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Short report

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**BCR-ABL1 induces aberrant splicing of *IKAROS* and lineage infidelity
in pre-B lymphoblastic leukemia cells**

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Abbreviations: *IGH*, immunoglobulin heavy-chain, SAGE, serial analysis of gene
expression; siRNA, short-interfering RNA

Running title: Lineage infidelity in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells

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Abstract

Pre-B lymphoblastic leukemia cells carrying a *BCR-ABL1* gene rearrangement exhibit an undifferentiated phenotype. Comparing genome-wide gene expression profiles of normal B cell subsets and *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells by SAGE, the leukemia cells show loss of B lymphoid identity and aberrant expression of myeloid lineage-specific molecules. Consistent with this, *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells exhibit defective expression of *IKAROS*, a transcription factor needed for early lymphoid lineage commitment. As shown by inducible expression of BCR-ABL1 in human and murine B cell precursor cell lines, BCR-ABL1 induces the expression of a dominant-negative *IKAROS* splice variant, termed IK6. Comparing matched leukemia sample pairs from patients before and during therapy with the BCR-ABL1 kinase inhibitor STI571 (Imatinib), inhibition of BCR-ABL1 partially corrected aberrant expression of IK6 and lineage-infidelity of the leukemia cells. To elucidate the contribution of IK6 to lineage infidelity in *BCR-ABL1*⁺ cell lines, IK6-expression was silenced by RNA interference. Upon inhibition of IK6, *BCR-ABL1*⁺ leukemia cells partially restored B lymphoid lineage commitment. Therefore, we propose that BCR-ABL1 induces aberrant splicing of *IKAROS*, which interferes with lineage identity and differentiation of pre-B lymphoblastic leukemia cells.

The *BCR-ABL1* gene rearrangement resulting from the t(9;22)(q34;q11) translocation represents the most frequent recurrent genetic aberration in B lymphoid leukemia in adults (Look, 1997). *BCR-ABL1* fusion genes encode constitutively active tyrosine kinase molecules mostly of a molecular weight of either 190 or 210 kD (p190 or p210, Laurent et al. 2001). *BCR-ABL1* kinase activity is required and sufficient to drive malignant transformation of B cell precursors in mice (Huettner et al., 1998). Pre-B lymphoblastic leukemia cells carrying a *BCR-ABL1* gene rearrangement typically exhibit a differentiation block at the pre-B cell stage of development (Klein et al., 2004; Klein et al., 2005).

Early B cell development is guided by a number of transcription factors including PAX5, E2A, EBF and IKAROS (Busslinger, 2004). In order to search for downstream targets of *BCR-ABL1* that contribute to a differentiation block in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells, genome-wide gene expression profiles of this leukemia type were compared with normal hematopoietic progenitor populations by serial analysis of gene expression (SAGE; Velculescu et al., 1995).

BCR-ABL1 interferes with B lymphoid differentiation in BCR-ABL1⁺ pre-B lymphoblastic leukemia cells

Comparing genome-wide gene expression profiles of leukemia cells from two leukemia cases of *BCR-ABL1*⁺ pre-B lymphoblastic leukemia with normal pre-B cells by SAGE, a number of myeloid lineage-specific genes including transcription factors (*MLF2*, *MZF1*, *AML1*, *GATA1*), surface receptors (*CSF3R*, *CSF1R*, *CD14*, *CD11A*) and signaling molecules (*IRAK1*, *MYD88*) were upregulated in the pre-B lymphoblastic leukemia cells as in normal myeloid progenitor cells (Figure 1). Hence, the pattern of gene expression in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells as compared to their normal pre-B cell counterpart is skewed to the myeloid lineage. Consistent with this, transcription factors and signaling molecules involved in early lymphoid differentiation (*IKAROS*, *E2A*, *IL7R α* , *RAG1*, *RAG2*, *TdT*) and B lymphoid lineage commitment (*EBF*, *PAX5*) were silenced in *BCR-ABL1*⁺ pre-B

lymphoblastic leukemia cells (Figure 1). These findings lead to the hypothesis that lymphoid lineage commitment is impaired in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells.

We next studied whether derangement of lymphoid lineage commitment and B cell differentiation in the *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells are indeed owing to BCR-ABL1 kinase activity. To this end, we induced BCR-ABL1 expression in a murine B lymphoid cell line carrying an inducible *BCR-ABL1*-transgene (Klucher et al., 1998, Figure 2A, B). BCR-ABL1 expression was induced by addition of doxycycline as monitored by measurement of BCR-ABL1 mRNA expression and Western blot analysis (Figure 2B). Induced expression of BCR-ABL1 resulted in upregulation of mRNA levels for the myeloid lineage-specific genes *Csfr1* and *Gata1* and downregulation of mRNA levels for *Il7rα*, which is critical for early lymphoid development. Of note, inducible BCR-ABL1 expression also resulted in aberrant splicing of *IKAROS*, leading to the expression of a dominant-negative form of Ikaros (IK6; Figure 2A). These findings indicate that BCR-ABL1 can promote lineage infidelity in the murine B cell precursor line. However, mRNA levels for *Oct2*, *Obf1* and *Pax5* remained unchanged.

Attenuation of lymphoid lineage commitment was also linked to BCR-ABL1 kinase activity in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells directly isolated from leukemia patients (Figure 2C, D). From three leukemia patients, matched sample pairs of leukemia cells were available before and during treatment with the BCR-ABL1 kinase-inhibitor **STI571 (Imatinib)**. Comparing leukemia cell samples before and during STI571-therapy by flow cytometry, inhibition of BCR-ABL1 kinase activity resulted in downregulation of the myeloid antigens GM-CSFRα, IL3Rα and CD13 (Figure 2C). Conversely, STI571-treated leukemia cells upregulated IL7Rα surface expression. In agreement with this, we found that inhibition of BCR-ABL1 kinase activity during STI571-therapy results in increased mRNA levels of *IL7Rα* while mRNA levels of myeloid transcription factors *AML1*, *GATA1* and *CEBPα* were downregulated (Figure 2D).

