BCR-ABL Tyrosine Kinase Sustained EVI1 Expression in Chronic Myeloid Leukaemia

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Running title: Novel mechanism of *EVI*1 gene activation in CML

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Abstract: EVI1 oncogene expression correlates with chronic myeloid leukaemia (CML) progression. Here we show that the knockdown of EVI1 (E) and MDS1-EVI1 (ME) isoforms reduces cell division at low cell density, inhibits colony forming cells (CFC) by 34% and moderately reduces BCR-ABL mRNA and protein expression but not tyrosine kinase catalytic activity in K562 cells. We also show that both *E* and *ME* are expressed in CD34+ selected cells of both CML chronic phase (CML-CP), and non CML (normal) origin. Furthermore, *EVI*1 mRNA and protein expression are repressed by imatinib mesylate treatment of CML-CP CD34+ cells, K562 and KY01 cell lines but has no effect in non CML BCR-ABL –ve CD34+ cells. Together these results suggest that BCR-ABL tyrosine kinase catalytic activity regulates *EVI*1 gene expression in CML-CP progenitor cells and that the BCR-ABL oncoprotein partially mediates its biological activity through EVI1. *EVI*1 gene expression in CML-CP progenitor cells would provide an *in vivo* selective advantage, contributing to CML pathogenesis.

Key Words: EVI1, BCR-ABL, tyrosine kinase, imatinib, Chronic Myeloid Leukaemia

Introduction: Chronic Myeloid Leukaemia (CML) is a disorder of haemopoietic stem cells (HSC) [Hamilton *et al*, 2010], characterised by the Philadelphia (Ph) chromosome [Kurzock *et al*, 1988]. The balanced translocation t(9;22) creates a novel fusion gene *BCR-ABL* [Ben-Neriah *et al*, 1986] which encodes a spatially [Wetzler *et al*, 1993] and functionally [Konopka *et al*, 1984] de-regulated tyrosine kinase, BCR-ABL. BCR-ABL inappropriately activates the MAPK, PI3K and JAK-STAT signal transduction pathways [Pendergast *et al*, 1993; Skorski *et al*, 1997; Carlesso *et al*, 1996] contributing to abnormal myeloid cell proliferation, differentiation, transformation and survival [Smith *et al* 2003].

CML usually progresses through three stages, designated chronic (CP), accelerated (AP) and terminal blast crisis (BC) phases [Irvine *et al*, 2010]. CML-CP and CML-BC phases resemble myeloproliferative disorder and acute leukaemia, respectively; the transition between stages is unpredictable, non-time limited and inevitable unless treated [Elrick *et al* 2005]. Imatinib mesylate (IM; Glivec®, Novartis Pharma), a rationally designed tyrosine kinase inhibitor (TKI) that selectively inhibits BCR-ABL tyrosine kinase catalytic activity, is the currently favoured therapeutic agent that successfully manages the majority of patients with CML-CP [Valent *et al*, 2010]**,** although it is much less effective when administered at advanced stages (CML-AP/BC) [O'Hare *et al*, 2006].

The t(9:22) translocation is a CML disease-initiating progenitor cell genetic change, the mutation being present in cells at all stages of the disease [Melo and Barnes, 2007]. Disease progression requires the acquisition of new genetic abnormalities and various genes have been implicated in the majority of cases (reviewed in Melo and Barnes, 2007). Indeed, enhanced expression of Ecotropic Viral Integration site 1 (*EVI*1), a proto-oncogene located on chromosome 3q26, is frequently observed in CML-BC [Russell *et al*, 1993; Carapeti *et al*, 1996; Ogawa *et al*, 1996; DeWeer *et al*, 2008]. The *EVI1* gene encodes a zinc finger transcription factor with important roles both in normal development and leukemogenesis [Weiser, 2007]. EVI1 belongs to the positive regulatory (PR) domain family and is expressed as multiple naturally occurring alternatively spliced variants [Huang, 1999; Alzuherri *et al*, 2006]. One form, designated EVI1 (E), encodes the originally described protein [Morishita *et al*, 1990a] whereas another results from splicing of the coding region of the *MDS*1 gene with exon 2 of the *EVI*1 gene (encoding the PR domain) translating MDS1-EVI1 (ME) protein [Fears *et al*, 1996]. Both proteins contain two domains of 7 (ZF1) and 3

(ZF2) repeats of the zinc finger motif [Morishita *et al*, 1988], function as DNA binding transcription factors [Weiser, 2007] and contribute to the progression of acute leukaemia [Morishita *et al*, 1992]. Enhanced expression of EVI1 in CML-BC implicates this transcription factor in disease progression [Ogawa *et al* 1996].

Previous studies have shown that normal human CD34+ haemopoietic cells express *EVI*1 [Gerhardt *et al* 1997]. Furthermore, this gene has been shown to have a role in self-renewal, proliferation and the repopulating capacity of murine HSC in *Evi*1 null mice [Yuasa *et al*, 2005]. However, EVI1's role in CML has not been fully determined. *EVI*1 over-expression in CML-BC has been found in both the presence or absence of chromosome 3q26 abnormalities [Morishita *et al*, 1990b]. Some chromosome 3q26 translocations generate enhanced expression of intact *EVI*1 including t(3:9;17;22), t(3;7), t(2;3), inv(3) and t(3;8) [DeWeer *et al*, 2008; Henzan *et al*, 2004; Stevens-Kroef *et al*, 2004; Suzukawa *et al*, 1997; Lin *et al*, 2009] whereas others create novel fusion proteins involving *TEL* t(3;12) [Nakamura *et al*, 2002] or *RUNX*1 t(3;21) [Mitani *et al*, 1994]. Many of the same genetic changes are also present in poor prognosis acute myeloid leukaemia.

Between 60-70% of CML-BC cells express *EVI*1 in the absence of detectable gross cytogenetic abnormalities but in these cases it is unclear if expression is a marker or a driver of disease [Ogawa *et al* 1996]. These and other studies suggest that *EVI*1 is not expressed in CML-CP mononuclear cells from bone marrow or peripheral blood, but CML-CP CD34+ cells have not been previously examined. In this study we have investigated *EVI*1 gene expression, the effect of IM treatment and the biological activity of this gene in primary CML-CP CD34+ progenitor cells as well as CML derived cell lines.

Materials & methods:

Cell culture K562 and human embryonic kidney (HEK) 293T cells were cultured at 37°C in 5% CO_2 in complete medium (CM) comprising RPMI 1640 or DMEM respectively supplemented with 10% foetal calf serum (FCS), 2.5mM glutamine, 50mg/mL penicillin, 50units/mL streptomycin (all sourced from Lonza Group Ltd, Basel, Switzerland), and 200mg/mL Geneticin® (for HEK293T cells only; Invitrogen, Paisley, UK). Lentivirus infected cells were selected and maintained in CM and 2μg/mL puromycin (Sigma-Aldrich, St Louis, MO, USA). TKI treated cells were incubated with CM supplemented with 5μM IM (LC labs, Woburn, MA, USA). Hydroxyurea (HU) treated cells were incubated in CM supplemented with 400μM HU (Sigma-Aldrich). For Colony-Forming Cell (CFC) assays, 1000 cells were plated in 1.5mL Methocult® (StemCell Technologies SARL, Grenoble, France) in 30mm petridishes and cultured at 37°C, 5% CO_2, 12 days. For IM treated CFC assays, Methocult® was supplemented with 5μM IM. Colonies were counted using an inverted microscope (CK2, Olympus UK Ltd, Southend-on Sea, UK).

Preparation of total cellular RNA, cDNA synthesis and real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) RNA was prepared from cells at exponential growth phase semi-confluent cultures of cells by the Trizol method (Invitrogen). 500ng of total cellular RNA was used to synthesise cDNA with the Superscript® III 1st strand synthesis supermix for qPCR according to the manufacturer's instructions (Invitrogen). 5% of the cDNA reaction was used for qPCR using ABsolute Blue QPCR mix (ABgene, Epsom, UK), gene specific oligonucleotide primers and dual labelled probes, 95°C, 15 min followed by 40 cycles 95°C, 15 sec, 60°C, 1 min in an OPTICON 2 DNA engine (MJ Research INC, Waltham, MA, U.S.A).

The efficiencies of the qRT-PCR reactions were calculated by using the formula Efficiency = $-1+10^{(-1/slope)}$ against the standard curve of each assay over a gradient of template concentration with each gene. The efficiency for *E*, *ME* and *GAPDH* primers/probes were 112, 115 and 110%. Relative expression levels of *E* and *GAPDH* or *ME* and *GAPDH* were determined using the arithmetic comparative $2^{-\Delta\Delta Ct}$ method [Livak *et al*, 2001] and were determined relative to the calibrators *E* or *ME* respectively in K562 or CD34+ cells as described.

