls fraction, we used fold change analysis (cut-off level of 1.5, $p<0.05$) (Chang*, et al* 2008, Hu*, et al* 2008, Ohlsson Teague*, et al* 2009, Pradervand*, et al* 2009, Tzur*, et al* 2008, Wang*, et al* 2009). Significant differences ($p<0.05$) in miRNA expression between

normal bone marrow and *EVI1* deregulated leukemia were identified with the Rank Product algorithm (Breitling*, et al* 2004).

Modulation of microRNA expression

To evaluate the phenotypic role of specific differentially expressed miRNAs, Kasumi-3, UCSD-AML1, NB-4 and ME-1 cells (2 x 10^6) were electroporated with 100 nM of the precursor *miR-449a* (PM11127; Applied Biosystems, Belgium) or 100 nM of the anti-miRNAs *miR-213* and *miR-107* (AM10381 and AM10056; Applied Biosystems, Belgium), according to the above described electroporation conditions for Kasumi-3 and UCSD-AML1. The NB-4 and ME-1 cells were electroporated at 300 V, 1000 µF (exponential decay pulse). As a control, cells were electroporated with 100 nM of a scrambled precursor or a scrambled anti-miRNA (Applied Biosystems, Belgium), respectively.

Cell viability and apoptosis assays

Upon electroporation of cells, cell viability and apoptosis were determined using the CellTiter-Glo assay (Promega, Belgium) and Caspase-Glo 3/7 assay (Promega, Belgium), respectively, according to the manufacturer's descriptions. Effects on apoptosis were confirmed using flow cytometry with AnnexinV staining (Bender Medsystems, Belgium).

Chromatin immunoprecipitation (ChIP)

ChIP was carried out on two AML cell lines, the TF-1 (*EVI1* over-expressing) and MOLM-13 (*EVI1* negative). ChIP samples were prepared using 10^7 cells of each cell line per condition. Chromatin was fragmented with a Bioruptor (Diagenode, Belgium) for 30 minutes (30 seconds pulses, 30 seconds pauses). ChIP was carried out according to the manufacturer's protocol (High Cell ChIP kit, Diagenode, Belgium, Catalog # kch-mahigh-G16) using anti-EVI1 (Cell Signalling Technology, Catalog #2593) or an equal amount of IgG isotype as negative control (Cell Signaling Technology, Catalog #2729) or anti-RNA Polymerase II (Millipore, Catalog #20-296) as positive control. Real-time PCR was used to amplify immunoprecipitated DNA using Power Sybr Green (Applied Biosystems) and a 7500 real-time PCR instrument (Applied biosystems). PCR results were calculated using the ΔΔCt method. The amount of immunoprecipitated DNA in each experiment is represented as signal relative to the amount of input and was calculated with the quantitative real-time PCR. Primers used for the ChIP experiment covered the potential

EVI1 binding site in the *miR-449a* promoter region (-4875bp upstream of transcription initiation site; forward 5' TCTGCAGATATGGATCAATTCAA 3', reverse 5'GAAACAAGACATGCCAACCA-3') and regions covering previously described direct EVI1 target genes *PBX1* (Shimabe*, et al* 2009), *FAM83B* and *CRHBP* (Lugthart, *et al* 2011) as positive controls.

Luciferase reporter experiments

Sixty-three basepair fragments containing the predicted target sites of *miR-449a* in the *NOTCH1* and *BCL2* 3' untranslated regions (UTR), as well as mutant target sites with 3 point mutations, were cloned into the *NotI* and *XhoI* cloning sites of the PsiCheck-2 vector (Promega, Belgium). For this purpose, synthetic oligonucleotides (Supplementary Table 4) were annealed for 3 min at 90°C and 1h at 37°C. Ligation into the PsiCheck-2 vector was performed using a T4 ligase (Promega, Belgium). Subsequently, competent *E. coli* cells were transformed with the *NOTCH1* and *BCL2* predicted binding site constructs and DNA was isolated using the High Pure Plasmid isolation kit (Roche, Belgium).

HEK293 cells (80 000 cells) were transfected with 200 ng of the *NOTCH1* or *BCL2* 3'UTR PsiCheck-2 constructs in combination with 100 ng of the precursor *miR-449a* (Applied Biosystems, Belgium) using the DharmaFECT Duo transfection reagent (Dharmacon, USA). After 24h incubation, luciferase activity was assayed using the Dual-Glo luciferase assay system (Promega, Belgium) on a FLUOstar OPTIMA (BMG labtechnologies GmbH, Germany).

Target gene expression analysis

To investigate the expression of candidate *miR-449a* target genes *NOTCH1* and *BCL2,* RT-qPCR on Kasumi-3 and UCSD-AML1 cells treated with a precursor *miR-449a* or with a scrambled precursor miRNA was performed as previously described (Poppe*, et al* 2006, Vandesompele*, et al* 2002). The reference genes *RPL13A*, *GAPDH* and *YWHAZ* were used for normalization as described above, and the qBasePlus software version 1.2 (Biogazelle, Belgium) was used for the calculations (Hellemans*, et al* 2007). Primer sequences for all tested genes are listed in Supplementary Table 3. Effects of precursor *miR-449a* electroporation on NOTCH1 and BCL2 protein levels was measured using Western blotting as described above.

Statistical analysis

For the identification of differentially expressed miRNAs, we used fold change analysis (cut-off level of 1.5, p<0.05) (Chang, *et al* 2008, Hu, *et al* 2008, Ohlsson Teague, *et al* 2009, Pradervand, *et al* 2009, Tzur, *et al* 2008, Wang, *et al* 2009) and the Rank Product algorithm (Breitling, *et al* 2004). Statistical analysis of differences in cell survival/apoptosis was calculated by the Student's t-test. Values were considered statistically significant at $p < 0.05$.

Results

Identification of EVI1 regulated microRNAs

In order to discover possible miRNAs that participate in *EVI1* mediated transformation, we performed miRNA expression analysis in *EVI1* knock down models and crossed these data with miRNA profiles of 38 *EVI1* rearranged patient samples and 6 normal bone barrow samples.

RNAi mediated knock down of *EVI1* was performed in the *EVI1* overexpressing cell lines Kasumi-3 and UCSD-AML1 and *EVI1* knock down was confirmed on mRNA and protein level (Supp Fig 1A, B). Differentially expressed miRNAs were identified according to a fold change analysis with a cut-off of 1.5 (p<0.05) (Supp Table 5).

Next, rank product analysis (p<0.05) was used to compare miRNA profiles of *EVI1* rearranged samples and 6 normal bone marrow samples, which lead to the identification of 25 down regulated and 24 up regulated miRNAs in *EVI1* samples compared to normal bone marrow (Supp Table 6).

Three down regulated (*miR-190*, *miR-215* and *miR-449a*) and 3 up regulated miRNAs (*miR-213*, *miR-187* and *miR-107*) were identified as differentially expressed in both the *in vitro* knock down models and the primary patient samples. Of these overlapping miRNAs, three (*miR-449a*, *miR-213* and *miR-107*) were selected for further functional analysis, based on a known role in tumorigenesis (Noonan, *et al* 2009, Roldo, *et al* 2006, Wong, *et al* 2008). Figure 1 represents the overall strategy for miRNA selection used in this study. Figure 2 shows the expression of *miR-449a*, *miR-213* and *miR-107* in primary leukemia samples, normal bone marrow and the *EVI1* knock down models.

Modulation of miR-449a, miR-213 and miR-107 expression decreases cell viability and increases apoptosis of EVI1 overexpressing leukemic cells

In order to evaluate a potential functional role of these miRNAs in *EVI1* overexpressing leukemic cells, we modulated the expression levels of *miR-449a* with a precursor miRNA or *miR-213* and *miR-107* with anti-miRNAs and measured the effects on cell viability and apoptosis.

Electroporation of anti-miRNAs directed against *miR-213* and *miR-107* as single agents resulted in a significant decrease in cell viability in the Kasumi-3 cell line (Fig 3A). Interestingly, the largest impact on cell viability was noticed after administration of the precursor *miR-449a*, with a decrease of 95% for Kasumi-3 at the 96h time point (Fig 3A). Electroporation of Kasumi-3 with the precursor *miR-449a* and anti-miRNAs *miR-213* and *miR-107* resulted in an increase in caspase-3 and/or caspase-7 activity, 48h after electroporation, indicative of induction of apoptosis. Again, the largest effects were seen after modulation of *miR-449a* expression (Fig 3B). Similar results both for cell viability as well as induction of apoptosis were obtained for the UCSD-AML1 cell line (Fig 3C, D).

To confirm the specificity of the observed effects on cell viability and apoptosis in *EVI1* deregulated cells, modulation of the expression levels of *miR-449a*, *miR-213* and *miR-107* were also performed in two cell lines without *EVI1* rearrangement, namely NB-4 and ME-1. *MiR-449a*, *miR-213* or *miR-107* were expressed at similar levels in these two cell lines as compared to normal bone marrow samples (data not shown). For these cell lines, no significant effects were observed on cell viability or apoptosis upon re-expression of *miR-449a* or inhibition of *miR-213* and *miR-107* (Supp Fig 2A-D).

Taken together, the above findings suggest that low *miR-449a* expression and high *miR-213*/*miR-107* expression are critical for the survival of *EVI1* deregulated cell lines. Given the fact that the most prominent functional effects were achieved upon modulation of *miR-449*, we decided to select this particular miRNA for further analysis.