BCR-ABL1 induces defective expression of IKAROS

Previous studies showed that loss of functional *IKAROS* prevents normal B cell development (Kirstetter et al. 2002, Georgopoulos et al. 1994). Since the expression of a dominant negative splice variant IK6 can inhibit B cell differentiation (Tonnellet et al. 2001), lineage infidelity in human pre-B lymphoblastic leukemia cells may result from BCR-ABL1-induced derangement of *IKAROS* pre-mRNA splicing leading to aberrant expression of IK6. Furthermore, the expression of dominant negative *IKAROS* splice variants lacking the DNA-binding domain was recently reported in both childhood and adult B cell lineage acute lymphoblastic leukemia (Sun et al., 1999; Nakase et al., 2000). Studying *IKAROS* isoform expression in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia, expression of dominant-negative IK6 was detected in 6 of 7 primary cases (Cases XIII to XIX in Klein et al., 2004) and two of three cell lines (in BV173 and SUP-B15 but not in Nalm1 cells).

As shown by us and others, BCR-ABL1 can induce aberrant splicing of various genes (Perrotti and Calabretta, 2001) including *SLP65* (Jumaa et al., 2003; Klein et al., 2004), *PYK2* (Salesse et al., 2004) and BTK (Feldhahn et al., 2005). Consistent with BCR-ABL1-induced derangement of *IKAROS* pre-mRNA splicing, patient-derived leukemia cells express dominant-negative IKAROS (IK6), before but not during extended therapy with the BCR-ABL1 kinase inhibitor STI571 (Figure 2D).

To directly analyze the effect of BCR-ABL1 expression on *IKAROS* pre-mRNA splicing in human pre-B lymphoblastic leukemia cells, 697 cells that carry an *E2A-PBX1* but not a *BCR-ABL1* gene rearrangement and only exhibit expression of full-length *IKAROS*, were transiently transfected with expression vectors encoding either GFP only or BCR-ABL1 and GFP (Figure 3A). For both transfections, GFP⁺ and GFP⁻ cells were sorted and separately analyzed for the expression of *IKAROS* splice variants. Expression of the BCR-ABL1 kinase in transfected cells was verified by Western blot analysis (Figure 3B). As shown in Figure 3A, induced expression of BCR-ABL1 results in aberrant splicing of *IKAROS* leading to the expression of IK6.

Inhibition of BCR-ABL1 kinase corrects splicing and nuclear localization of IKAROS in pre-B lymphoblastic leukemia cells

Treatment of the leukemia cells with STI571 for four days induced selective outgrowth of differentiating subclones that downregulate IL3R α expression and exhibit surface IgM expression (not shown), which indicates the pre-B to immature B cell transition (Klein et al., 2005). We studied mRNA expression of *IKAROS* in undifferentiated leukemia cells and in IgM⁺ MACS-enriched differentiating subclones. Whereas undifferentiated leukemia cells predominantly express dominant negative IK6, expression of functional IKAROS transcripts was largely restored in IgM⁺ differentiating subclones (Figure 3C).

While nuclear localization of IKAROS is critical for its function as a transcription factor, in two untreated leukemia cell lines expressing either p190 or p210 BCR-ABL1 proteins, IKAROS protein is localized in the cytoplasm, which is consistent with the expression of non-DNA binding IK6 (Figure 3D). However, differentiating subclones that were MACS-enriched for IgM-expression, exhibit a nuclear pattern of staining for IKAROS, which is consistent with expression of DNA-binding forms of IKAROS (Figure 3D).

Silencing of dominant negative IK6 partially restores B lymphoid lineage commitment

Expression of dominant-negative IK6 can effectively block B cell differentiation of hematopoietic progenitor cells from umbilical cord blood (Tonnellet et al., 2001). In the absence of functional IKAROS, hematopoietic stem cells cannot give rise to lymphoid cells and are exclusively diverted into the myeloid lineages (Georgopoulos et al., 1994).

In order to provide evidence for a possible link between the expression of IK6 and lineage infidelity in pre-B lymphoblastic leukaemia cells, we silenced IK6 expression in two *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cell lines (SUP-B15 and BV173). Three different siRNA duplexes against IK6 and one non-targeting siRNA duplex were used in transfection experiments. All siRNA duplexes were labeled with a Cy2 fluorochrome to identify transfected cells. Cy2⁺ cells carrying siRNAs were sorted by FACS and subjected to RT-PCR

analysis. In both cell lines, IK6 expression was significantly diminished (Figure 4). Using cells transfected with a non-targeting siRNA duplex as a reference, mRNA levels for the V(D)J-recombinase molecule *RAG1* and the B cell specific component of the surrogate light chain $\lambda 5$ were increased, while mRNA levels of the myeloid transcription factor *GATA1* were reduced in parallel with IK6. However, no differences were detected for the mRNA expression levels of *PAX5* (Figure 4). Specificity of the IK6-targeting siRNA duplexes were controlled by co-amplification of GAPDH, mRNA levels of which remained stable (Figure 4). We conclude that BCR-ABL1-induced expression of IK6 contributes to lineage infidelity observed in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells. However, we cannot exclude that also other molecules than IK6 also contribute BCR-ABL1-induced loss of lineage determination.