Oligonucleotides Gene specific oligonucleotides were synthesised and supplied by Integrated DNA Technologies (Leuven, Belgium)

5' Human *EVI*1: 5'CTTCTTGACTAAAGCCCTTGGA 3'

3' Human *EVI*1 and *MDS*1-*EVI*1: 5'GTACTTGAGCCAGCTTCCAACA 3'

5' Human *MDS*1-*EVI*1: 5'GAAAGACCCCAGTTATGGATGG 3'

5' FAM, 3' TAMRA Human *EVI*1 and MDS1-EVI1probe: 5'CTTAGACGAATTTT ACAATGTGAAGTTCTGCATAG 3'

5' Human *GAPDH*: 5'CACATGGCCTCCAAGGAGTAA 3'

3' Human *GAPDH*: 5'TGAGGGTCTCTCTCTTCCTCTTGT 3'

5' 6-FAM, 3' TAMRA Human *GAPDH* probe: 5'CTGGACCACCAGCCCCAGCA AG 3'.

5' Human *BCR-ABL*: 5'TCCGCTGACCATCAAYAAGGA3'

3' Human *BCR-ABL*: 5'CACTCAGACCCTGAGGCTCAA3'

5' FAM, 3' IOWA BLACK Human *BCR-ABL* probe: 5'CCCTTCAGCGGCCAGTA GCATCTGA3'

Preparation of plasmid DNA pLKO.1 plasmids (Sigma-Aldrich) HB11 (*EVI*1 shRNA CCGGGCACTACGTCTTCCTTAAATACTCGAGTATTTAAGAAGACGTAGTCT TTTT), HB14 (*EVI*1 shRNA CCGGTGCAGGGTCACTCATCTAAAGCTCGAGCT TTAGATGAGTGACCCTGCATTTTT) and NT (MISSION® Non-target shRNA control vector) were prepared by affinity chromatography using Nucleobond® PC500EF gravity flow columns according to manufacturer's instruction (Macherey-Nagal GmbH & Co. Kg, Düren, Germany).

Production of lentivirus and infection of K562 Cells 4-5x10^6 HEK293T cells were cultured in CM supplemented with 10% tetracycline-free FCS (Clontech Laboratories Inc., Mountain View CA, USA) as described, and 3μg of pLKO.1 recombinant Lentivirus plasmid DNA, Lenti-X™ HT packaging system (Clontech Laboratories Inc.) plasmid DNA transfected using the Lentiphos™ HT system (Clontech Laboratories Inc.) according to the manufacturer's instructions. Virus containing cell supernatants were passed through a 0.45μm cellulose acetate filter (Nalgene company, Rochester, NY, USA) and viral titres determined using the Lenti-X™ qRT-PCR Titration Kit (Clontech Laboratories Inc.). 2x10^5 K562 cells were transduced at a multiplicity of infection of 40:1 with recombinant Lentivirus in CM supplemented with 4μg/mL polybrene in 6 well plates. Plates were centrifuged 1200g

(Allegra™ X-22R, Beckman Coulter, Inc. Brea, CA, USA) 60 min, 20°C and cultured at 37°C, 5% CO_2, 24h. Transduced cells were selected in CM supplemented with 2μg/mL puromycin (Sigma-Aldrich).

Western blotting Protein extracts, SDS polyacrylamide gel electrophoresis and Western blotting were performed as described previously [Bartholomew *et al*, 1997] with either α-EVI1 (C50E12, Cell Signaling Technology, New England Biolabs, Hitchin, UK) or α-GAPDH (CA5, Fitzgerald Industries, North Acton, MA, USA) and diluted 1/1000 or 1/5000. α-c-ABL (2862), α-Phospho-CrKL (Tyr207) and α-CrKL (32H4) were each obtained from Cell Signaling Technology and diluted 1/1000. Anti-phosphotyrosine (4G10) was obtained from Millipore (Temecula, CA, U.S.A) and diluted 1/1000. Appropriate HRP conjugated anti-rabbit or anti-mouse (Sigma-Aldrich) IgG secondary antibodies were used at 1/5000 dilutions and detection was performed by enhanced chemiluminescence (Pierce, Rockford, IL, USA). Relative protein quantification was determined by densitometric analysis using Image Lab™ Software v3.0 (Bio-Rad laboratories Ltd, Hemel Hempstead, UK.).

Single cell proliferation assay $1x10^6$ cultured cells were incubated with 1/10 diluted FITC-labelled CD45 antibody (BD Biosciences, San Jose, CA, USA) for 15mins, washed with PBS (supplemented with 2% FCS). 50μg/mL propidium iodide (PI; Sigma-Aldrich) was added and cells immediately washed with PBS/2% FCS. Washed cells were re-suspended in 500μL of PBS and single FITC+ / PI negative cells isolated by cell sorting (BD FACS Aria, BD Biosciences) were dispensed into each well of a 96 well tissue culture plate by the automatic cell dispensing unit (ACDU). Cells were monitored by visual inspection using an inverted light microscope (CKX41, Olympus UK Ltd) to confirm presence of single cells and daily for 4 days for evidence of cell division. All cells dividing at least once were classified as proliferating.

Preparation of primary CD34+ cells Leukapheresis samples were obtained with informed consent as part of the routine assessment of untreated, newly diagnosed patients with CML-CP. Non-CML leukapheresis collections were processed as Ph-negative controls. Samples were enriched to >90% CD34+ progenitors by positive selection (CliniMACS®, Miltenyi Biotec, Bergisch Gladbach, Germany) and cryopreserved. CD34+ cells were cultured at 37°C in 5% CO_2 in IMDM (Invitrogen) supplemented with serum substitute (bovine serum albumin, insulin and transferrin:

'BIT', StemCell Technologies), glutamine, penicillin/streptomycin and 5 growth factors (IL-3, IL-6, flt3-L, and SCF from StemCell Technologies; G-CSF from Chugai Pharma Europe Ltd) as previously described [Jørgensen *et al*, 2005].

Results:

EVI1 knock down in K562 cells Lentiviral vectors encoding non-target (NT) control shRNA and shRNAs targeting *EVI*1 (HB11 & HB14), were used to create lentivirus particles by transient transfection of HEK293T cells as described in Materials and methods, and generated virus titres of $6x10^8$/mL (NT), $1.2x10^9$/mL (HB11) and $3x10^9$/mL (HB14). HB11 is complimentary to part of exon 7 of *EVI*1 that is alternatively spliced to generate *EVI*1 Δ324 and therefore should not knockdown (KD) production of this isoform (Fig.1A). HB14 is complimentary to sequences present in all naturally occurring transcripts (3' untranslated region) and therefore should KD all isoforms (Fig.1A).

Total cellular RNA and proteins derived from puromycin selected cell populations from independent cultures of K562 cells infected with each lentivirus were examined by qRT-PCR and Western blot analysis to investigate the success of *EVI*1 KD. The results show 60-70% KD of *EVI*1 gene expression (*E* and *ME* transcripts) in K562 cells with HB11, HB14 or a combination of HB11 and HB14, relative to NT control cells (Fig.1B). Western blot analysis showed that production of both 145kDa (E) and 88kDa (EVI1 Δ324) EVI1 isoforms were significantly repressed (80-90%) by HB14 or the HB11/HB14 combination whereas only the E protein is repressed by HB11 (70-80%) (Fig.1C), consistent with the qRT-PCR data and the anticipated specificity of the two shRNAs. The ME protein was not detected. Western blot analysis for GAPDH detected similar levels of the 35kDa protein showing equal loading of total cell protein lysates in each case (Fig. 1C).

EVI1 KD has no significant impact on BCR-ABL kinase activity To characterise *EVI*1 KD K562 cells, we investigated if there was an effect on BCR-ABL at the message, protein or catalytic activity (via CrKL phosphorylation) levels. qRT-PCR results showed a slight reduction of *BCR-ABL* gene expression in *EVI*1 KD K562 with HB11, HB14 and HB11/14 infected cells but not in NT control cells (Fig. 2A). The same trend was seen by Western blot analysis of BCR-ABL protein levels with α-c-ABL antibodies (Fig. 2B). However, BCR-ABL tyrosine kinase catalytic activity was unaffected by EVI1 KD as shown by uniform phosphorylation levels of the BCR-ABL substrate CrKL in all cells examined (Fig. 2B). The abundance of total CrKL

protein was similar in all cells and equal levels of GAPDH confirmed similar protein levels in the cell extracts examined (Fig. 2B).