MiR-449a is down regulated in a regulable EVI1 overexpression model

To further confirm *EVI1* mediated transcriptional regulation of *miR-449a*, we used a tetracycline inducible U937 *EVI1* overexpression model (Konrad, *et al* 2009). Using this model, *miR-449a* was shown to be rapidly down regulated following overexpression of the *EVI1* oncogene (Fig 4A). The results from this validated *EVI1* regulable model system are in accordance with our previous observations in the *EVI1* knock down cell line models and EVI1 rearranged patient samples, thus further supporting the robustness of *miR-449a* repression in *EVI1* overexpressing leukemic cells.

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MiR-449a is a direct target of the transcriptional repressor EVI1

The short time interval between *EVI1* upregulation and subsequent *miR-449a* downregulation in the U937 *EVI1* overexpression model (Fig 4A) is suggestive for a direct regulation of transcription of the *miR-449a* gene by the *EVI1* transcription factor. To test this hypothesis, we identified a potential EVI1 binding site 4875 bp upstream of the miR-449a transcription start site using MatInspector 37 (Fig 5A) and performed EVI1 ChIP analysis. Despite extensive experimental efforts, we were not able to optimize the ChIP protocol in Kasumi-3 or UCSD-AML1 cell lines, and we therefore performed ChIP assays in the *EVI1* overexpressing cell line TF-1 and the *EVI1* negative MOLM-13 cell line as negative control. In this analysis, we showed specific DNA binding of EVI1 to the promoter of *miR-449a* similar to previously described direct EVI1 target genes *PBX1* (Shimabe*, et al* 2009), *FAM83B* and *CRHBP* (Lugthart, *et al* 2011) (Fig 5B).

NOTCH1 and BCL2 are direct miR-449a targets

In order to identify *miR-449a* target genes, we interrogated 4 different miRNA target prediction algorithms (DIANA-microT, miRanda, PicTar, Targetscan), which rendered a list of 1607 candidate genes. This list was narrowed down to 15 genes by selecting for those genes predicted by at least 2 or more algorithms and those genes with known functions in cell viability, cell cycle regulation, apoptosis or differentiation (Supp Table 7). Next, RT-qPCR expression analysis of these candidate genes revealed decreased *NOTCH1* and *BCL2* mRNA levels in Kasumi-3 and UCSD-AML1 cells following treatment with a *miR-449a* precursor, as compared to control cells. This effect was not observed using housekeeping genes, ruling out possible broad off-target effects (Fig 6A). NOTCH1 and BCL2 modulation upon *miR-449a* re-expression was also confirmed on the protein levels (Fig 6B).

Next, we used luciferase assays to confirm that *NOTCH1* and *BCL2* are *bona fide miR-449a* targets. The decrease in luciferase activity was rescued by mutation of 3 critical bases in the predicted binding sites for *miR-449a* in the 3' UTR of *NOTCH1* and *BCL2* (Fig 6C, D). Finally, we used the tetracycline regulable U937 *EVI1* overexpression model (Konrad*, et al* 2009) to show additional evidence of increased *NOTCH1* and *BCL2* expression upon *EVI1* overexpression (Fig 4B), as expected from *miR-449a* repression in this model (Fig 4A).

MiR-449a exerts effects on cell viability and apoptosis partially through repression of NOTCH1 and BCL2 expression

To evaluate the role of *NOTCH1* and *BCL2* in relation to the observed phenotypic effects upon modulation of *miR-449a* expression, we performed knock down of *NOTCH1* and *BCL2* by use of siRNAs in Kasumi-3 and UCSD-AML1 and evaluated the effects on cell viability and apoptosis. Knock down of *NOTCH1* and *BCL2* was confirmed at the mRNA level using RT-qPCR and at the protein level using Western blotting (Supp Fig 3A-D). A decrease in cell viability and an increase in caspase-3 and/or caspase-7 activity, indicative of apoptosis, was observed in Kasumi-3 following *NOTCH1* and *BCL2* knock down, respectively. The most prominent effects were observed for *BCL2* knock down (Figure 7A-B). Similar results were obtained for UCSD-AML1 (data not shown). Taken together, these results suggest that the observed effects on cell viability and apoptosis by *miR-449a* re-expression are at least in part due to suppression of the expression of its target genes *NOTCH1* and *BCL2*.

Discussion

Leukemias over expressing the *EVI1* oncogene are responsible for ≥10% of all myeloid leukemias and are characterized by a poor prognosis. Recently, it was shown that transcription factors can directly regulate the expression of miRNAs, as well as of mRNAs, adding another dimension to the transcriptional control of downstream pathways (Schulte*, et al* 2008). As miRNAs are involved in processes such as proliferation, apoptosis and differentiation (Medina and Slack 2008), pathways that are deregulated in cancer, identification of miRNAs involved in *EVI1* pathogenesis could offer novel therapeutic strategies and further insight into leukemic initiation and progression.

In this study, we identified a distinct series of up- and down regulated miRNAs following expression profiling of 366 miRNAs using stem loop RT-qPCR of 38 *EVI1* rearranged and overexpressing patient samples, normal bone marrow samples, and two *EVI1* cell line knock down model systems. Upon modulation of three selected miRNAs (*miR-449a*, *miR-213* and *miR-107*), a decrease in cell viability and an increase in apoptosis was observed, with the most prominent effects observed after precursor *miR-449a* electroporation. For the latter miRNA, we further validated its expression in relation to *EVI1* expression levels using a tetracycline regulable *EVI1* overexpression model system. *MiR-449a* expression was indeed suppressed in less than 12 hours after

switching on *EVI1* expression, suggesting a direct regulation. In concordance with these data, we confirmed that *miR-449a* is a direct transcriptional target of EVI1.

Other evidence in the literature highlights the importance of *miR-449a* in cancer. Recently, the *miR-449a* promoter was shown to be hypermethylated in sarcoma (Yang*, et al* 2009). *MiR-449a* was also described as down regulated in prostate cancer and re-expression studies of *miR-449a* in prostate cancer cells resulted in cell cycle arrest and apoptosis (Noonan*, et al* 2009), similar to the results obtained in this study. In addition, a recent study showed that the tumor suppressor function of *miR-449a/b* might in part be mediated through regulation of Rb/E2F1 activity and subsequently *miR-449a/b* inhibits *CDK6* and *CDC25A*, resulting in a negative feedback loop. Furthermore, the expression of *miR-449a/b* was shown to be epigenetically silenced through histone H3 Lys27 trimethylation. This would suggest that escape from Rb/E2F1 regulation through an aberrant epigenetic event contributes to E2F1 deregulation and unrestricted proliferation in human cancer (Noonan*, et al* 2010, Yang*, et al* 2009).

In this study, we show for the first time that *BCL2* and *NOTCH1* are direct *miR-449a* targets. *BCL2* is an anti-apoptotic gene capable of antagonizing the p53 pathway, and has been described as over expressed in different types of lymphoma and AML (Nagy*, et al* 2003, Thomadaki and Scorilas 2006). The *NOTCH1* gene, which is part of a type 1 transmembrane protein family, is involved in regulation of self-renewal, apoptosis and differentiation. Up regulation of *NOTCH1* has already been described in T-cell acute lymphoblastic leukemia (T-ALL), where *NOTCH1* activating mutations occur in about 50% of the patients (Palomero and Ferrando 2008). Interestingly, the *C. elegans EVI1* homolog *EGL-43* is known to act downstream of *NOTCH* signalling in cell fate specification (Hwang*, et al* 2007). These results indicate that the relationship between *NOTCH1* and *EVI1* might be highly context dependent, differing from normal developing cells to cancer cells.

An important failsafe program against tumorigenesis is provided by the gatekeeper protein p53, and it is generally accepted that this barrier must be overcome during the ontogenesis of a tumour. Interestingly, both *NOTCH1* (Beverly*, et al* 2005, Rosati*, et al* 2009, Secchiero*, et al* 2009) and *BCL2* (Thomadaki and Scorilas 2006) have been implicated in suppression of p53 activity in leukemic cells. Therefore, our findings position repression of *miR-449a* as a means by which *EVI1* leukemic cells circumvent the p53 anti-tumour barrier and it is tempting to speculate that this suppression of the p53 pathway relies on up regulation of both *BCL2* and *NOTCH1* expression.

Interestingly, as *EVI1* is associated with a stem cell phenotype (Goyama*, et al* 2008, Takeshita*, et al* 2008), regulation of *NOTCH1*, which is required for self-renewal, through an *EVI1-miR-449a* axis, could lead to survival of leukemic stem cells which are resistant to chemotherapeutics (Misaghian*, et al* 2009). Therefore, targeting *NOTCH1* might aid in eliminating those cells that are likely to cause disease relapse (Drewa*, et al* 2008).

In conclusion, our study uncovered *miR-449a* as a crucial direct target of *EVI1* involved in the pathogenesis of *EVI1* rearranged leukemias and unravels *NOTCH1* and *BCL2* as important novel targets of *miR-449a*. Selective restoration of *miR-449* expression, gamma secretase inhibitors targeting *NOTCH1* or small molecule inhibitors targeting *BCL2* may prove valuable as new therapeutic strategies in this poor prognostic subgroup of AML patients.