Taken together, these findings show that BCR-ABL1 kinase activity i.) induces lineage infidelity in pre-B lymphoblastic leukemia cells ii.) interferes with IKAROS pre-mRNA splicing and iii.) that aberrant expression of IK6 contributes to lineage infidelity. Therefore, we propose that defective lineage commitment and B cell differentiation in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells can occur as a consequence of BCR-ABL1-induced derangement of *IKAROS*-expression.

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References

- Busslinger M. Transcriptional control of early B cell development. *Annu Rev Immunol.* 2004; 22: 55-79.
- Feldhahn N, Klein F, Mooster JL, Hadweh P, Sprangers M, Wartenberg M, Bekhite MM, Hofmann WK, Herzog S, Jumaa H, Rowley JD, Müschen M. Mimicry of a constitutively active pre-B cell receptor in acute lymphoblastic leukemia cells. *J Exp Med.* 2005; 201: 1837-1852.
- Feldhahn N, Schwering I, Lee S, Wartenberg M, Klein F, Wang H, Zhou G, Wang SM, Rowley JD, Hescheler J, Krönke M, Rajewsky K, Küppers R, Müschen M. Silencing of B cell receptor signals in human naive B cells. *J Exp Med.* 2002; 196:1291-305.
- Georgopoulos K, Bigby M, Wang JH, Molnar A, Wu P, Winandy S, Sharpe A. The Ikaros gene is required for the development of all lymphoid lineages. *Cell.* 1994; 79: 143-56
- Huettner CS, Zhang P, Van Etten RA, Tenen DG. Reversibility of acute B-cell leukaemia induced by BCR-ABL1. *Nat Genet.* 2000; 24: 57-60.
- Jumaa H, Bossaller L, Portugal K, Storch B, Lotz M, Flemming A, Postila V, Riikonen P, Pelkonen J, Niemeyer CM, Reth M. Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukaemia. *Nature.* 2003; 423: 452-456.
- Klein F, Feldhahn N, Lee S, Wang H, Ciuffi F, von Elstermann M, Toribio ML, Sauer H, Wartenberg M, Barath VS, Krönke M, Wernet P, Rowley JD, Müschen M. T lymphoid differentiation in human bone marrow. *Proc Natl Acad Sci U S A.* 2003; 100: 6747-52.
- Klein F, Feldhahn N, Harder L, Wang H, Wartenberg M, Hofmann WK, Wernet P, Siebert R, Müschen M. The BCR-ABL1 kinase bypasses selection for the expression of a Pre-B cell receptor in Pre-B acute lymphoblastic leukemia cells. *J Exp Med.* 2004; 199: 673-85.

- Klein F, Feldhahn N, Mooster JL, Sprangers M, Hofmann WK, Wernet P, Wartenberg M, Müschen M. Tracing the pre-B to immature B cell transition in human leukemia cells reveals a coordinated sequence of primary and secondary *IGK* gene rearrangement, *IGK* deletion, and *IGL* gene rearrangement. *J Immunol.* 2005; 174: 367-375.
- Klucher KM, Lopez DV, Daley GQ. Secondary mutation maintains the transformed state in BaF3 cells with inducible BCR/ABL expression. *Blood.* 1998; 91: 3927-34.
- Laurent E, Talpaz M, Kantarjian H, Kurzrock R. The BCR gene and philadelphia chromosome-positive leukemogenesis. *Cancer Res.* 2001;61(6): 2343-55.
- Look AT. Oncogenic transcription factors in the human acute leukemias. *Science.* 1997; 278: 1059-1064.
- Müschen M, Lee S, Zhou G, Feldhahn N, Barath VS, Chen J, Moers C, Krönke M, Rowley JD, Wang SM. Molecular portraits of B cell lineage commitment. *Proc Natl Acad Sci U S A.* 2002; 99: 10014-9.
- Nakase K, Ishimaru F, Avitahl N, Dansako H, Matsuo K, Fujii K, Sezaki N, Nakayama H, Yano T, Fukuda S, Imajoh K, Takeuchi M, Miyata A, Hara M, Yasukawa M, Takahashi I, Taguchi H, Matsue K, Nakao S, Niho Y, Takenaka K, Shinagawa K, Ikeda K, Niiya K, Harada M. Dominant negative isoform of the Ikaros gene in patients with adult B-cell acute lymphoblastic leukemia. *Cancer Res.* 2000; 60: 4062-5.
- Perrotti D, Calabretta B. Post-transcriptional mechanisms in BCR/ABL leukemogenesis: role of shuttling RNA-binding proteins. *Oncogene.* 2002; 21: 8577-83.
- Salesse S, Dylla SJ, Verfaillie CM. p210 BCR/ABL-induced alteration of pre-mRNA splicing in primary human CD34⁺ hematopoietic progenitor cells. *Leukemia.* 2004; 18: 727-733.

- Sun L, Heerema N, Crotty L, Wu X, Navara C, Vassilev A, Sensel M, Reaman GH, Uckun FM. Expression of dominant-negative and mutant isoforms of the antileukemic transcription factor Ikaros in infant acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A*. 1999; 96: 680-5.
- Tonnelle C, Bardin F, Maroc C, Imbert AM, Campa F, Dalloul A, Schmitt C, Chabannon C. Forced expression of the Ikaros 6 isoform in human placental blood CD34⁺ cells impairs their ability to differentiate toward the B-lymphoid lineage. *Blood*. 2001; 98:2673-80.
- Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science*. 1995; 270: 484-487.