EVI1 KD reduced K562 CFC and single cell proliferation

To investigate the impact of *EVI*1 KD on K562 cells, single parental K562, NT control and HB14 infected cells were sorted by fluorescence activated cell sorting (FACS) into the wells of a 96 well cell culture plate and cell division monitored. The results showed that a significantly reduced proportion of the *EVI*1 KD K562 single cells were able to proliferate (Fig.3) and if they did divide, they turned over less frequently (*data not shown*) than parental K562 or NT control cells. These data suggest the proliferation capacity of *EVI*1 KD K562 is reduced.

We next investigated the functional effect of *EVI*1 KD by examining the number of CFC in semi-solid culture media. The results showed a significant reduction in CFC (34%; $p<0.0001$) in all *EVI*1 KD cell populations (Fig. 4A) relative to NT control cell populations (Fig. 4A). Interestingly, all the colonies produced by the KD cell populations were not only fewer in number but also significantly reduced in size (Fig. 4C &D). These data suggest that *EVI*1 KD significantly reduces K562 CFC activity.

EVI1 expression in CD34+ cells Previous studies have shown detectable *EVI*1 gene transcripts in CML-BC but not CML-CP patient cells [Ogawa *et al*, 1996]. To see if *EVI*1 expression was detectable in CML-CP, we examined CD34+ selected cells derived from the peripheral blood of 3 newly diagnosed patients (demographics shown in Table 1) and in one allogeneic normal donor CD34+ as well as CD34+ cells from 2 non-CML patients (collectively designated 'non-CML') as controls. *E* and *ME* expression were normalised to *GAPDH*, then further scaled to *GAPDH* normalised *E* or *ME* calibrator respectively in normal CD34+ cells (sample 010). Both *E* and *ME* isoforms were readily detected in CD34+ cells, of both CML and non-CML origin. The relative abundance of both transcripts was similar in CML-CP CD34+ cells compared to the non-CML CD34+ cells (Fig. 5A,B). These data show that *E* and *ME* gene transcripts are readily detected in primitive CML-CP CD34+ cells.

EVI1 expression in CML-CP cells and CML cell lines is repressed by IM IM treatment has been shown to dramatically reduce the survival of p210 BCR-ABL+

cells *in vitro*. As EVI1 is a putative survival factor, we were interested to know if switching off the kinase survival pathway with IM would have any impact on the *EVI*1 expression in CML-CP CD34+ cells. Interestingly, the results showed a time dependent reduction of both *E* and *ME* gene expression in IM treated cells relative to untreated cells (Fig. 6A,B). The results also showed *E* and *ME* expression reduced independently of IM when CML-CP CD34+ cells (donor 289) were cultured for 12h or more (Fig. 6A,B). However, there was a statistically very significant reduction of *E* and *ME* in IM treated cells, relative to cells cultured in growth medium alone, at both 12 (*E* $p < 0.0008$, *ME* $p < 0.0086$) and 24h (*E* $p < 0.0001$, *ME* $p < 0.0009$) (Fig. 6A,B). *E* and *ME* expression was also examined in non-CML CD34+ cells (donor 019) + and – IM. IM treatment has no effect on *EVI*1 expression in these BCR-ABL –ve CD34+ cells although once again expression is slightly decreased following 12 hrs or more of cells in culture in the presence or absence of the TKI (Fig. 6 C,D).

To confirm the effect of IM on *EVI*1 gene and protein expression we examined its impact in CML derived cell lines K562 and KY01. Both *E* and *ME* isoforms were dramatically reduced after 6 h and continued to be repressed further for the time points shown (>90% after 24 h, Fig. 7A) in K562. The same IM mediated repression of *E* and *ME* isoforms was also observed in another CML derived cell line, KYO1 (Fig. 7B). Western blot analysis detected the 145kDa E protein in both cell lines (Fig. 7C), The abundance of the 145kDa E protein rapidly diminished within 6 h of IM treatment and further reduced after 24 h (Fig. 7C, 70%-90% reduction after 24hrs), consistent with the qRT-PCR data. IM treatment for 24hrs inhibits phosphorylation of BCR-ABL (Fig. 7D). Furthermore, inhibition of BCR-ABL catalytic activity is observed after 6hrs IM treatment and is sustained for at least 24hrs, as observed by dramatically reduced phosphorylation of the CrKL substrate protein (Fig. 7E). Cell counting of trypan blue (Sigma-Aldrich) treated cells demonstrated that 5μM IM treatment inhibited K562 proliferation but had no effect on cell viability over the 24hr period examined (*data not shown*).

To see if the impact of IM on EVI1 was specific, or a more general consequence of inhibiting cell proliferation, we examined the effect of hydroxyurea (HU) in K562 cells. Western blot analysis shows no major change in the 145ka E protein following treatment of K562 cells with HU for up to 24hrs (Fig. 7C).

Our data show that IM rapidly inhibits *EVI*1 gene expression in primary CML-CP CD34+ cells and CML derived cell lines. Furthermore EVI1 KD significantly reduces K562 CFC activity (Fig.4A); this compares with an 80% reduction in K562 CFC achieved following IM (5μM) treatment (Fig. 4B). Together these data suggest *EVI*1 repression could partially mediate the cellular response to IM.

Discussion:

We have shown that EVI1 has a role in cell proliferation in Ph+ cells. KD of EVI1 in K562 cells reduced their proliferative capacity in CFC as well as single cell proliferation assays. These observations were the same for KD either of all EVI1 isoforms (with HB14 treated cells) or when EVI1Δ324 expression only was retained (HB11 treated cells), showing this latter truncated protein, which lacks transforming activity [Kilbey and Bartholomew, 1998] cannot compensate for reduced E and ME isoforms. It is possible that our studies underestimate the impact of EVI1 repression due to partial KD only since other studies, including *Evi*1 K.O mice show a more severe effect on HSC numbers [Yuasa *et al*, 2005] and an almost complete cell cycle arrest is reported in *EVI*1 KD K562 cells by Lugthart *et al* (2011).

The ME 185kDa protein is not detected in K562 or KY01 cells using α-EVI1 antibody despite qRT-PCR data demonstrating RNA expression of this isoform. It is currently unclear if the ME protein is not produced in K562 cells or is at levels below the detection limits of our assays. This study might have underestimated the abundance of *E* encoding transcripts as there are multiple transcription initiation sites (1a, 1b, 1c and 3L) which are not detected by the specific primers and probe set used here [Aytekin *et al*, 2005; Lugthart *et al*, 2008]. Expression of *E* alone is associated with poor prognosis acute leukaemia [Barjesteh *et al*, 2003]. Therefore higher levels of E relative to ME would be likely to contribute to disease progression. A similar observation has been made for another PR family gene *RIZ* where the shorter PR domain deleted forms are always found in chromosome 1p36 linked malignancies, consistent with the view that the long PR domain containing forms have tumour suppressor activity [Huang, 1999].

We also show here that *EVI*1 expression is not only seen in K562 and KY01 cell lines but in primary CML-CP cells also whereas previous reports have suggested it is only observed in CML-BC [Ogawa *et al*, 1996]. It is likely that the discrepancy between our results and previous data are due to increased sensitivity of detection resulting from the analysis of a CD34+ subpopulation of peripheral blood mononuclear cells, which express the highest levels of *EVI*1. Both CD34+ non-CML cells and CD34+ CML cells express similar levels of both *E* and *ME* gene transcripts. Previous studies show both isoforms are present in normal tissues [Fears *et al*, 1996; Wimmer *et al*, 1998]. Only one of the samples in this study, donor 010, was normal but the relative

abundance of both transcripts was similar here to that of the other non-CML and CML samples examined. Therefore, these data show: i) that CD34+ cells express high levels of *EVI*1 and ii) that both transcripts are present in CML cells at similar levels to normal and non CML cells.

In this study we observed that EVI1 KD had no effect on BCR-ABL kinase activity, so we next switched–off the oncogenic catalytic kinase by treatment with the TKI, IM. IM mediated inhibition of BCR-ABL resulted in a rapid repression of both *ME* and *E EVI1* gene expression in CML CD34+, K562 and KY01 cells. This effect is likely to be specific as IM treatment has no effect on *EVI*1 expression in BCR-ABL -ve non-CML CD34+ cells (Fig.6C,D). Western blot analysis showed a dramatic reduction of the 145kDa E protein in IM-treated K562 and KY01 cells. This repression was not a consequence of general inhibition of cell proliferation but BCR-ABL specific as treatment with the anti-metabolite, hydroxyurea did not alter E protein levels. Furthermore, this was not the result of general cell toxicity as trypan blue exclusion studies show the cells retained viability for at least 24hrs of IM treatment and there was no impact on cellular levels of numerous other proteins examined, including CrKL, GAPDH, STAT5, ERK1/2, AKT and BCR-ABL (*data not shown*).