At the time of submission of our report, also other studies have investigated the role of *EVI1* in miRNA regulation in myeloid leukemias. Dickstein and colleagues demonstrated that *miR-124* is silenced by induced *EVI1* expression in a murine MDS model (Dickstein*, et al* 2010), and in line with these results, Vazquez and colleagues found decreased expression of *miR-124a* in myeloid cell lines and AML patients with *EVI1* overexpression (Vazquez*, et al* 2010). In another study, it was shown that *miR-1-2* and *miR-133-a-1* expression was correlated with *EVI1* expression in AML cell lines and AML patient samples (Gomez-Benito*, et al* 2010). Although we were able to detect *miR-133a* up regulation in *EVI1* rearranged patients compared to normal bone marrow samples, we did not find significant correlation of *EVI1* expression with the other reported miRNAs (e.g. *miR-124a* and *miR-1-2).* However, in depth comparison of the different studies is complicated by the fact that the datasets in the above mentioned studies are not fully accessible, and conclusions from these comparisons should be carefully interpreted as the different datasets were generated from different organisms, model systems and patient samples. In conclusion, based on a large cohort of *EVI1* rearranged patient samples and *EVI1* model systems, we found a strong correlation with *EVI1* expression for several miRNAs, and we were able to demonstrate a functional role for *miR-449a* in *EVI1* pathogenesis.

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FIGURE LEGENDS

Figure 1. Overall strategy for microRNA selection. The expression of 384 small RNAs was determined for 38 *EVI1* rearranged patient samples, 6 normal bone marrow samples and *EVI1* knock down model systems of the *EVI1* rearranged cell lines Kasumi-3 and UCSD-AML1. Analysis of the microRNA profiles lead to the identification of 25 downregulated and 24 upregulated microRNAs in *EVI1* samples compared to normal bone marrow ($p<0.05$). Three downregulated (*miR-190*, *miR-215* and *miR-449a*) and 3 upregulated microRNAs (*miR-213*, *miR-187* and *miR-107*) were identified as differentially expressed in both the cell line models and patient samples. Of these overlapping microRNAs, three (*miR-449a*, *miR-213* and *miR-107*) were selected for further functional analysis, based on a known role in tumorigenesis (*miR-213* and *miR-107*) or homology to microRNAs with an established role in cancer (*miR-449a*). *MiR-449a* was selected for further analysis, as the most prominent functional effects were achieved upon modulation of this microRNA.

Figure 2. *MiR-449a*, *miR-213* and *miR-107* expression in *EVI1* rearranged patient samples and EVI1 knockdown models

Expression levels of A) *miR-449a*, B) *miR-213* and C) *miR-107*, in 38 *EVI1* deregulated patient samples, 6 normal bone marrow samples and two *EVI1* rearranged cell lines Kasumi-3 and UCSD-AML1 (with or without *EVI1* siRNA electroporation, triplicate) as determined with RT-qPCR. Red bars indicate the median expression.

Figure 3. *MiR-449a* re-expression or inhibition of *miR-213* or *miR-107* results in decreased cell viability and increased apoptosis in the Kasumi-3 and UCSD-AML1 cell lines

Kasumi-3 (A,B) or UCSD-AML1 (C,D) cells were electroporated with precursor *miR-449a*, *anti-miR-213* or *anti-miR-107* and effects on cell viability (A+C) and caspase-3 and caspase-7 activity (B+D) were measured 96h (cell viability) or 48h (apoptosis) after electroporation. Measurements were performed in duplicate for each condition within one biological experiment. Viability or caspase-3 and caspase-7 activity of cells treated with a scrambled precursor or anti-miRNA was set to 100% or 1, respectively.

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Figure 4. *MiR-449a*, *NOTCH1* and *BCL2* expression in a tetracycline inducible U937 *EVI1* overexpression model

Expression of *miR-449a* (A) and *NOTCH1* and *BCL2* (B) in a tetracycline regulable *EVI1* overexpression model system was determined using RT-qPCR. U937T EVI1-HA cells were transferred to media without tetracycline and RNA was extracted at the indicated time points thereafter. The expression of *miR-449a*, *NOTCH1* or *BCL2* in cells transduced with an empty vector was set to 100%. Each RT-qPCR reaction was performed in duplicate for all conditions in a single experiment.

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Figure 5. *MiR-449a is a direct target of EVI1*

A) Schematic representation of the predicted EVI1 binding site in the promoter region of *miR-449a*. B) Specific DNA binding of EVI1 to the promoter region -4875 bp of the transcription start site of *miR-449a* was detected by ChIP. Real time PCR was performed on fragmented chromatin precipitated by anti-EVI1 antibody (black bars) or normal Rabbit IgG (grey bars) from an *EVI1* positive (*EVI1*$^{+}$) TF-1 cell line and the *EVI1* negative (*EVI1*$^{-}$) MOLM-13 cell line. Primers were designed to amplify the predicted EVI1 binding site in the promoter region *miR449*. Primers directed to the promoter region of the *EVI1* positive target genes *PBX1*, *FAM83B* and *CRHBP* were used as positives controls. The ratio of immunoprecipitated DNA versus input material (in %) is shown.

Figure 6. *NOTCH1* and *BCL2* are direct targets of *miR-449a*

A) Kasumi-3 cells were electroporated with the precursor *miR-449a* and the mRNA expression of *NOTCH1*, *BCL2*, *YWHAZ, RPL13A* and *GAPDH* was determined using RT-qPCR. For each time point, expression of these genes in a control fraction treated with a scrambled precursor was set to 100%. Each RT-qPCR reaction was performed in duplicate for all conditions in a single experiment. B) Kasumi-3 was electroporated with the precursor *miR-449a* and the protein levels of NOTCH1 and BCL2 were evaluated using Western blotting. As a loading control, the blots were stained with an α-tubulin antibody. C) The *NOTCH1* and *BCL2* 3'UTR *miR-449a* putative target sites (www.targetscan.org). D) Luciferase activity upon transfection of HEK293 cells with a vector containing the wild-type 3'UTR *NOTCH1 miR-449a* binding site or a vector containing the wild-type *BCL2 miR-449a* binding site in combination with the precursor *miR-449a*. Also the luciferase activity of the mutated constructs in combination with the precursor *miR-449a* was determined. Luciferase activity of cells transfected with the

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empty vector was set to 1 and data were normalized accordingly. The luciferase experiment shown is representative for two independent biological assays; in each experiment, luciferase measurements were performed in duplicate for each condition.

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Figure 7. Knockdown of *NOTCH1* or *BCL2* results in decreased cell viability and increased apoptosis

Kasumi-3 was electroporated with *NOTCH1* or *BCL2* siRNAs and A) cell viability or B) caspase-3 and caspase-7 activity was measured 96h (cell viability) or 48h (apoptosis) after electroporation. Viability or caspase-3 and caspase-7 activity of cells treated with a scrambled siRNA was set to 100% or 1, respectively. Measurements were performed in duplicate for each condition within one biological experiment.

Figures

Figure 1. Overall strategy for microRNA selection.

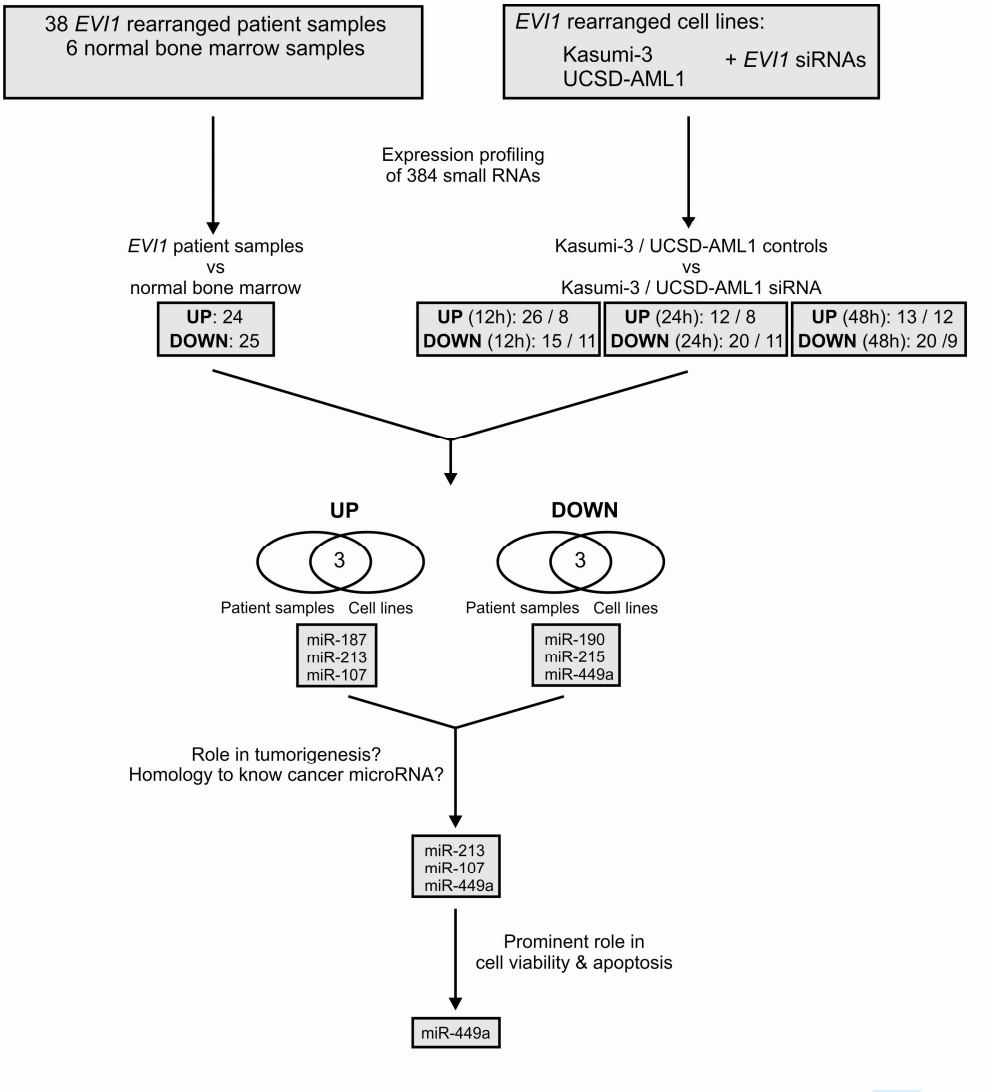

Figure 2. *MiR-449a*, *miR-213* and *miR-107* expression in *EVI1* rearranged patient samples and *EVI1* knockdown models.