Figure legends

Figure 1: *SAGE-Analysis of differentially expressed genes in BCR-ABL1⁺ pre-B lymphoblastic leukemia cells and normal pre-B cells*

cDNA-synthesis, SAGE analysis, cloning and sequencing of SAGE concatemers was carried out as previously described (Müschen et al., 2001; Feldhahn et al., 2001; Klein et al., 2003). A total of 592,000 SAGE tags were collected for 10 SAGE profiles. All SAGE libraries were normalized to 100,000 tags. Leukemia cells from two cases of *BCR-ABL1⁺* pre-B lymphoblastic leukemia (case II, p190 and case IX, p210, Klein et al., 2004) and normal bone marrow populations and mature B cell subsets were compared by SAGE. Upregulated genes are depicted in red, downregulated genes in green. For each gene, a proposed function with a reference is given. SAGE extracts a 14-bp fragment of any transcript in the analyzed cell population as a unique identifier (SAGE-tag) of an expressed gene using the reference database UniGene. By counting the number of SAGE-tags per 100,000, the representation of a transcript within the transcriptome can be quantified. Normal bone marrow populations analyzed include CD34⁺ hematopoietic progenitor cells (HSC), CD15⁺ myeloid progenitor cells (CMP), CD7⁺ CD10⁺ T lymphoid progenitor cells (TLP) and CD10⁺ CD19⁺ pre-B cells (pre-B). In addition, CD19⁺ CD27⁻ naïve B cells (NBC), CD20⁺ CD77⁺ germinal centre B cells (GCB), CD19⁺ CD27⁺ memory B cells (MBC) and CD19⁺ CD138⁺ plasma cells (PC) were analysed. In both leukemia cases (p190 and p210), the cells harbor an *V_HDJ_H* gene rearrangement on one allele and an *DJ_H* gene rearrangement on the second allele (Klein et al., 2004). However, *IGK* and *IGL* loci were in germline configuration in both cases (not shown). An *IGH VDJ*-gene rearrangement in the absence of *IGK* or *IGL* gene rearrangement defines a pre-B cell stage of development. We therefore sorted SAGE-data based on the ratio of SAGE-tag frequencies in *BCR-ABL1⁺* pre-B lymphoblastic leukemia cells to normal pre-B cells. SAGE data were graphically visualized using the Cluster and Treeview software (<http://rana.lbl.gov/>) and sorted according to the ratio between SAGE-tag counts in pre-B cells and in *BCR-ABL1⁺* ALL cases.

Figure 2: *BCR-ABL1 kinase activity results in lineage infidelity and differentially regulates the expression of lymphoid versus myeloid-specific genes*

A B lymphoid derivative of the murine cell line TONB210 (Klucher et al., 1998) carrying an inducible *BCR-ABL1* transgene under the control of a doxycycline-dependent promoter was used for inducible expression of a *BCR-ABL1* transgene. The cells were cultured in the presence or absence of 1 $\mu\text{g/ml}$ doxycycline (DOX). In the absence of BCR-ABL1 expression the cells remain viable in the presence of 2 ng/ml IL-3. BCR-ABL1 expression was induced by addition of 1 $\mu\text{g/ml}$ doxycycline and verified by Western blot analysis (B). EIF4e was used as a loading control. mRNA levels for human BCR-ABL1 and murine *Csfr1*, *Gata1*, *Pax5*, *Ikaros* splice variants, *Il7r α* , *Oct2*, *Obf1* and *Hprt* were monitored (A). From three leukemia patients, matched leukemia sample pairs (Patients I to III correspond to cases XIII, XIV and XVIII in Klein et al., 2004) before (white histograms) and during (grey histograms) therapy with the BCR-ABL1 kinase inhibitor STI571 were analyzed by flow cytometry for surface expression of GM-CSFR α , IL3R α , CD13 and IL7R α (C). One representative case out of three is shown. These patient samples were also subjected to RT-PCR analysis for mRNA expression of *IKAROS* splice variants, *PAX5*, *IL7R α* and the myeloid transcription factors *AML1*, *GATA1*, *CEBP α* and normalized for cDNA amounts and leukemia cell content by amplification of *BCR-ABL1* and *GAPDH* cDNA fragments (D). Specificity of the inhibitory effect of STI571 on BCR-ABL1 with respect to B lymphoid versus myeloid lineage markers was tested using three *MLL-AF4*⁺ leukemia cell lines (BEL1, RS4;11 and SEM) that did not carry a *BCR-ABL1* gene rearrangement yet exhibited a mixed lineage (lymphoid/ myeloid) phenotype. These three cell lines were cultured in the presence or absence of 10 $\mu\text{mol/l}$ STI571 for 48 hours. STI571 had no effect on expression levels (carried out by FACS analysis) of myeloid-lineage related surface molecules GM-CSFR α , IL3R α and CD13 and the lymphoid lineage marker IL7R α (not shown). Primers used for semiquantitative RT-PCR analysis are listed in supplementary Table 1.

Figure 3: *BCR-ABL1 induces aberrant splicing of IKAROS in human pre-B lymphoblastic leukemia cells*