As was seen for K562 cell line, IM mediated inhibition of BCR-ABL results in a rapid decline in *EVI*1 gene expression at both the mRNA and protein level in primary CML-CP cells. *EVI*1 expression also declines by 50% following culture of primary CML-CP cells for 12-24 h. This reduction is not as great as in the presence of IM and might reflect maturation of cells or *ex-vivo* culture not adequately replicating *in vivo* conditions. A similar partial decline in *EVI*1 expression is also observed in non-CML CD34+ cells cultured for 12 hrs or more.

Inhibition of *EVI*1 expression by IM demonstrates for the first time that its expression is regulated in BCR-ABL+ cells by the catalytic activity of this aberrant kinase. Regulation of mRNA expression is rapid, suggesting it is a direct response to inhibition of tyrosine kinase mediated signaling from the BCR-ABL protein. Several studies have identified many potential BCR-ABL target genes previously but *EVI*1 was not described [Håkansson *et al*, 2008; Nunoda *et al*, 2007; Bianchini *et al*, 2007]. This study represents the first report of a signal transduction pathway that regulates *EVI*1 gene expression. Although BCR-ABL transmits an aberrant signal, these results suggest *EVI*1 expression is modulated by one or more of the three major pathways,

JAK-STAT, MAPK or PI3K activated by this promiscuous kinase [Pendergast *et al*, 1993; Skorski *et al*, 1997; Carlesso *et al*, 1996]. The particular pathway involved is currently under investigation. The molecular basis of BCR-ABL mediated *EVI*1 regulation should facilitate how expression of this developmentally important gene is controlled in HSC and other tissues where it is normally expressed [Yuasa *et al*, 2005; Goyama *et al*, 2008, Hoyt *et al*, 1997].

These data establish a link between BCR-ABL kinase catalytic activity and *EVI*1 gene expression. We propose that BCR-ABL positively regulates *EVI*1 gene expression. The level of *EVI*1 gene expression is not elevated by BCR-ABL kinase relative to expression levels observed in non-CML and normal primitive haemopoetic cells. However, it is deregulated since the mechanism regulating *EVI*1 gene expression in normal CD34+ cells is distinct from that in CML-CP CD34+ cells since in the latter expression is dependent upon BCR-ABL catalytic activity. The BCR-ABL kinase might activate a pathway that normally regulates *EVI*1 production in primitive haemopoietic cells. Since BCR-ABL kinase is constitutively active, it will continuously stimulate the pathway leading to sustained de-regulated *EVI*1 gene expression. It is unlikely that *EVI*1 expression is repressed by inhibition of other receptors (PDGFRα, PDGFRβ or c-KIT) that are known to be inactivated by IM [Fabbro *et al*, 1999] because IM has no effect on BCR-ABL –ve CD34+ cells (Fig. 6C,D).

Maintenance of EVI1 expression in primitive HSC is likely a selective advantage. Retroviral tagging studies show that proviral insertions are frequently seen in the *EVI*1 locus in dominant non-malignant HSC clones retrieved from transplant recipients [Kustikova *et al*, 2005] and primary myeloid CD34+ *ex-vivo* cultures are enriched for viral insertions in this gene [Sellers *et al*, 2010]. Furthermore, EVI1 is required for the survival and proliferation of HSC [Yuasa *et al*, 2005; Goyama *et al*, 2008]. The EVI1 KD studies in K562 cells described above support this notion as these cells show a reduced proliferative capacity in CFC and single cell proliferation assays. Our results suggest that BCR-ABL tyrosine kinase catalytic activity sustains *EVI*1 gene expression in CML-CP CD34+ cells and that this gives cells a selective advantage in a manner analogous to retroviral insertion. It is possible the impact of *EVI*1 KD in primary CML-CP cells would be even greater than we see with K562

cells which is an immortal cell line with a number of additional genetic abnormalities. We are currently investigating this.

Sustained expression of EVI1 could be one of the mechanisms by which BCR-ABL contributes a selective advantage to primitive haemopoietic cells in CML, resulting in an increased production of mature cells in peripheral blood. In this case gross chromosome abnormalities are not required as the BCR-ABL mutation causes de-regulation of *EVI*1 gene expression. Interestingly, *EVI*1 translocations are frequently observed in CML patients treated with TKI inhibitors that progress to blast crisis [Paquette *et al*, 2011] and enhanced *EVI*1 expression is a predictor of poor prognosis in TKI resistant chronic phase CML [Daghistani *et al*, 2010]. This suggests that inhibition of BCR-ABL kinase may select for cells that de-regulate *EVI*1 expression by alternative mechanisms. Mutations causing elevated levels of EVI1, E relative to ME, or its fusion proteins which can occur in CML-BC might be necessary for other EVI1 mediated biological activities including inhibition of terminal cell differentiation. Indeed, previous studies show that EVI1 mediated inhibition of granulocyte differentiation is dependent on the level of expression [Khanna-Gupta *et al*, 1996]. Our results suggest BCR-ABL mediated *EVI*1/*MDS*1-*EVI*1 gene expression represents a novel mechanism of de-regulating this gene in leukaemia.

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Conflict of interest: The authors declare no conflict of interest.

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Tables:

Table 1

Shows sex, age and diagnosis of donor CD34+ cell samples used for qRT-PCR analysis. Not available N/A.

Table 1

Patient ID	Sex	Age	Diagnosis
010	M	45	Normal donor
011	F	51	Multiple myeloma
012	F	61	Mantle cell lymphoma
019	M	62	Mantle cell lymphoma
249	N/A	N/A	CML
255	F	46	CML
257	M	47	CML
289	F	47	CML

Figure legends:

Fig. 1 *EVI*1/*MDS*1-*EVI*1 knockdown in K562 cells. (**A**) Shows a schematic representation of the four indicated natural *EVI*1 isoforms. The position of shRNA homology for HB11 and HB14 are indicated by thick horizontal lines showing that HB11 is complimentary to sequences located in exon 7 present in all transcripts except EVI1Δ324 and HB14 is complimentary to sequences located in the 3' untranslated region of all transcripts. The coding exons 3 to 16 are numbered and indicated by boxes as described previously [Alzuherri *et al*, 2006]. The *EVI*1 exon 2 and *MDS*1 are also boxed and indicated for *MDS*1-*EVI*1. Splice variant deleted exons are shown in black boxes. Both introns and 3' untranslated regions are shown by horizontal lines that connect exons. (**B**) Shows histogram of *EVI*1 (white bars) or *MDS*1-*EVI*1 (black bars) mRNA levels normalised for *GAPDH* mRNA for the indicated cell populations relative to the K562 calibrator for *EVI*1 (GAPDH normalised *EVI*1) or MDS1-*EVI*1 (*GAPDH normalised MDS*1-*EVI*1) respectively, as determined by qRT-PCR. Columns are the mean of an experiment performed in quadruplicate and the error bars the standard deviation. (**C**) shows Western blot analysis of the indicated whole cell protein extracts using α-EVI1 and α–GAPDH antibodies. The position of the 145kDa EVI1, 88kDa Δ324 and 35kDa GAPDH proteins are indicated by arrow.

Fig. 2 BCR-ABL expression and catalytic activity in K562, NT, HB11, HB14 and HB(11+14) cells. (**A**) Shows histogram of *BCR-ABL* mRNA levels normalised for *GAPDH* mRNA relative to *GAPDH* normalised *BCR-ABL* mRNA in K562 cells (calibrator) for the indicated cell populations, determined by qRT-PCR. Columns are the mean of an experiment performed in quadruplicate and the error bars the standard deviation. (**B**) shows Western blot analysis of the indicated whole cell protein extracts using α-c-ABL, α-Phospho-CrKL (Tyr207), α-CrKL and α–GAPDH antibodies. The position of the 145kDa EVI1, 88kDa Δ324 and 35kDa GAPDH proteins are indicated by arrow.

