NUMB inactivation confers resistance to imatinib in chronic myeloid leukemia cells

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ABSTRACT

Chronic myeloid leukaemia (CML) progresses from a chronic to a blastic phase, where the leukemic cells are proliferative and undifferentiated. The CML is nowadays successfully treated with BCR-ABL kinase inhibitors as imatinib and its derivatives. NUMB is an evolutionary well-conserved protein initially described as a functional antagonist of NOTCH function. NUMB is an endocytic protein associated with receptor internalization, involved in multiple cellular functions. It has been reported that MSI2 protein, a NUMB inhibitor, is upregulated in CML blast crisis, whereas NUMB itself is downregulated. This suggest that NUMB plays a role in the malignant progression of CML. Here we have generated K562 cells (derived from CML in blast crisis) constitutively expressing a dominant negative form of NUMB (dnNUMB). We show that dnNUMB expression confers a high proliferative phenotype to the cells. Importantly, dnNUMB triggers a partial resistance to imatinib in these cells, antagonizing the apoptosis mediated by the drug. Interestingly, imatinib resistance is not linked to p53 status or NOTCH signaling, as K562 lack p53 and imatinib resistance is reproduced in the presence of NOTCH inhibitors. Taken together, our data support the hypothesis that NUMB activation could be a new therapeutic target in CML.

INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasia usually diagnosed as an indolent chronic phase characterized by the accumulation of differentiated myeloid cells, followed by an accelerated phase where myeloid cell expansion increases, and finally progresses into a blast crisis phase where the expanded myeloid cells are more immature and aggressive. The molecular hallmark of all CML is expression of the BCR-ABL tyrosine kinase. This fusion oncoprotein is the result of the 9; 22 translocation giving rise to the Philadelphia chromosome [1, 2]. BCR-ABL inhibitors as imatinib and other more recently introduced inhibitors are the frontline drugs in CML therapy [2, 3].

The available data indicate that CML is a hematopoietic stem cell malignancy where BCR-ABL leads to a progressive block of differentiation and increased genetic instability [1, 4, 5]. Although constitutive expression of BCR-ABL is sufficient to recapitulate the chronic phase, the molecular basis underlying the transition from a chronic to an aggressive blast crisis phase remains poorly understood.

NUMB is an evolutionarily well-conserved protein, initially described as a NOTCH inhibitor [6, 7]. In subsequent studies, Numb was shown to be an endocytic protein associated with receptor internalization and clathrin dependent endosomal association [8-

12]. Numb plays a critical role in cell fate determination, displaying a complex pattern of functions, controlling asymmetric cell division, cell adhesion and migration, acting on several signaling pathways, as preTCR and Hedgehog [13, 14]. Interestingly, there is also evidence of a reduction in NUMB expression in some cancers [15-18].

The RNA-binding protein MSI2 (Musashi 2) promotes NOTCH signaling by binding to the mRNA of Numb, a negative regulator of NOTCH signaling. Recent reports demonstrated that MSI2 is upregulated in blast crisis CML, and correspondingly, NUMB was downregulated. Moreover, exogenous expression of NUMB inhibited leukemogenesis [19]. Thus, the MSI2–NUMB signaling axis plays a role in the malignant progression of CML and the promotion of a more immature aggressive myeloid leukaemia [19, 20]. Additionally, this stem cell regulator, MSI2, has been recently described as a prognosis factor in acute myeloid leukemia, being involved in the maintenance and engraftment of HSC [21], but whether this function is independent of NUMB is under debate [22].

To better define the role of NUMB in CML, we engineered a human CML cell line, K562, carrying a dominant negative NUMB form (dnNUMB). This is a truncated NUMB protein containing the N-terminal phosphotyrosine-binding region (PTB) described as an activator of NOTCH signaling [23, 24]. We describe here that NUMB loss of function increased K562 proliferation and confers partial resistance to imatinib, and these effects are Notch-independent.