697 pre-B lymphoblastic leukemia cells carrying an *E2A-PBX1* gene rearrangement were transiently transfected through electroporation with an expression vector encoding only GFP (pMIG_GFP) or BCR-ABL1 and GFP (pMIG_BCR-ABL1/GFP). Expression of the BCR-ABL1 kinase was identified by Western blot analysis (B). EIF4e was used as loading control. 24 hours after electroporation, for each transfection, GFP⁺ and GFP⁻ cells were sorted, subjected to RNA isolation, cDNA synthesis and analyzed for the expression of *IKAROS* splice variants (A). cDNA amounts were normalized for *COX6B* mRNA levels. *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells (BV173) were cultured in the presence or absence of 10 μmol/l STI571 for four days. Differentiating IgM⁺ subclones were enriched by MACS as previously described (Klein et al., 2004). Undifferentiated *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells (IgM⁻) and differentiating subclones (IgM⁺) were subjected to RT-PCR analysis for *IKAROS* isoform expression (C). IK6 expression (IKAROS splice variant lacking exons 3 to 6) was identified by sequence analysis. (Sequence data is available from EMBL/GenBank under accession number, pending). cDNA amounts were normalized by amplification of BCR-ABL1 fusion transcripts. Localization of IKAROS (D) was studied by confocal laser microscopy in *BCR-ABL1*⁺ pre-B lymphoblastic leukemia cells (BV173 and SUP-B15) expressing either a p210 or p190 BCR-ABL1 fusion molecule, respectively. For localization of IKAROS, undifferentiated and MACS-enriched IgM⁺ differentiating subclones were stained with propidium iodide (nucleus) and with anti-IKAROS antibodies. Nuclear or cytoplasmic localization of IKAROS was analyzed using primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) together with anti-rabbit IgG-Cy2 (Jackson Immunoresearch). Cells were fixed with 0.4% para-formaldehyde and incubated for 10 minutes in 90% methanol on ice and subjected to confocal laser-scanning microscopy as previously described (Klein et al., 2003).

Figure 4: *Specific silencing of IK6 partially restores lineage determination in BCR-
ABL1⁺ pre-B leukemia cells.*

BCR-ABL1⁺ pre-B lymphoblastic leukemia cell lines (SUP-B15 and BV173) were transfected either with a pool of siRNA duplicies targeting IK6 or a non-targeting siRNA duplex serving as a negative control. Three different IK6 siRNAs were designed and synthesized (MWG Biotech, Ebersberg, Germany) to target the junction of exon2 and exon7, which defines the *IKAROS* splice variant IK6 (sequence data available from EMBL/GenBank under accession number, pending). The control non-targeting siRNA duplex did not match a known mRNA sequence. The IK6-specific siRNA duplexes were designed according to the guidelines described by Dr. Thomas Tuschl at <http://www.rockefeller.edu/labheads/tuschl/sirna.html>. All siRNA duplices were labeled with Cy2 using an siRNA labeling kit (Ambion, Austin, TX) according to the manufacturer's protocol. siRNAs were applied to the cells with a final concentration of 100 nmol/l for each siRNA, using Oligofectamine (Invitrogen) in Opti-MEM1 medium (Invitrogen) according to the manufacturer's instructions. After 24 hours, leukemia cells were re-transfected with labeled siRNAs and subsequently incubated for further 24 hours. The silencing effect of siRNAs for *IKAROS* isoform IK6 was controlled by RT-PCR analysis of IK6 in Cy2⁺ sorted cells. siRNA transfected cells were subjected to RT-PCR analysis as previously described and investigated for the expression of *IKAROS*, *IK6*, *PAX5*, *RAG1*, *λ5*, *GATA1* and *GAPDH*. Oligonucleotides used for semiquantitative RT-PCR analysis and siRNA oligonucleotides are listed in supplementary Table 1.

Figure 1: *SAGE-Analysis of differentially expressed genes in BCR-ABL1⁺ B cell precursor leukemia cells and normal pre-B cells*

HSC	CMP	TLP	p190	p210	pre-B	NBC	GCB	MBC	PC	UniGene	Gene name	Proposed function	Reference
										81994	<i>GYPC</i>	erythroid/ myeloid differentiation	Robinson et al., 1981
										2175	<i>CSF3R</i>	GCSF-receptor	Dong et al., 1996
										181002	<i>MSF1</i>	related to myeloid leukemia	Osaka et al., 1999
										85289	<i>CD133</i>	hematopoietic stem cell antigen	Yin et al., 1997
										182018	<i>IRAK1</i>	IL1 signaling in myeloid cells	Cao et al., 1996
										79026	<i>MLF2</i>	myeloid leukemia factor 2	Kuefer et al., 1996
										169832	<i>MZF1</i>	myeloid zinc finger gene 1	Hromas et al., 1991
										112255	<i>NUP98</i>	related to myeloid leukemia	Ahuja et al., 1999
										174142	<i>CSF1R</i>	colony-stimulating factor 1 receptor	Gisselbrecht et al., 1987
										460463	<i>IL3Rα</i>	Shared subunit with GM-CSFR	Kitamura et al., 1991
										1817	<i>MPO</i>	myeloperoxidase	Weil et al., 1988
										89633	<i>PML</i>	related to myeloid leukemia	Cleary, 1996
										1239	<i>CD13</i>	myeloid differentiation antigen	Look et al., 1986
										129914	<i>AML1</i>	implicated in myelopoiesis	Tanaka et al., 1995
										153837	<i>MNDA</i>	myeloid nuclear differentiation antigen	Briggs et al., 1994
										83731	<i>CD33</i>	myeloid differentiation	Peiper et al., 1988
										31551	<i>CBFA2T2</i>	related to myeloid leukemia	Kitabayashi et al., 1998
										765	<i>GATA1</i>	myeloid differentiation	Qian et al., 2002
										174103	<i>CD11A</i>	macrophage differentiation	Springer et al., 1985
										196352	<i>NCF4</i>	oxidative burst in myeloid cells	Zhan et al., 1996
										279751	<i>SIGLEC8</i>	myeloid inhibitory receptor	Kikly et al., 2000
										82116	<i>MYD88</i>	IL1 signaling in myeloid differentiation	Adachi et al., 1998
										272537	<i>TdT</i>	generation of junctional diversity	Kung et al., 1975
										89414	<i>CXCR4</i>	SDF1 receptor, pre-B cells	Nagasawa et al., 1996
										25648	<i>CD40</i>	Proliferation of B cell precursors	Hasbold et al., 1994
										96023	<i>CD19</i>	B cell co-receptor	Carter & Fearon, 1992
										285823	<i>IGHCM</i>	IGH μ chain	Raff et al., 1976
										170121	<i>CD45</i>	Antagonizes SHP1, CSK	Pani et al., 1997
										101047	<i>E2A</i>	Initiation of <i>IGH</i> gene rearrangement	Bain et al., 1994
										79630	<i>Igα</i>	ITAM signaling chain	Flaswinkel et al., 1995
										74101	<i>SYK</i>	B cell receptor signaling	Kurosaki et al., 1994
										237868	<i>IL7Rα</i>	Lymphoid differentiation	Uckun et al., 1991
										66052	<i>CD38</i>	Ligation causes tyrosine phosphorylation	Kitanaka et al., 1999
										167746	<i>BLNK</i>	Linker in B cell receptor signaling	Fu et al., 1998
										82132	<i>IRF4</i>	<i>IGK</i> and <i>IGL</i> gene rearrangement	Lu et al., 2003
										2407	<i>OBF1</i>	<i>IGK</i> gene transcription	Casellas et al., 2002
										22030	<i>PAX5</i>	Required for pro- to pre-B cell transition	Nutt et al., 1999
										158341	<i>TAC1</i>	Growth control of early B cells	Yan et al., 2001
										54452	<i>IKAROS</i>	Critical for early B cell development	Kirstetter et al., 2002
										1521	<i>IgMBP2</i>	Signal transduction through Ig α	Grupp et al., 1995
										3631	<i>IgαBP1</i>	Involvement in B cell receptor signaling	Fukita et al., 1993
										159494	<i>BTK</i>	Critical for (pre-) B cell receptor signaling	Cheng et al., 1994
										192861	<i>SPIB</i>	<i>IGK</i> and <i>IGL</i> gene rearrangement	Su et al., 1996
										73958	<i>RAG1</i>	V(D)J recombination	Menetski & Gellert, 1990
										159376	<i>RAG2</i>	V(D)J recombination	Schatz et al., 1989
										1101	<i>OCT2</i>	Regulates <i>IGH</i> gene transcription	Staudt et al., 1988
										192824	<i>EBF</i>	Required for B lymphopoiesis	Lin & Grosschedl, 1995