Fig. 3 K562 and HB14 KD single cell proliferation assay. Histogram shows the percentage undivided single cells at 0 to 4 days of culture. At day 0 all single cells in

96 well plates are undivided, giving a value of 100%. Black bars are K562 cells, grey bars NT cells and white bars HB14. All K562 and NT cells have divided at least once by day 4 compared to only 50% of HB14 cells. The results shown are the mean of an experiment performed twice.

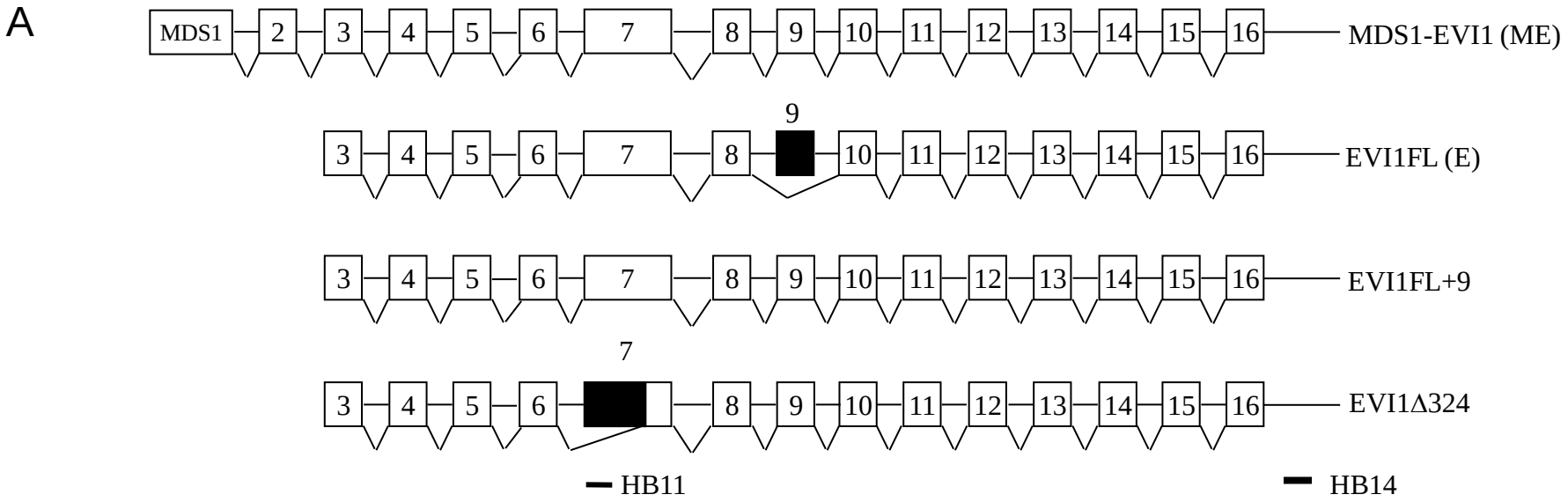
Fig. 4 Colony forming activity of K562 and derivative EVI1 KD cells. (**A**) Shows the number of CFC per 1000 cells plated for the indicated cell populations. Each CFC assay was performed in triplicate for the indicated cell population and the results shown are a typical example of an experiment performed four times. *** *p<0.0001.* (**B**) The same as (**A**) for K562 cells + or – 5μM IM. (**C**) Image shows typical colonies formed by the indicated cell populations. (**D**) Histogram showing mean colony diameter of indicated cell population as a % of mean K562 colony diameter. Error bars are the standard deviation. n=20 for the total number of each cell colony type analysed.

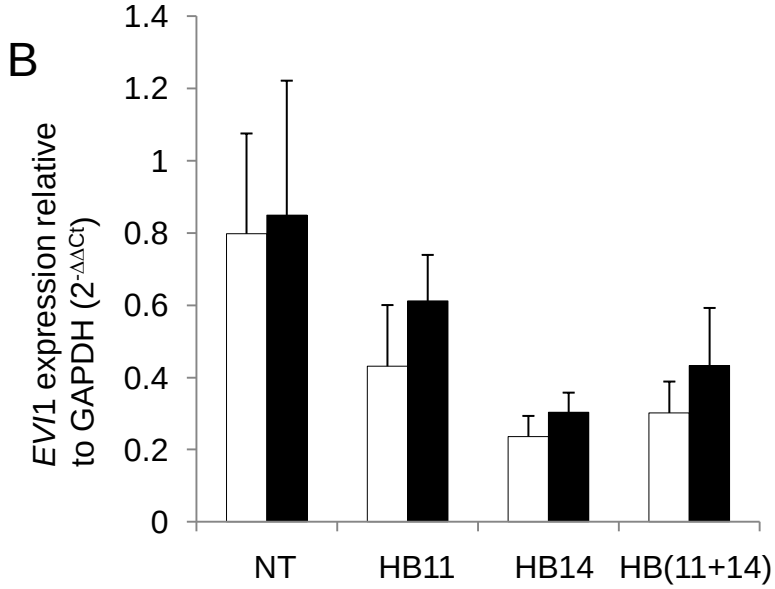
Fig. 5 *EVI*1 (white bars) and *MDS*1-*EVI*1 (black bars) gene expression in the indicated donor Ph- CD34+ non-CML cells (donor 011,012) and Ph+ CD34+ CML-CP cells (donor 249,255,257). Shows histograms of *EVI*1 and *MDS*1-*EVI*1 mRNA levels normalised for *GAPDH* mRNA relative to *GAPDH* normalised *EVI*1 or *MDS*1-*EVI*1 mRNA respectively of calibrator cells (Ph- CD34+ normal cells 010), determined by qRT-PCR. Columns are the mean of an experiment performed in quadruplicate and the error bars the standard deviation.

Fig. 6 *EVI*1 (white bars) and *MDS*1-*EVI*1 (black bars) gene expression in donor 289 Ph+ CML-CP cells and Ph- donor 019 cells exposed to 5μM IM for various times. Shows histograms of *EVI*1 (**A,C**) and *MDS*1-*EVI*1 (**B,D**) mRNA levels normalised for *GAPDH* mRNA relative to *GAPDH* normalised *EVI*1 (A,C) or *MDS*1-*EVI*1 (B,D) mRNA in calibrator cells (0 hrs, untreated donor 289 [A,B] or 019 [C,D]) respectively, in the presence (+) or absence (-) of 5μM IM for 6, 12 and 24h determined by qRT-PCR. Columns are the mean of an experiment performed in quadruplicate and the error bars the standard deviation.

Fig. 7 Shows *EVI*1 and *MDS*1-*EVI*1 mRNA levels in K562 (**A**) and KY01 (**B**) treated with 5μM IM for the time indicated. Analysis as described in legend to figure 1 with untreated 0hr K562 (**A**) or KY01 (**B**) as calibrator. **(C)** shows Western blot analysis of whole cell protein extracts derived from cells treated with either 5μM IM (K562 and KY01) or 400μM HU for the indicated time using α-EVI1 and α–GAPDH antibodies. Also shown are Western blot analysis of whole cell protein extracts derived from IM treated (+) or untreated (-) cells for the indicated times with α-phosphotyrosine, α-c-ABL and α-GAPDH **(D)** and α-pCrKL, α-CrKL and α-GAPDH **(E)** antibodies.

Fig. 1


A
MDS1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
MDS1-EVI1 (ME)
EVI1FL (E)
EVI1FL+9
EVI1Δ324
HB11
HB14


B
EVI1 expression relative to GAPDH (2-ΔΔCt)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NT
HB11
HB14
HB(11+14)