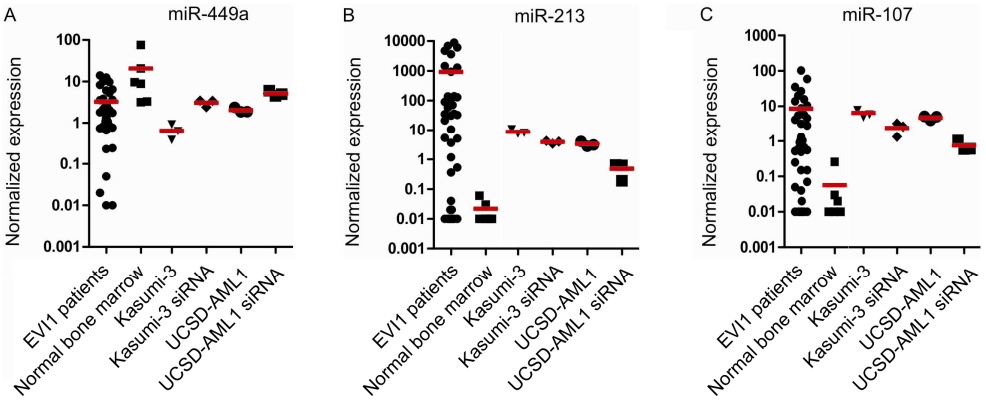

Figure 3. *MiR-449a* re-expression or inhibition of *miR-213* or *miR-107* results in decreased cell viability and increased apoptosis in the Kasumi-3 and UCSD-AML1 cell lines

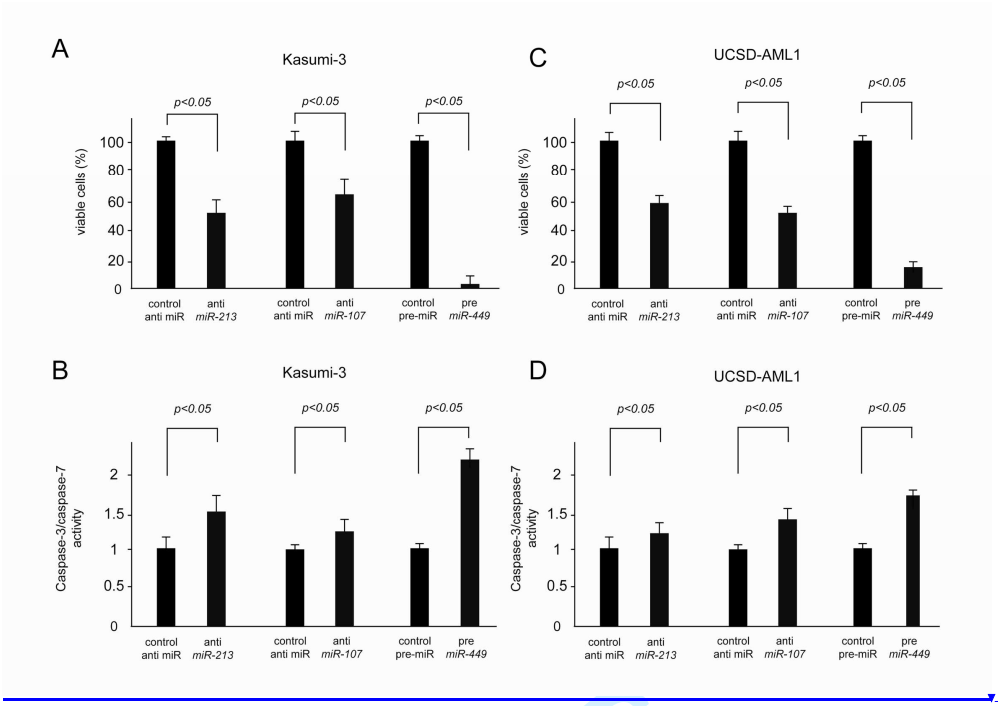

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Figure 4. *MiR-449a*, *NOTCH1* and *BCL2* expression in a tetracycline inducible U937 *EVI1* overexpression model