Figure 2: *BCR-ABL1 kinase activity results in lineage infidelity and differentially regulates the expression of lymphoid versus myeloid-specific genes*

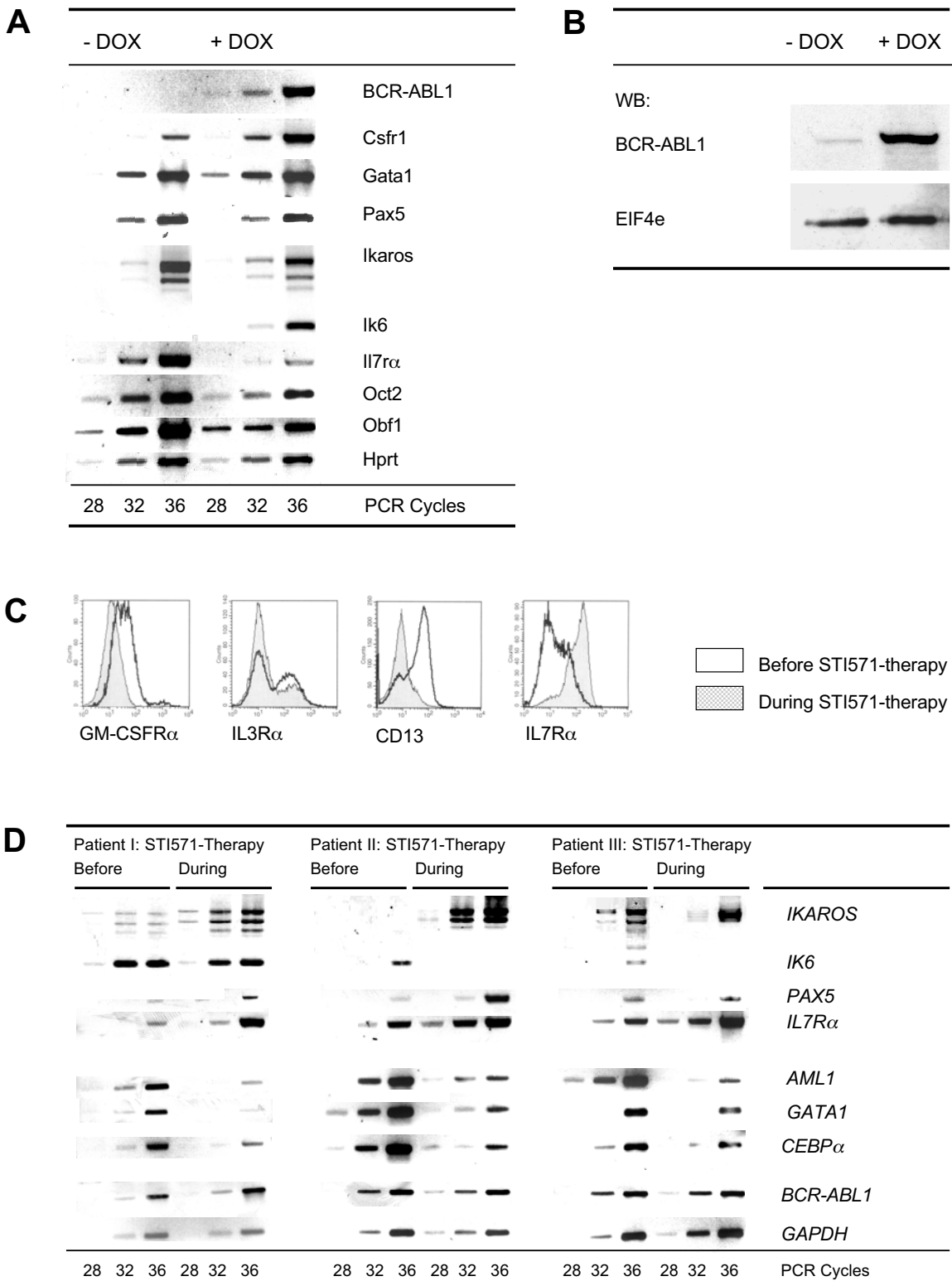


Figure 3: *BCR-ABL1 induces aberrant splicing of IKAROS in human pre-B lymphoblastic leukemia cells*