RESULTS

We first set out to generate K562 cells with constitutive expression of dominant-negative NUMB (dnNUMB). We used as dnNUMB the region of 163 amino acids comprising the PTB domain [23] (Fig. 1A). We added a Myc tag at the N-terminus and the Myc-PTB was inserted in the SEWP lentiviral vector backbone [25] to generate the SdnNUMBWP lentiviral vector The SEWP lentiviral vector expressing enhanced green fluorescence protein (eGFP) was used as control. The major features of these vectors are depicted in Fig 1B. We transduced K562 cells with SdnNUMBWP lentiviral particles to generated the KdnNUMB cell line and dnNUMB ectopic expression in the transduced cell lines was analyzed by intracellular flow cytometry using an anti-Myc tag antibody. As a control we also transduced cells with lentivirus driving GFP expression (KGFP). With increasing volumes of viral supernatant we obtained increasing percentages of transduced cells reaching up to 85 % of the cells expressing dnNUMB protein (not shown). We selected a pool of transduced cells and the expression of dnNUMB was assessed by western blot (Fig. 1C). It is expected that the expression of dnNUMB would result in over-activation of NOTCH. Thus, to test whether KdnNUMB cells expressed a functional dnNUMB protein, we determined the mRNA levels of several NOTCH target genes. For this purpose, total RNA from KdnNUMB and control KGFP

cells was extracted and mRNA levels of HES1, E2A, CCND1, CDK2, BCLX (BCL2L1) and MYC were analyzed by RT-qPCR. The result showed increased expression of NOTCH target genes in KdnNUMB cells (Fig.1D). This indicates that dnNUMB is impairing NOTCH signaling in K562 cells through the functional inactivation of NUMB.

We next asked whether inhibition of NUMB exerted an effect on K562 cell proliferation. The results showed that expression of dnNUMB resulted in an increased proliferation rate of KdnNUMB cells, as demonstrated by cell counting (Fig. 2A) and thymidine incorporation into DNA (Fig. 2B). In an independent approach, we labeled K562 cells expressing the dominant negative NUMB as well as control cells with bromodeoxyuridine (BrdU) for 24 h. The results showed that K562 cells expressing dnNUMB proliferated more than control K562 cells (Fig. 2C). To confirm the above results in lentivirally transduced cells we also transfected K562 with a plasmidic dnNUMB expression vector (pCEFL-dnNUMB). Several stable dnNUMB-transfectant clones were selected and the dnNUMB overexpression confirmed by immunoblot (Fig. 2D). The proliferation of two of them was tested by BrdU incorporation and the results showed that these clones proliferated faster than the control cells transfected with the empty vector (Fig 2E). Altogether the results show that dnNUMB elevated the proliferation rate in K562 cells. The hyperproliferation of K562 due to dnNUMB was a striking finding given that parental K562 cells already show a high proliferation capacity.

In view of the association of NUMB-Mushashi with CML progression, we assayed the effect of dnNUMB on the response to imatinib, the front-line drug used in this leukemia. In parental K562 cells imatinib at concentrations ≥0.5 μM readily induces cell death, as previously reported [26, 27]. In contrast, KdnNUMB cells were partially resistant to the antiproliferative effect of imatinib, as assessed by viable cell counting (Fig. 3A). Cell cycle analysis also demonstrated a higher fraction of KdnNUMB in S phase as compared to control cells. This result was reproduced in the presence of imatinib and other BCR-ABL inhibitors as dasatinib and nilotinib (Fig. 3B). The KdnNUMB cells also showed a reduced imatinib-induced apoptosis, as assessed by the cleavage of poly (ADP-ribose) polymerase (PARP) after immunoblot analysis (Fig. 3C) and the fraction of sub-G1 cells as assessed by cell cytometry (Fig. 3D). We asked if this resistance to imatinib was due to the up-regulation of BCLX, a NOTCH target gene. BCLX protein expression was determined by immunoblot in cells untreated and treated with imatinib. The results show that BCLX levels were similar in KGFP and KdnNUMB cells, and that imatinib treatment reduces these levels This reduction is even higher in KdnNUMB cells (Fig. 3E). Therefore, the partial resistance to imatinib of KdnNUMB cells was not due to increased BCLX levels. As previously reported [28, 29], K562 does not express BCL2 (Fig. 3E), and therefore BCL2 cannot be involved in imatinib resistance.