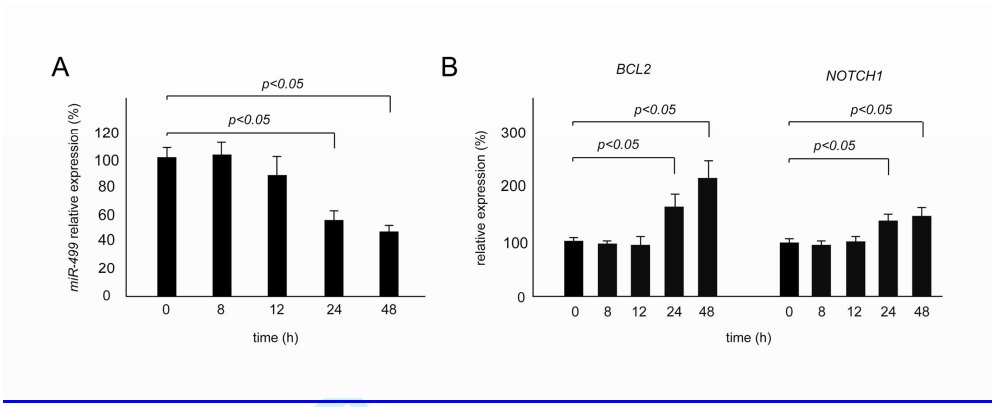


Figure 5. *MiR-449a* is a direct target of *EVI1*

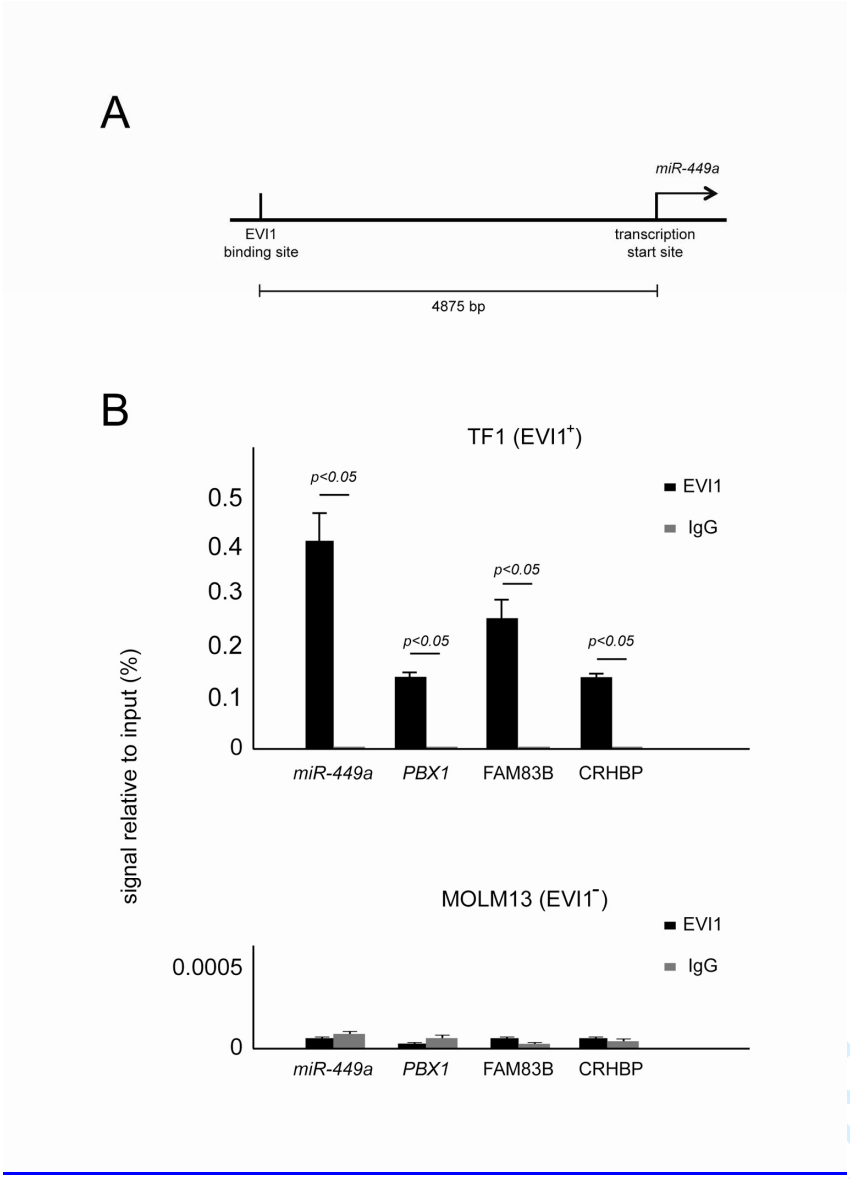


Figure 6. *NOTCH1* and *BCL2* are direct *miR-449a* targets

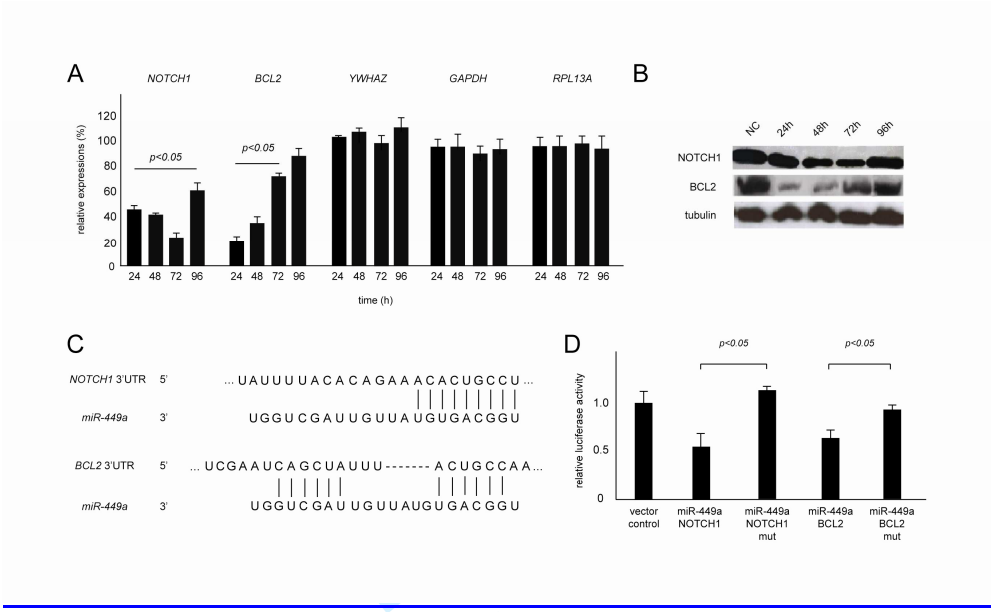
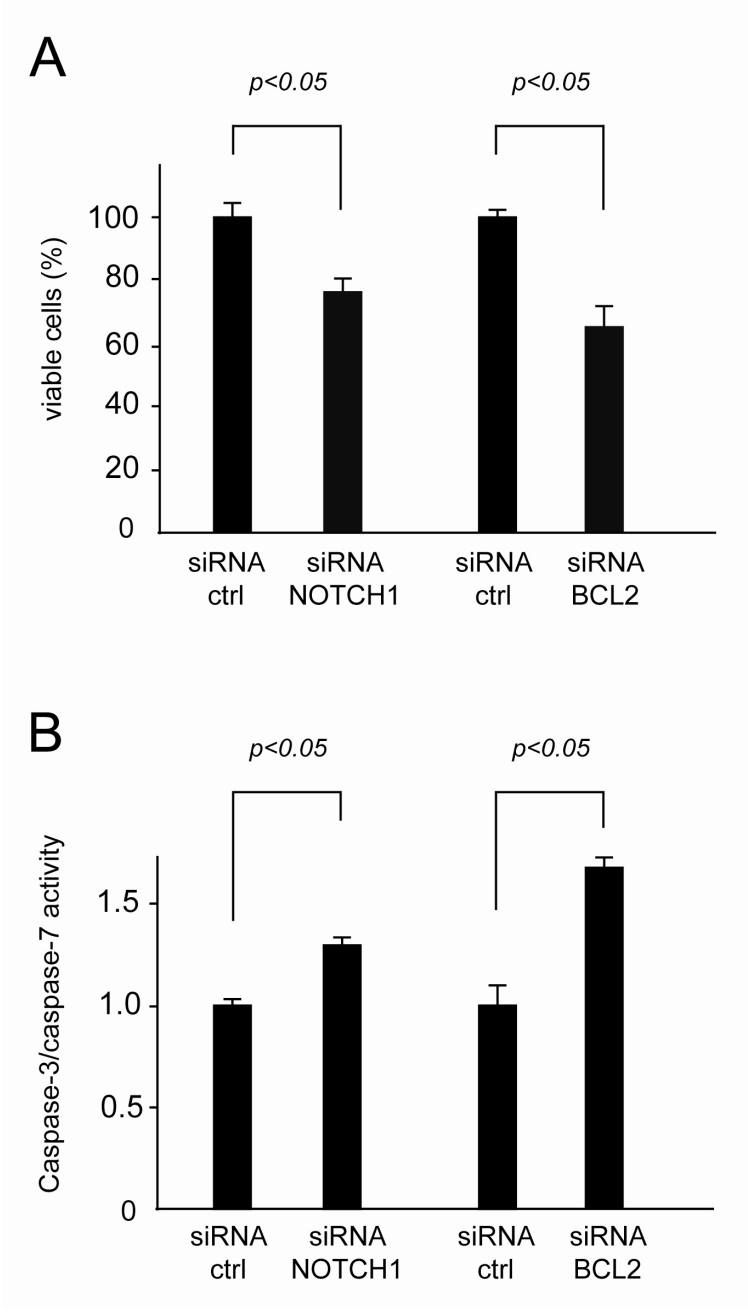


Figure 7. Knockdown of *NOTCH1* or *BCL2* results in decreased cell viability and increased apoptosis



Supplementary files

Legends to supplementary figures

Supplementary Figure 1. Introduction of *EVI1* siRNAs results in efficient *EVI1* knockdown on mRNA and protein level

Two *EVI1* overexpressing cell lines Kasumi-3 and UCSD-AML1 were electroporated with a combination of two *EVI1* targeting siRNAs and RT-qPCR and Western blotting were performed. A) RT-qPCR with *EVI1* specific primers (measurements were performed in duplicate). The expression of *EVI1* mRNA in cells treated with a scrambled siRNA duplex (NTC) was set to 100%. B) Western blotting with a monoclonal *EVI1* antibody. The 145 kDa protein band corresponding to the full length *EVI1* protein is displayed. As a loading control, the blots were treated with an α-tubulin antibody.

Supplementary Figure 2. Introduction of miR-449a or anti-miR-213 and anti-miR-107 does not significantly alter cell viability and apoptosis in non EVI1 rearranged cell line models

NB-4 (A and C) and ME-1 (B and D) cells were electroporated with the precursor miR-449a or a combination of the anti-miR-213 and anti-miR-107 and effects on A) and B) cell viability and C) and D) caspase-3 and caspase-7 activity were measured. Viability or caspase-3 and caspase-7 activity of cells treated with a scrambled precursor or anti-microRNA was set to 100% or 1, respectively.

Supplementary Figure 3. Introduction of *NOTCH1* or *BCL2* siRNAs results in efficient *NOTCH1* or *BCL2* knockdown

Kasumi-3 and UCSD-AML1 cells were electroporated with A-B) *NOTCH1* siRNAs or C-D) *BCL2* siRNAs and RT-qPCR for *NOTCH1* (A) or *BCL2* (C) expression was performed. The expression of *NOTCH1* or *BCL2* in cells treated with a scrambled siRNA duplex (NTC) was set to 100%. (B) and (D) Western blotting with a polyclonal NOTCH1 or a polyclonal BCL2 antibody was performed. As a loading control, the blots were treated with an α-tubulin antibody.

Supplementary Table 1. Patient and cell line characteristics: diagnosis, age at diagnosis and karyotype

Case	Diagnosis[1]	Age at diagnosis	Karyotype[2]
1	AML	64	45,XY,**inv(3)(q21q26)**,-7[13]
2	AML	69	45,XX,**inv(3)(q21q26)**,del(5)(q22q32),-7[14]
3	AML	51	46,XY,**inv(3)(q21q26)**[7]/ 46,XY[8]
4	MDS	71	45,XX,**t(3;8)(q26;q23)**,-7[8]
5	MDS	66	46,XY,**inv(3)(q21q26)**[8]/ 46,XY[2]
6	AML	72	43,XY,inv(2)(p25q34),**inv(3)(q21q26)**,add(10)(p15),t(11;16)(q10;p10),-17,-18,-21[15]
7	AML	56	45,X,add(x)(p22),t(1;3)(p32;p13),**inv(3)(q21q26)**,-5,del(11)(p14),+mar[15]
8	AML	70	46,XY,**t(3;6)(q26;q25)**,del(5)(q22q34)[16]
9	AML	48	46,XX,**inv(3)(q21q26)**[4]/ 46,XX[6]
10	AML	30	46,XX,**t(3;12)(q26;p13)**,del(7)(q11q35)[13]/ 46,XX[3]
11	AML	52	46,XY,**t(2;3)(p22;q26)**[15]
12	AML	66	45,XY,**inv(3)(q21q26)**,-7[20]
13	CML-BC	79	46,XY,t(9;22)(q34;q11)[5]/ 46,sl,**t(3;21)(q26;q22)**[10]
14	CML-BC	36	46,XY,t(9;22)(q34;q11)[6]/ 46,sl,**inv(3)(q21q26)**[24]
15	AML	45	48,XY,**add(3)(q26)**,+8,del(8)(q24),t(9;22)(q34;q11),+16,add(16)(p13)[9]/ 54,sl,+4,+del(8)(q24),+10,+12,+20,+der(22)t(9;22)[21]
16	MDS	71	45,XX,**inv(3)(q21q26)**,-7[10]