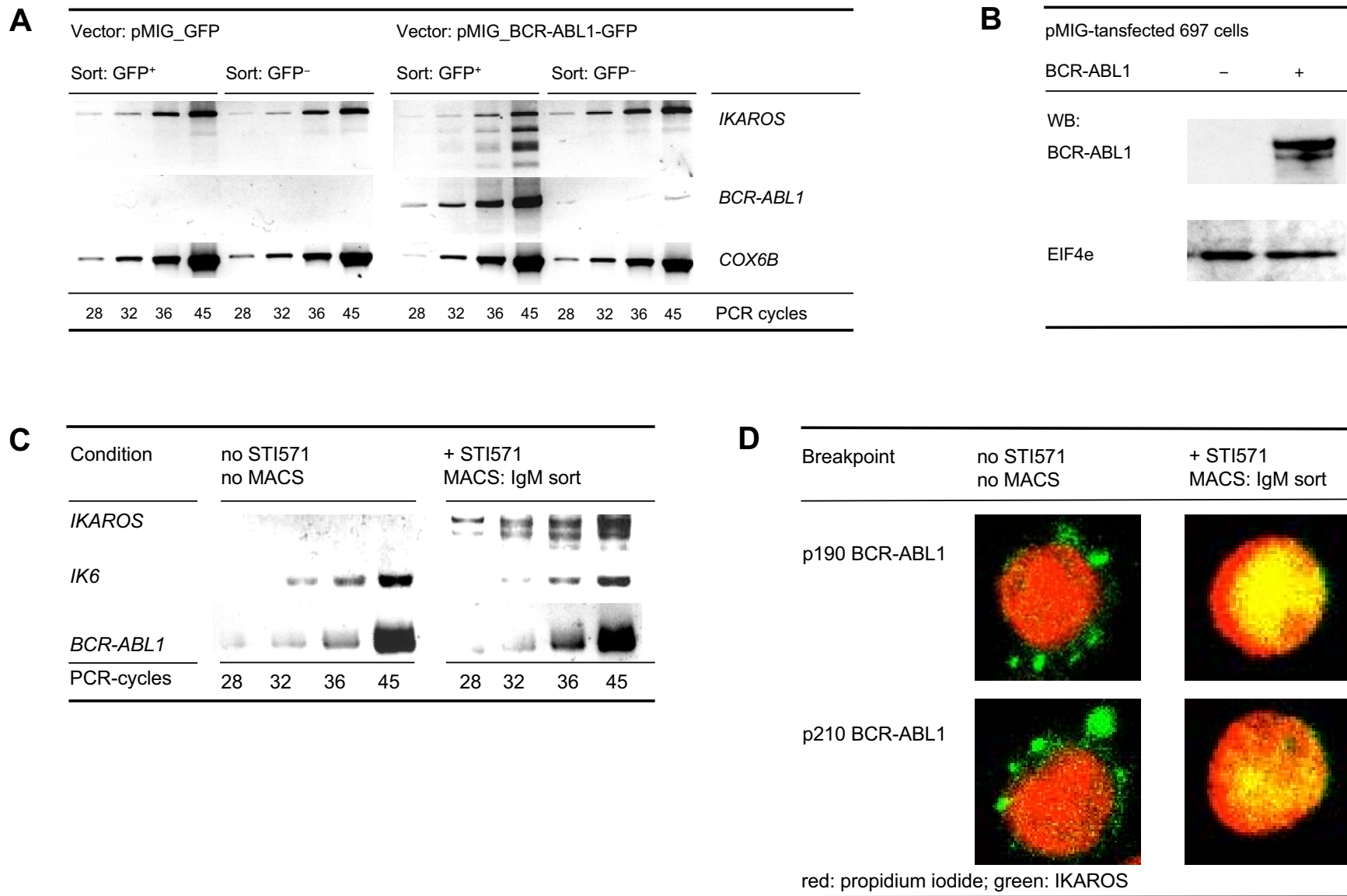
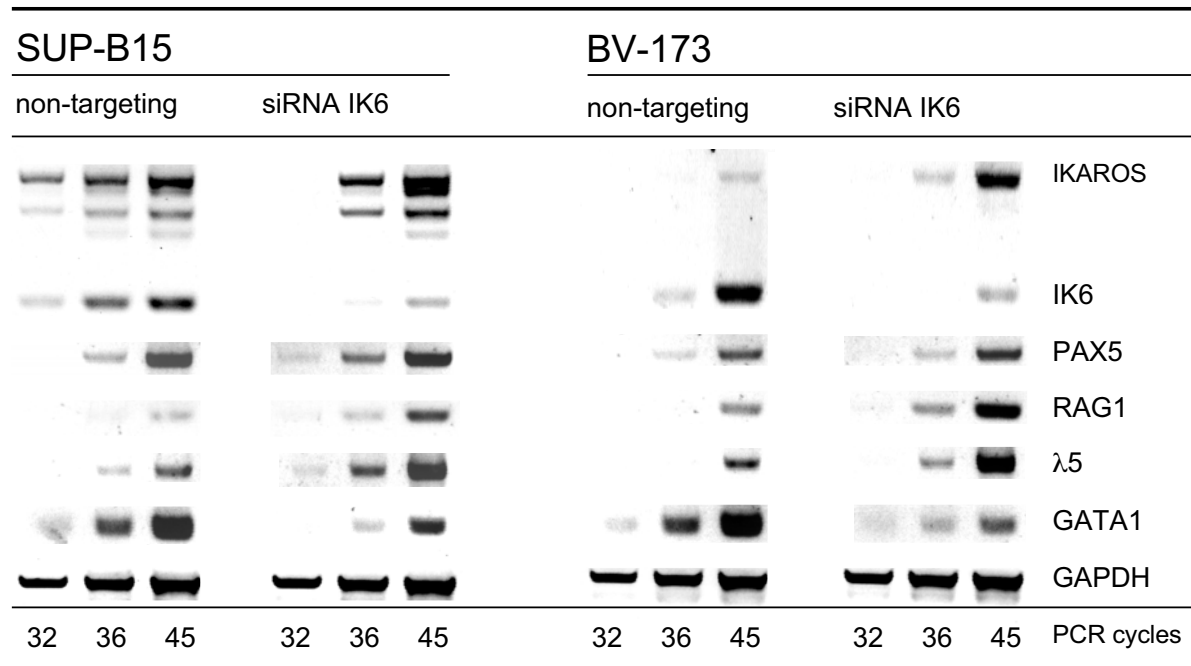