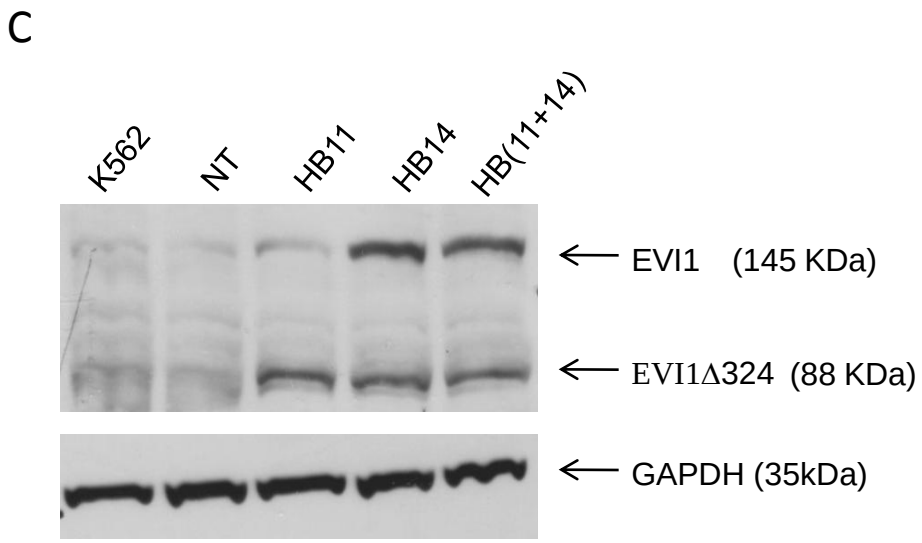
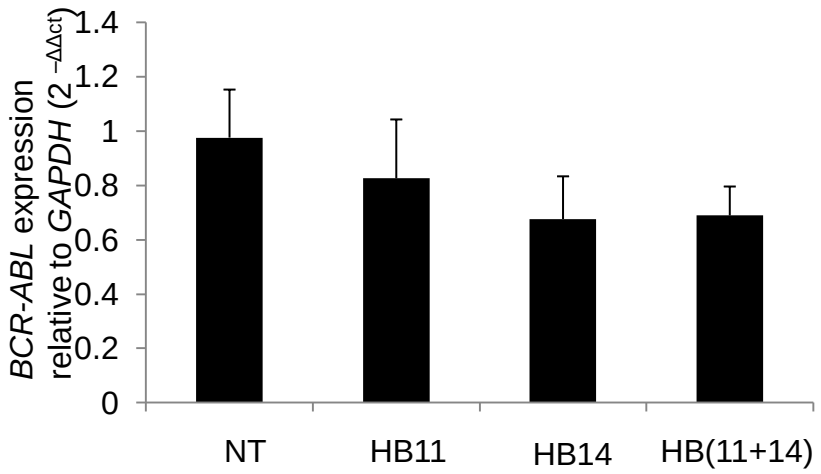

C
K562
NT
HB11
HB14
HB(11+14)
EVI1 (145 KDa)
EVI1Δ324 (88 KDa)
GAPDH (35kDa)

Fig. 2

A


BCR-ABL expression relative to GAPDH (2 –ΔΔct)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NT
HB11
HB14
HB(11+14)

B

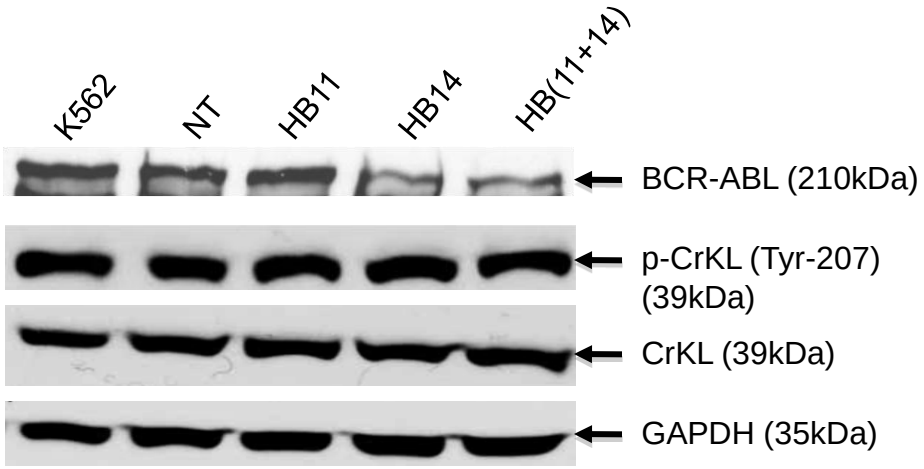

K562
NT
HB11
HB14
HB(11+14)
BCR-ABL (210kDa)
p-CrKL (Tyr-207) (39kDa)
CrKL (39kDa)
GAPDH (35kDa)

Fig. 3

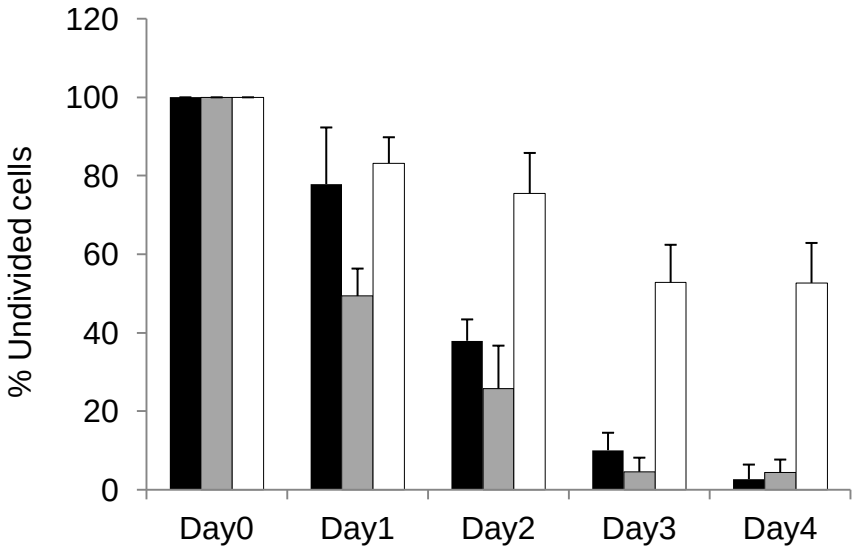
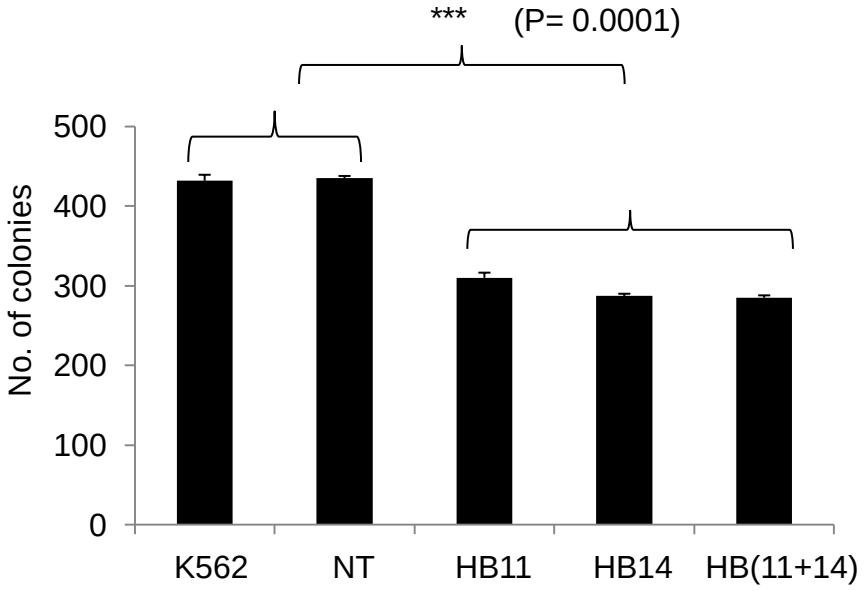
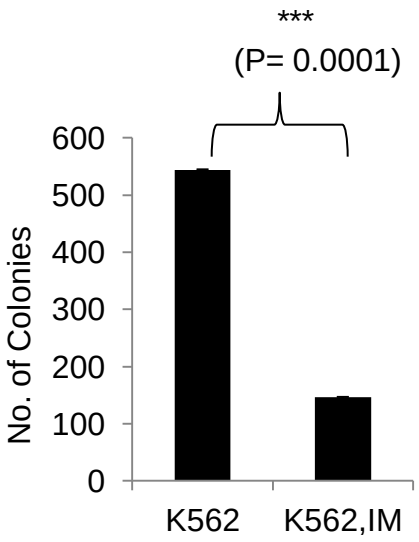