17	AML	42	45,XX,-7[1]/ 45,XX,sl,**t(3;21)(q26;q22)**[6]/ 46,XX[18]
18	AML	26	47,XY,t(1;6)(p22;q27),**t(3;7)(q26;p15)**,add(7)(p21),+8,t(14;15)(q24;q25)[12]/ 47,XY,inv(1)(p21q44),+8,add(13)(q34),t(16;17)(p12;q12)[8]
19	AML	52	45,X,-Y,**inv(3)(q21q26)**[5]/ 46,XY[5]
20	AML	35	46,XX,**t(3;14)(q26;q32)**[1]/ 45,sl,-7[21]
21	AML	47	46,XY,t(9;22)(q34;q11)[1]/ 46,sl,**inv(3)(q21q26)**[5]/ 46,XY[14]
22	AML	77	46,XY,**t(3;12)(q26;p12)**[8]
23	AML	40	46,XX,**ins(3)(q21;q24q26~27)**[9]/ 46,XY[1]
24	AML	76	46,XX,t(9;22)(q34;q11)[6]/ 47,sl,add(2)(q33),**t(3;21)(q26;q22)**,+der(22)t(9;22)[10]/
25	AML	1	46,XX,**t(3;21)(q21;q22)**[9]/ 46,sl,add(2)(q3?),add(4)(q23)[7]/ 46,sl,add(1)(p36)[3]
26	MDS	73	46,XX,**inv(3)(q22q26),**t(3;10)(q13;q21)[8]
27	AML	54	47,XX,**t(3;21)(q26;q22)**,+8[8]/ 46,XX[2]
28	CML	78	46,XX,**inv(3)(q21q26)**[3]/ 46,XX[21]
29	MDS	49	46,XX,**inv(3)(q21q26)**[23]/ 46,XX[1]
30	AML	78	47,XXY,**ins(3)(q26q21q26)**[10]

			47,XXY,del(7)(q22q34)[13]
31	MDS	58	46,XX,**t(3;21)(q26;q22)**[25]
32	AML	40	46,XY,**t(3;3)(q21;q26)**[1]/ 46,sl,t(1;11)(p36;p11),del(6)(q15q23)[10]/ 45,sl,t(1;5)(q21q31?),t(9;16)(q22;q24),add(10)(q26),-14[3]/ 46,XY[12]
33	AML	82	46,XY,**t(3;3)(q21;q26)**[8]/ 45,X,-Y,sl[5]/ 46,XY[1]
34	AML	41	46,XY,**t(3;3)(q21;q26)**[19]
35	AML	69	46,XY,**t(3;3)(q21;q26)**,-7,+13[6]
36	AML	65	46,XX,**t(3;21)(q26;q22)**[10]
37	CML-BC	51	46,XX,**t(3;21)(q26;q21)**,t(9;22)(q34;q11)[9]/ 48,sl,+8,+der(22)t(9;22)[2]/ 49,sl,+8,+12,+der(22)t(9;22)[2]/ 49,sl,del(6)(q16q23),+8,+12,+der(22)t(9;22)[2]/ 50,sl,del(6),+8,+12,+2xder(22)t(9;22)[2]
38	CML-BC	57	50,XY,**t(3;9;22;17)(q26;q34;q11;q22)**,+8,+der(9)t(3;9;22;17),+10,+12,del(16)(q23)[15]
Kasumi-3	AML	/	46,XY,t(2;5)(p13;q33),**t(3;7)(q26;q22)**,del(5)(q15),-8,del(9)(q32),add(12)(p11),add(16)(q13),+mar[20]
UCSD-AML1	AML	/	45,XX,**t(3;3)(q21;q26)**,-7,t(2;22)(p13;q12)[20]

[1] AML = acute myeloid leukemia, MDS = myelodysplastic syndrome and CML-BC = chronic myeloid leukemia in blast crisis

[2] The chromosomal aberration implicating the *EVI1* locus is indicated with bold formatting. Karyotype nomenclature according to ISCN 2009.

Supplementary Table 2. *EVI1*, *NOTCH1* and *BCL2* siRNA sequences

siRNA name	Sequence 5' - 3'
EVI1 siRNA 1	AUUGAAGCCAGAUUCUGAAGAGGC
EVI1 siRNA 2	UUUCGAGGCUCAGUCAGCUUUGUCC
NOTCH1 siRNA 1	GCGACAAGGUGUUGACGUU
NOTCH1 siRNA 2	GAUGCGAGAUCGACGUCAA
NOTCH1 siRNA 3	GGACAUCACGGAUCAUAUG
NOTCH1 siRNA 4	GAACGGGGCUAACAAAGAU
BCL2 siRNA 1	GGGAGAACAGGGUACGAUA
BCL2 siRNA 2	GAAGUACAUCCAUUAUAAG
BCL2 siRNA 3	GGAGGAUUGUGGCCUUCUU
BCL2 siRNA 4	UCGCCGAGAUGUCCAGCCA

Supplementary Table 3. RT-qPCR primer sequences

Primer name	Sequence 5' - 3'	RTPrimerDB ID[1]
EVI1 F	GAAAATGGTAAAATGTTCAAAGACA	7845
EVI1 R	CACCAGTCCTGTTGAACCAA	
RPL13A F	CCTGGAGGAGAAGAGGAAAGAGA	6
RPL13A R	TTGAGGACCTCTGTGTATTTGTCAA	
YWHAZ F	ACTTTTGGTACATTGTGGCTTCAA	9
YWHAZ R	CCGCCAGGACAAACCAGTAT	
NOTCH1 F	GTGACTGCTCCCTCAACTTCAAT	4670
NOTCH1 R	CTGTCACAGTGGCCGTCACT	
BCL2 F	CATGTGTGTGGAGAGCGTCAA	7846
BCL2 R	GCCGGTTCAGGTACTCAGTCA	
GAPDH F	GTCGGAGTCAACGGATT	8114
GAPDH R	AAGCTTCCCGTTCTCAG	

[1] Identification number RTPrimerDB (www.rtprimerdb.org) [57]

Supplementary Table 4. Oligonucleotide sequences comprising predicted microRNA binding sites in 3'UTR of target genes

Oligonucleotide name[1]	Sequence 5' - 3'[2]
NOTCH1 F	*TCGA*TTATGTACTTTTATTTTACACAGAAACACTGCCTTTTTATTTATATGTACTGTTTTATC
NOTCH1 R	*GGCC*GATAAAACAGTACATATAAATAAAAAGGCAGTGTTTCTGTGTAAAATAAAAGTACATAA
BCL2 F	*TCGA*CAGGCAAAACGTCGAATCAGCTATTTACTGCCAAAGGGAAATATCATTTATTTTTTACA
BCL2 R	*GGCC*TGTAAAAAATAAATGATATTTCCCTTTGGCAGTAAATAGCTGATTCGACGTTTTGCCTG
NOTCH1 mut F	*TCGA*TTATGTACTTTTATTTTACACAGAAA**C**G**C**C**G**T**C**TTTTTATTTATATGTACTGTTTTATC
NOTCH1 mut R	*GGCC*GATAAAACAGTACATATAAATAAAAAG**A**C**G**G**C**GTTTCTGTGTAAAATAAAAGTACATAA
BCL2 mut F	*TCGA*CAGGCAAAACGTCGAATCAGCTATTTA**A**T**A**C**T**AAAGGGAAATATCATTTATTTTTTACA
BCL2 mut R	*GGCC*TGTAAAAAATAAATGATATTTCCCTTT**A**G**T**A**T**TAAATAGCTGATTCGACGTTTTGCCTG

[1] F = forward, R = reverse and mut = mutated

[2] Oligonucleotide comprises *Not1* or *Xho1* restriction site (Italics), 26 nucleotides left from target sequence, target sequence according to TargetScanHuman 5.1 (www.targetscan.org) (underlined, point mutations indicated in bold formatting) and 26 nucleotides right from target sequence

Supplementary Table 5. Up- and downregulated microRNAs in two *EVI1* knockdown model systems using a combination of two *EVI1* siRNAs

DOWN regulated microRNAs after siRNA treatment

Kasumi-3

12h time point		24h time point		48h time point	
MicroRNA ID[1]	Fold change[2]	MicroRNA ID[1]	Fold change[2]	MicroRNA ID[1]	Fold change[2]
hsa-let-7f	4.0971	**hsa-mir-213**	4.3519	hsa-mir-220	3.7943
hsa-mir-544	2.8722	hsa-mir-107	4.1011	hsa-mir-500	3.4824
hsa-mir-139	2.6045	hsa-mir-204	3.6594	hsa-mir-514	3.3594
hsa-mir-432*	2.5093	hsa-mir-508	3.4388	hsa-mir-517*	2.9009
hsa-mir-187	2.2751	hsa-let-7f	3.3384	hsa-mir-302d	2.8016
hsa-mir-526b*	2.0241	hsa-mir-509	3.1167	**hsa-mir-107**	2.5760
hsa-mir-213	2.0230	hsa-mir-199b	3.0701	hsa-mir-31	2.3323
hsa-mir-107	2.0210	hsa-mir-514	2.9235	**hsa-mir-213**	2.0765
hsa-mir-514	2.0010	hsa-mir-383	2.5816	hsa-mir-497	2.0351
hsa-mir-520b	1.9017	hsa-mir-505	2.5519	hsa-let-7f	2.0250
hsa-mir-539	1.7411	hsa-mir-539	2.4426	**hsa-mir-187**	2.0140
hsa-mir-656	1.7188	hsa-mir-503	2.3200	hsa-mir-501	1.7811
hsa-mir-302c	1.7093	**hsa-mir-187**	2.2423	hsa-mir-503	1.7569
hsa-mir-632	1.5419	hsa-mir-153	2.1006	hsa-mir-573	1.7494
		hsa-mir-107	2.0105	hsa-let-7e	1.6809
		hsa-mir-9*	1.8857	hsa-mir-30e-3p	1.6481
		hsa-mir-429	1.7556	hsa-mir-520h	1.5520
		hsa-mir-374	1.6690	hsa-mir-326	1.5351
		hsa-mir-7	1.6458	hsa-mir-580	1.5342
		hsa-mir-511	1.6211	hsa-mir-509	1.5327