Figure 4: *Specific silencing of IK6 partially restores lineage determination in BCR-ABL1⁺ pre-B leukemia cells*



Supplementary Table 1: *Oligonucleotides used for semiquantitative RT-PCR and RNA interference*

Human-specific primers:

BCR-ABL1	forward	5'-ACCTCACCTCCAGCGAGGAGGACTT-3'
BCR-ABL1	reverse	5'-TCCACTGGCCACAAAATCATACAGT-3'
COX6B	forward	5'-AACTACAAGACCGCCCCTTT-3'
COX6B	reverse	5'-GCAGCCAGTTCAGATCTTCC-3'
GAPDH	forward	5'-TTAGCACCCCTGGCCAAG-3'
GAPDH	reverse	5'-CTTACTCCTTGGAGGCCATG-3'
IKAROS	forward	5'-CCCCCTGTAAGCGATACTCCAGAT-3'
IKAROS	reverse	5'-GGCTTGGTCCATCACGTGGGA-3'
IL7RA	forward	5'-CAAAATGCTGATGGTTAGTAAG-3'
IL7RA	reverse	5'-ACGGGAACCCAGGAGACA-3'
CEBPA	forward	5'-AGTCAGACCAGAAAGCTAG-3'
CEBPA	reverse	5'-AAATGGTGGTTTAGCAGAGA-3'
AML1	forward	5'-AATGATGAAAACACTACTCGGCT-3'
AML1	reverse	5'-TTGGTCTGATCATCTAGTTTC-3'
GATA1	forward	5'-AGTCTTTCAGGTGTACCCAT-3'
GATA1	reverse	5'-AAAGAAGGTACTGGAAAAGTC-3'
PAX5	forward	5'-GAGGATAGTGGAACCTTGCTCAT-3'
PAX5	forward	5'-GTGAAGATGTCTGAGTAGTGCTG-3'
RAG1	forward	5'- TGCAGACATCTCAACACTTTGGCCAG-3'
RAG1	reverse	5'- TTTCAAAGGATCTCACCCGGAACAGC-3'
λ5	forward	5'- TAACTCAGTGACGCATGTGTTTG-3'
λ5	reverse	5'- GCTGGGAACCTATGAACATTCTG-3'

Mouse-specific primers:

mHprt	forward	5'-GGGGGCTATAAGTTCTTTGC-3'
mHprt	reverse	5'-TCCAACACTTCGAGAGGTCC-3'
mIkaros	forward	5'- CACAAGTCTGTTGATAACCTGAA-3'
mIkaros	reverse	5'- ACCAACATAAAAGAACAAATGCT-3'
mI17ra	forward	5'-CACCATTCTGAGTTTGTTC-3'
mI17ra	reverse	5'-TTTAAGATGCCTGGCTAGAAAT-3'
mObf1	forward	5'-AGCTCCCTGACCATTGAC-3'
mObf1	reverse	5'-CTGTCCCATCCCCCTGTAA-3'
mOct2	forward	5'-ATCGAGACGAATGTCCGCTT-3'
mOct2	reverse	5'-GTAGCTGGTCGGCTTTCC-3'
mPax5	forward	5'-AGAGAAAAATTACCCGACTCCTC-3'
mPax5	reverse	5'-CATCCCTCTTGCGTTTGTGGTG-3'
mCsfr	forward	5'-TCCCCCAGAGGTCAGTGTTAC-3'
mCsfr	reverse	5'-GCCAGTCCAAAGTCCCCAATC-3'
mGata1	forward	5'- TACTGAGATTCAGGCATGTATTG-3'
mGata1	reverse	5'- TCCAATTGACACTGACATTTATT-3'

siRNA-constructs for RNA interference

non-targeting siRNA	5'-UUGUACCUGAAUUUCGUCCCAC-3'
	3'-CAUGGAUUAAAGCAGGGUGUU-5'
IK6 siRNA1	5'-UUACAGAGUCGUGGGGGACAA-3'
	5'-UUGUCCCCCAGACUCUGUUU-3'
IK6 siRNA2	5'-UUUCGUGGGGGACAAGGGCCU-3'
	5'-AGGCCCUUGUCCCCCAGAUU-3'
IK6 siRNA3	5'-UUGAGUCGUGGGGGACAAGGC-3'
	5'-CCCUUGUCCCCCAGACUCUU-3'