Fig. 4

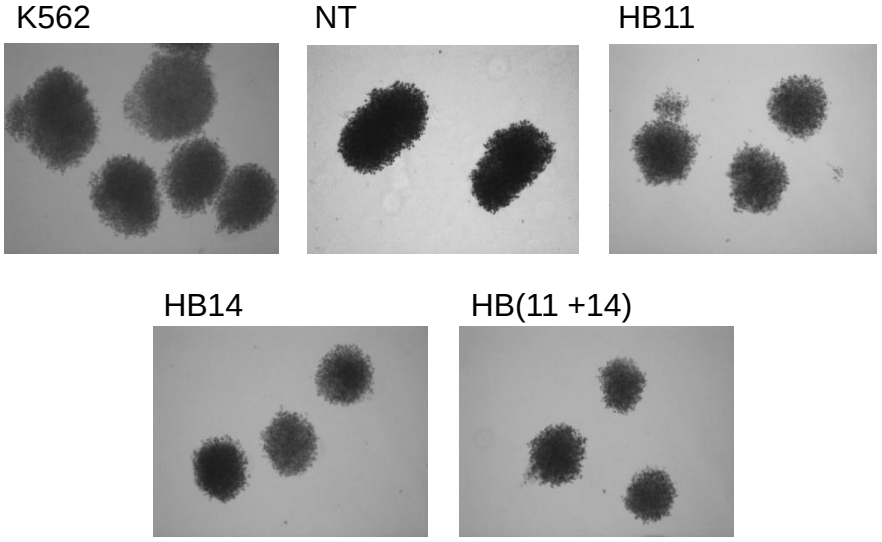
A


***
(P= 0.0001)
No. of colonies
500
400
300
200
100
0
K562
NT
HB11
HB14
HB(11+14)

B


***
(P= 0.0001)
No. of Colonies
600
500
400
300
200
100
0
K562
K562,IM

C


K562
NT
HB11
HB14
HB(11 +14)

D

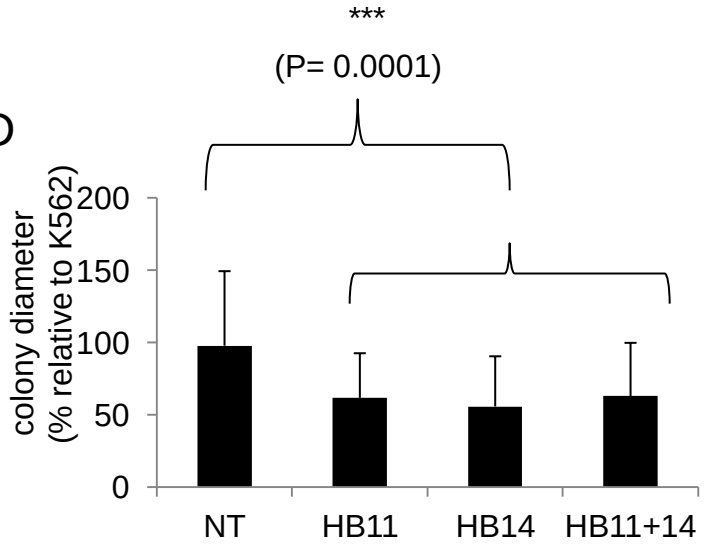

***
(P= 0.0001)
colony diameter
(% relative to K562)
200
150
100
50
0
NT
HB11
HB14
HB11+14

Fig. 5

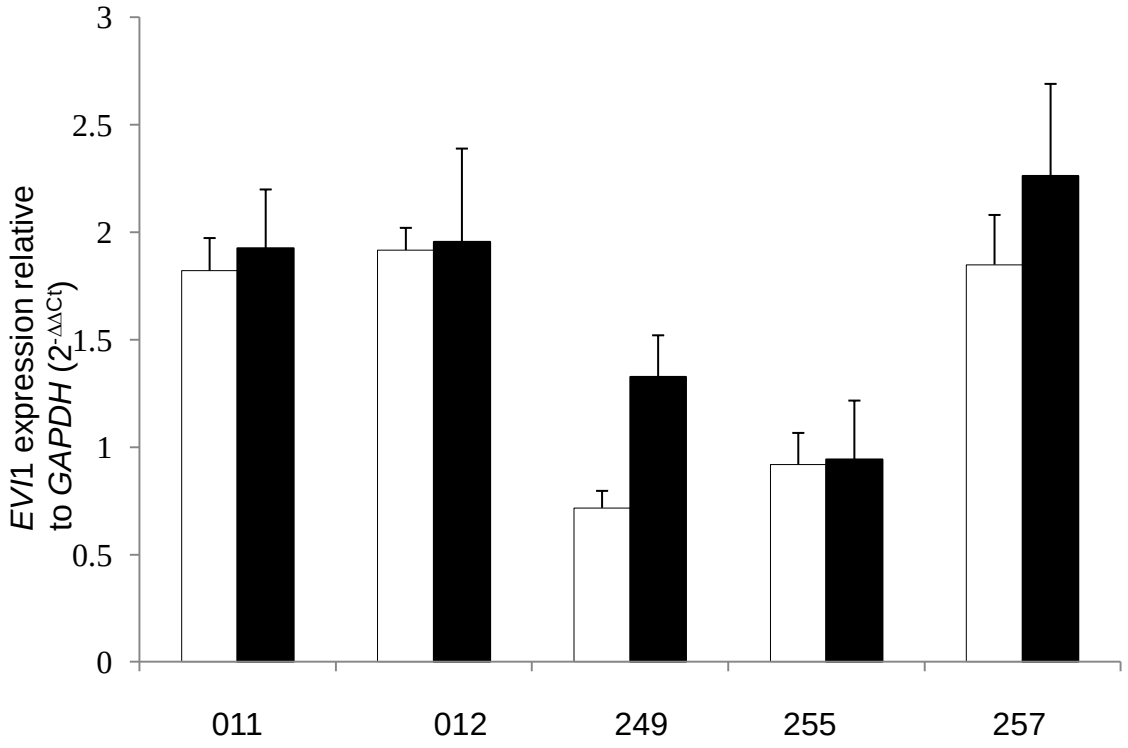

3
2.5
2
1.5
1
0.5
0
EVI1 expression relative to GAPDH (2-ΔΔCt)
011
012
249
255
257