UP regulated microRNAs after siRNA treatment

Kasumi-3

12h time point		24h time point		48h time point	
MicroRNA ID[1]	Fold change[2]	MicroRNA ID[1]	Fold change[2]	MicroRNA ID[1]	Fold change[2]
hsa-mir-141	4.2972	hsa-mir-139	7.5819	hsa-mir-410	6.6164
hsa-mir-23b	3.9814	hsa-mir-31	3.5201	**hsa-mir-449a**	3.7619
hsa-mir-432	3.7962	hsa-mir-520d*	2.4293	hsa-mir-521	2.6209
hsa-mir-501	3.5560	hsa-mir-551a	2.3453	hsa-mir-33	2.3291
hsa-mir-335	3.3066	hsa-mir-521	2.2647	**hsa-mir-215**	2.2260
hsa-mir-215	3.2656	**hsa-mir-215**	2.1589	hsa-mir-184	1.9912
hsa-mir-502	3.0249	**hsa-mir-449a**	2.1246	hsa-mir-520g	1.9140
hsa-mir-616	2.7049	hsa-mir-656	1.9532	hsa-mir-147	1.8868
hsa-mir-503	2.5061	hsa-mir-184	1.8250	hsa-mir-135b	1.8526
hsa-mir-135b	2.4798	hsa-mir-302d	1.6844	hsa-mir-651	1.7909
hsa-mir-379	2.3692	hsa-mir-432*	1.6026	hsa-mir-507	1.7821
hsa-mir-573	2.3425	hsa-mir-526b*	1.5285	hsa-mir-302a*	1.6546
hsa-mir-511	2.2638			hsa-mir-204	1.5740
hsa-mir-216	2.1783				
hsa-mir-449a	2.1773				
hsa-mir-410	2.0212				
hsa-mir-23a	1.9675				
hsa-mir-181a	1.9423				
hsa-mir-9*	1.9218				
hsa-mir-662	1.7580				
hsa-mir-449b	1.7091				
hsa-mir-34b	1.6528				
hsa-mir-148a	1.6388				
hsa-mir-452*	1.6163				
hsa-mir-100	1.5418				
hsa-mir-184	1.5227				

UCSD-AML1

12h time point		24h time point		48h time point	
MicroRNA ID[1]	Fold change[2]	MicroRNA ID[1]	Fold change[2]	MicroRNA ID[1]	Fold change[2]
hsa-mir-107	3.4511	hsa-mir-7	13.3526	hsa-mir-7	10.4877
hsa-mir-7	2.5165	hsa-mir-627	3.4528	**hsa-mir-107**	2.7899
hsa-mir-187	2.4155	hsa-mir-147	2.9738	hsa-mir-220	1.9857
hsa-mir-503	1.8455	hsa-mir-520a	2.7131	hsa-mir-373*	1.8556

UCSD-AML1

12h time point		24h time point		48h time point	
MicroRNA ID[1]	Fold change[2]	MicroRNA ID[1]	Fold change[2]	MicroRNA ID[1]	Fold change[2]
hsa-mir-190	3.7895	hsa-mir-489	4.1702	**hsa-mir-190**	2.5778
hsa-mir-139	2.5465	hsa-mir-662	3.4666	hsa-mir-139	2.4896
hsa-mir-216	1.7575	**hsa-mir-190**	3.4624	hsa-mir-662	2.0114
hsa-mir-449a	1.7357	hsa-mir-138	2.2722	hsa-mir-622	1.9575

hsa-mir-493-3p	1.8232	hsa-mir-302a	2.5379	hsa-mir-519d	1.7690	hsa-mir-520a	1.6558	hsa-mir-139	2.1097	hsa-mir-525	1.9323
hsa-mir-525	1.7266	hsa-mir-183	2.1515	hsa-mir-302c	1.7458	hsa-mir-518c*	1.6455	**hsa-mir-449a**	1.8212	hsa-mir-409-5p	1.9118
hsa-mir-220	1.6575	**hsa-mir-107**	2.1472	hsa-mir-323	1.7211	hsa-mir-558	1.5679	hsa-mir-657	1.7214	**hsa-mir-449a**	1.7933
hsa-mir-409-5p	1.6232	hsa-mir-148a	1.9461	hsa-mir-600	1.5646	hsa-mir-137	1.5434	hsa-mir-182*	1.6082	hsa-mir-519e*	1.7480
hsa-mir-373*	1.5547	hsa-mir-521	1.7831	hsa-mir-20a	1.5545					hsa-mir-520a	1.6991
hsa-mir-302d	1.5323	hsa-mir-520c	1.6711							hsa-mir-182*	1.6428
hsa-mir-652	1.5121	hsa-mir-96	1.6345							hsa-mir-558	1.5788
										hsa-mir-23a	1.5121

[1] Bold formatting indicates microRNAs that were also identified as being differentially expressed in *EVI1* deregulated patient samples compared to normal bone marrow.

[2] $p<0.05$

Supplementary Table 6. Up- and downregulated microRNAs in *EVI1* rearranged patients compared to normal bone marrow

DOWN regulated microRNAs			
MicroRNA ID[1]	Adjusted P-value	Fold change	Chromosomal position[2]
hsa-mir-627	< 0.0001	2.9174	15q15.1
hsa-mir-376a	< 0.0001	8.4942	14q32.31
hsa-mir-377	< 0.0001	14.8259	14q32.31
hsa-mir-182	< 0.0001	21.6981	7q32.2
hsa-mir-96	0.0004	23.2055	7q32.2
hsa-mir-302c	0.0004	25.0552	4q25
hsa-mir-373	0.0021	29.3798	19q13.41
hsa-mir-486	0.004	37.0995	8p11.21
hsa-mir-451	0.0093	39.1498	17q11.2
hsa-mir-519b	0.0086	42.764	19q13.41
hsa-mir-453	0.0084	42.9166	14q32.31
hsa-mir-190	0.0083	43.2435	15q22.2
hsa-mir-210	0.0098	43.7469	11p15.5
hsa-mir-519e	0.0095	44.7324	19q13.41
hsa-mir-555	0.0104	44.8523	1q22
hsa-mir-215	0.0162	45.9622	1q41
hsa-mir-411	0.0309	48.7512	14q32.31
hsa-mir-449b	0.0351	53.0335	5q11.2
hsa-mir-192	0.0333	54.2642	11q13.1
hsa-mir-449a	0.037	54.3671	5q11.2
hsa-mir-18b	0.0469	55.3124	xq26.2
hsa-mir-518c	0.0458	57.3651	19q13.41
hsa-mir-382	0.0471	57.9604	14q32.31
hsa-mir-566	0.0471	58.2334	3p21.31
hsa-mir-193b	0.0462	58.3512	16p13.12
UP regulated microRNAs			
MicroRNA ID[1]	Adjusted P-value	Fold change	Chromosomal position[2]
hsa-mir-213	< 0.0001	7.6312	1q31.3
hsa-mir-34a	< 0.0001	21.2414	1p36.23
hsa-mir-372	< 0.0001	22.5682	19q13.41
hsa-mir-202	< 0.0001	23.1649	10q26.3
hsa-let-7e	< 0.0001	25.1082	19q13.33
hsa-mir-369-3p	< 0.0001	30.8797	Xq28
hsa-mir-107	< 0.0001	31.0029	10q23.31
hsa-mir-187	< 0.0001	37.0609	18q12.2
hsa-mir-208	0.0009	39.0362	14q11.2
hsa-mir-133a	0.0008	39.0706	18q11.2
hsa-mir-519e	0.0009	41.9395	19q13.41
hsa-mir-496	0.0017	43.7542	14q32.31
hsa-mir-128b	0.005	47.5518	3p22.3
hsa-mir-33	0.0118	51.3368	22q13.2
hsa-mir-517	0.0119	51.5086	19q13.41
hsa-mir-223	0.0225	54.8395	Xq12
hsa-mir-23b	0.0242	55.6051	9q22.32
hsa-mir-196b	0.0241	55.9266	7p15.2

hsa-mir-133b	0.0319	58.1882	6p12.2
hsa-mir-527	0.0308	58.294	19q13.41
hsa-mir-551a	0.0365	59.5325	1p36.32
hsa-mir-219	0.0417	60.5877	6p21.32
hsa-mir-520c	0.0497	61.9214	19q13.41
hsa-mir-181a	0.0482	61.984	1q31.3