Fig. 6

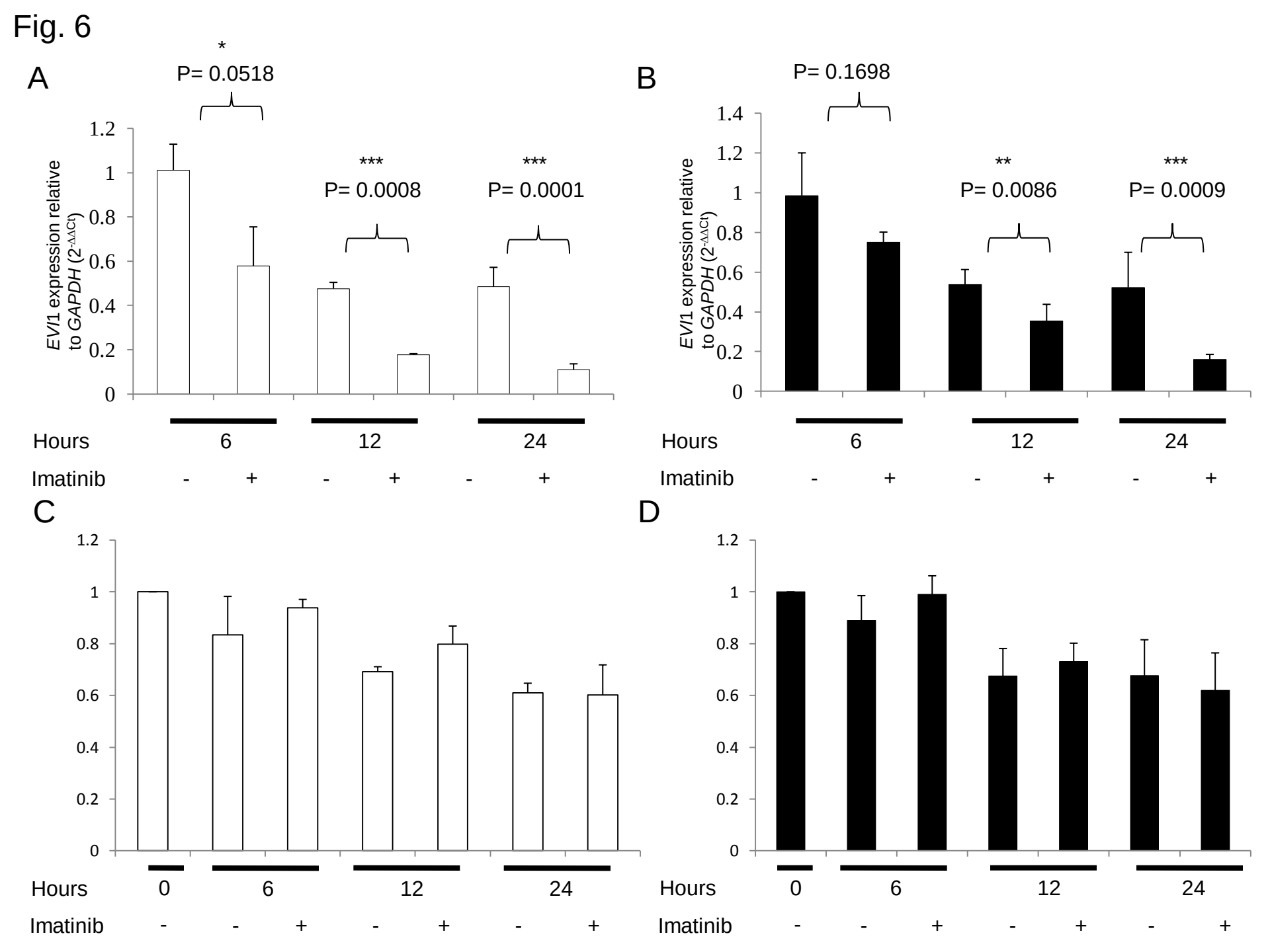
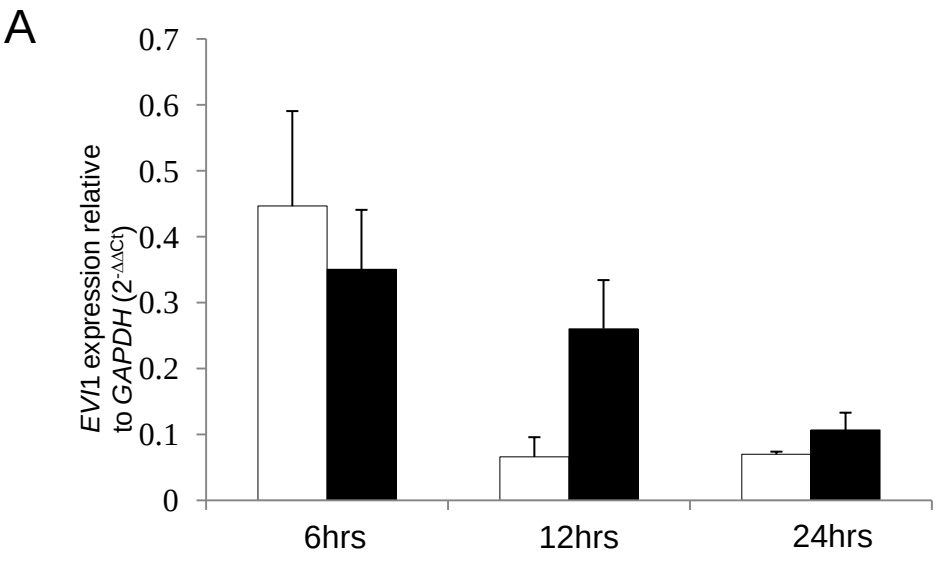
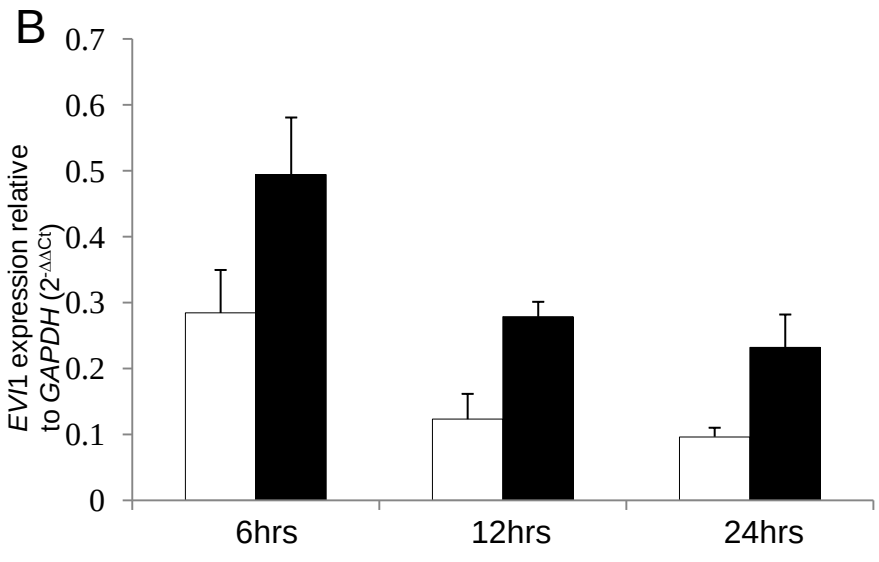
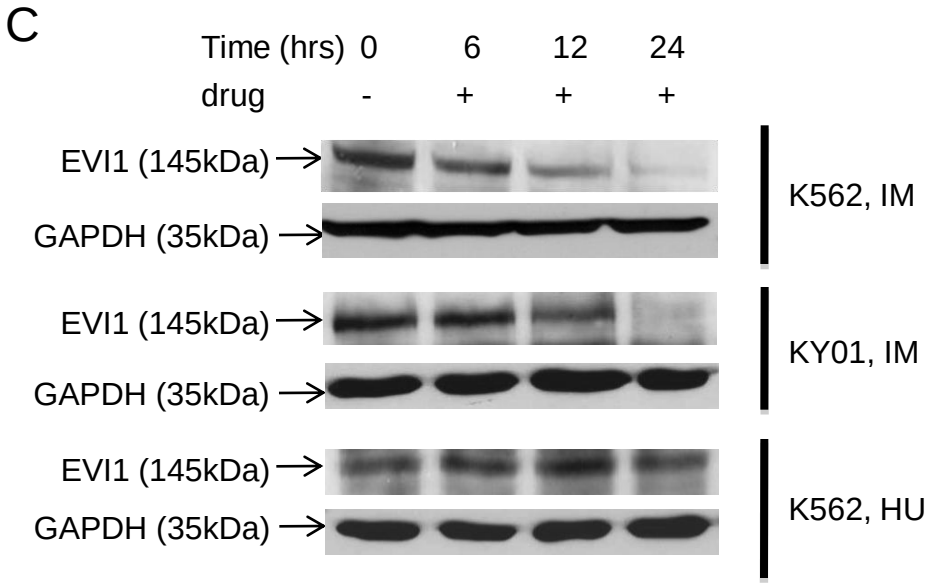
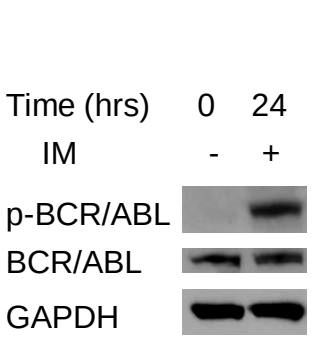

A
*
P= 0.0518
***
P= 0.0008
***
P= 0.0001
EVI1 expression relative to GAPDH (2-ΔΔCt)
1.2
1
0.8
0.6
0.4
0.2
0
Hours
6
12
24
Imatinib
- + - + - +
B
P= 0.1698
**
P= 0.0086
***
P= 0.0009
EVI1 expression relative to GAPDH (2-ΔΔCt)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Hours
6
12
24
Imatinib
- + - + - +
C
1.2
1
0.8
0.6
0.4
0.2
0
Hours
0
6
12
24
Imatinib
- - + - + - +
D
1.2
1
0.8
0.6
0.4
0.2
0
Hours
0
6
12
24
Imatinib
- - + - + - +

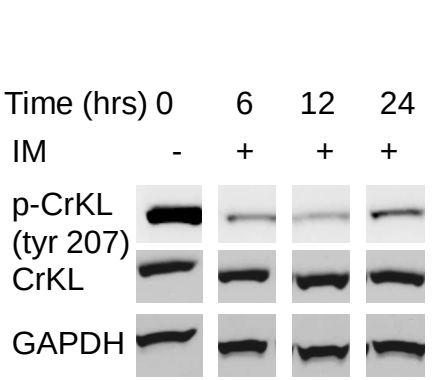
Fig. 7


A
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
EVI1 expression relative to GAPDH (2-ΔΔCt)
6hrs
12hrs
24hrs


B
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
EVI1 expression relative to GAPDH (2-ΔΔCt)
6hrs
12hrs
24hrs


C
Time (hrs) 0 6 12 24
drug - + + +
EVI1 (145kDa)
GAPDH (35kDa)
K562, IM
EVI1 (145kDa)
GAPDH (35kDa)
KY01, IM
EVI1 (145kDa)
GAPDH (35kDa)
K562, HU


D
Time (hrs) 0 24
IM - +
p-BCR/ABL
BCR/ABL
GAPDH


E
Time (hrs) 0 6 12 24
IM - + + +
p-CrKL (tyr 207)
CrKL
GAPDH