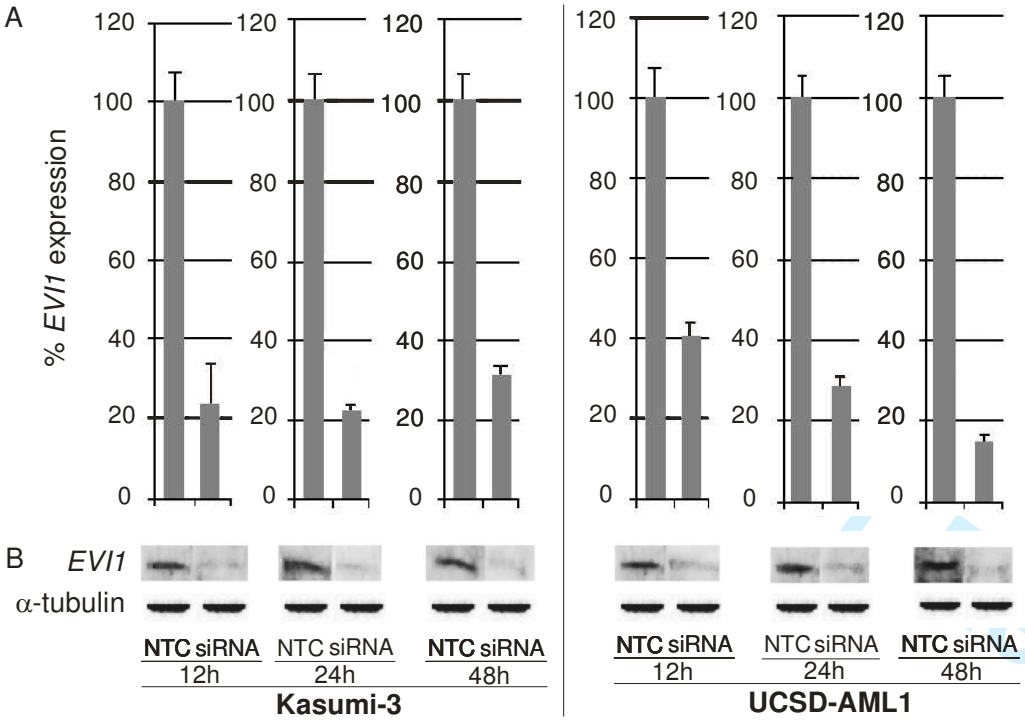
[1] Bold formatting indicates microRNAs that were also identified as being differentially expressed in *EVI1* overexpressing cell lines.

[2] Based upon the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway)

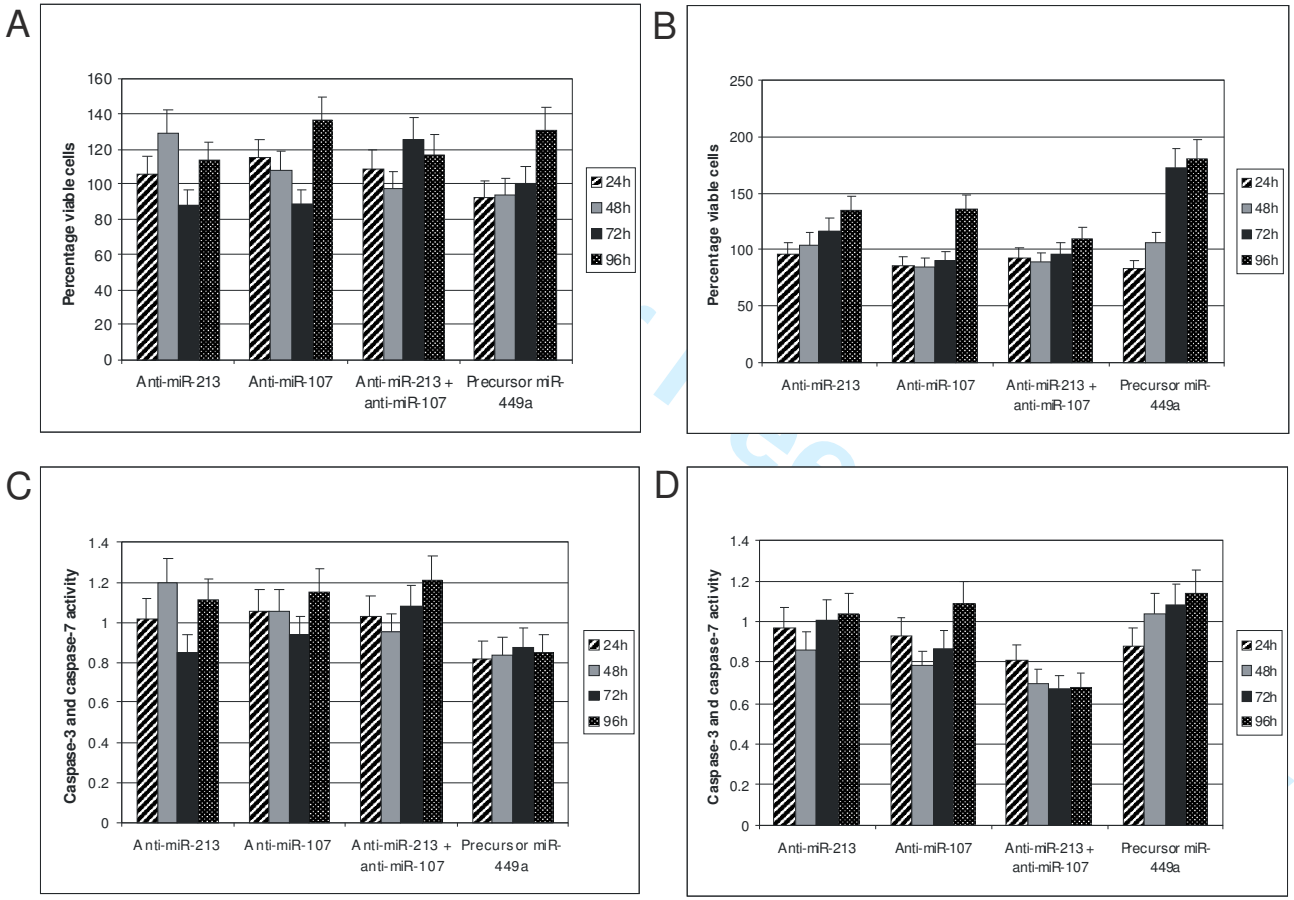
Supplementary Table 7. Selection of *miR-449a* target genes based on known function in cell viability, cell cycle regulation, apoptosis or differentiation

Gene name	Description
ACCN1	Amiloride-sensitive cation channel 1
BCL2	Apoptosis regulator Bcl-2
CCNE2	G1/S-specific cyclin-E2
DLL1	Delta-like protein 1
E2F5	Transcription factor E2F5
EFNB1	Ephrin-B1
FOXG1B	Forkhead box protein G1C
IGFBP3	Insulin-like growth factor-binding protein 3
JAG1	Jagged-1
NOTCH1	Neurogenic locus notch homolog protein 1
RARG	Retinoic acid receptor gamma-1
SEMA4C	Semaphorin-4C
SEMA5B	Semaphorin-5B
SGPP1	Sphingosine-1-phosphate phosphatase 1
UHRF2	Ubiquitin-like PHD and RING finger domain-containing protein 2

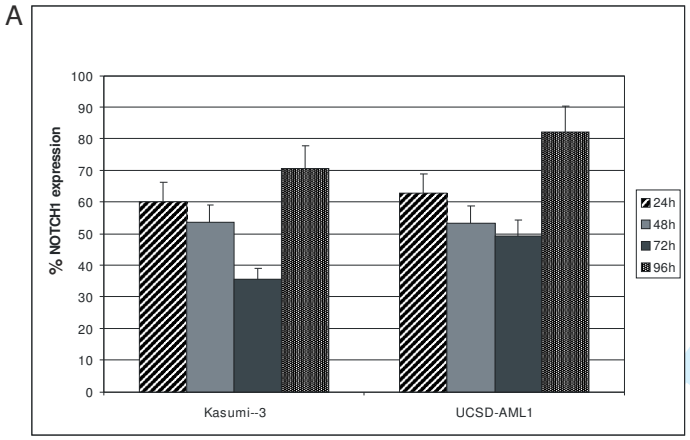
Supplementary Figure 1

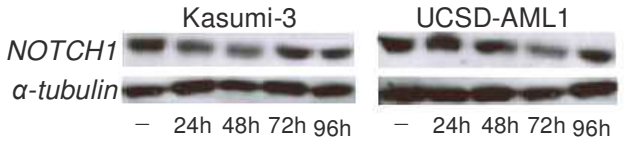


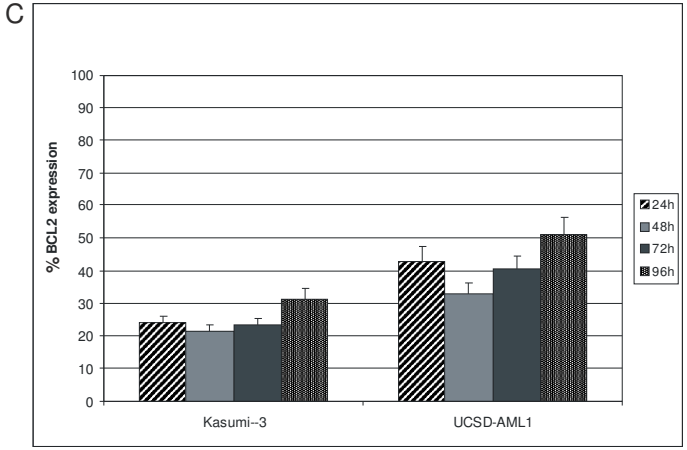
Supplementary Figure 2

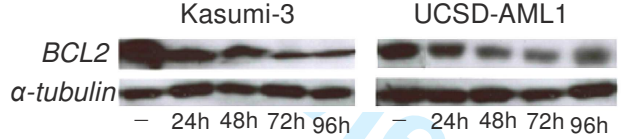


Supplementary figure 3









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