To explore the mechanism for dnNUMB-mediated resistance to imatinib we studied the BCR-ABL kinase activity in KdnNUMB cells and in vector-expressing cells. The levels of phosphorylated BCR-ABL (which undergoes auto-phosphorylation) and CRKL (a classical BCR-ABL kinase substrate) [30] were analyzed by immunoblot. Consistent with their partial resistance against imatinib, KdnNUMB cells showed a higher BCR-ABL activity (Fig. 4A). Imatinib, however, did not impair the activity of dnNUMB as assessed by the elevated expression of different Notch-target genes (Fig. 4b). We conclude that dnNUMB confers a partial resistance of BCR-ABL to the growth inhibitory activity of imatinib. It has been described that imatinib induces erythroid differentiation in K562 [31] and that NOTCH enhances erythroid differentiation in human and mouse hematopoietic cells [32, 33]. Therefore, we tested differentiation of KdnNUMB cells, and we found that mRNA expression of erythroid genes, as erythropoietin receptor (EPOR) and several globins, was elevated in KdnNUMB cells as compared with control cells (Fig. 4C). The KdnNUMB cells also showed increased differentiation when treated with imatinib (Fig. 4C). The same result was found when γ-globin levels were determined by immunoblotting (Fig. 4D). The fraction of hemoglobinized cells as assessed by the benzidine assay was also higher in KdnNUMB cells with respect to control cells (Fig. 4E). K562 can also be differentiated into the megakaryocytic lineage by staurosporine [34] but dnNUMB showed no effect on this differentiation (not shown).

Finally, we asked whether dnNUMB effect is a consequence of its activating effect on NOTCH or due to other effects, as NUMB is not exclusively involved in NOCTH signaling but also mediates the internalization processes for many receptors. To address this question we used a γ-secretase inhibitor (DAPT) which specifically blocks NOTCH signaling. As expected, the levels of NOTCH Intracellular Domain (NICD), the product of active cleaved NOTCH was increased in KdnNUMB cells with respect to parental cells, as shown by immunoblot (Fig. 5A). The effect of DAPT on both cell lines was demonstrated by the decreased levels of NICD in DAPT-treated cells KGFP and KdnNUMB cells with respect to untreated cells (Fig. 5A). We next compared the effects of DAPT on cell proliferation of dnNUMB-expressing cells and the control cells. We assayed the dose-dependent effect of DAPT (Fig. 5B) ~~and along 72 h of treatment (Fig. 5D).~~ and the results showed that DAPT decreased the fast growing phenotype of KdnNUMB cells, but much less than in control cells. This result is consistent with the fact that dnNUMB activates the NOTCH pathway, as ~~confirmed~~ previously shown (Fig. 1D). The NOTCH inhibitor did not modify the resistance to imatinib shown by KdnNUMB cells (Fig. 5C). Moreover, DAPT did not significantly modify the expression of CDK2, HES1 and MYC mRNA (Fig. 6A) or BCLX protein (Fig. 6B) in KdnNUMB cells with respect to control cells in the presence of imatinib. Altogether, these

data show that the hyper-proliferative effect of NUMB inhibition and resistance to imatinib in CML cells is not primarily mediated by NOTCH.

DISCUSSION

Here we describe an increased proliferative rate in the K562 cell line carrying a dominant negative form of NUMB. It was previously reported that repression of NUMB could be essential for maintenance of blast crisis CML [20]. However, in contrast with that report, our results show that the pro-proliferative effect associated with this repression of Numb does not depend on p53, as K562 cells are deficient in p53 [35].

This stimulation in the proliferation could be explained by the up-regulation of several targets of NOTCH pathway observed in the K562 cells expressing dnNUMB, which include proteins that stimulate cell cycle (e.g., Cdk2, Myc, and E2A). However, it seems to be independent of NOTCH-mediated signaling, as the treatment with DAPT, a γ-secretase inhibitor~~, didn't have,~~ did not exert a relevant effect on the proliferative behavior of KdnNUMB cells. This is consistent with reports demonstrating that Notch inhibition does not impair K562 growth [36] and with the phenotype of dnNUMB transgenic mice, which, unexpectedly, is different from that of activated *Notch* transgenic mice [37, 38]. Our results suggest that pathways other than NUMB-NOTCH are being inhibited in KdnNUMB. It is conceivable that NUMB acts as endocytic adaptor for internalization of receptors required for CML cells proliferation, and dnNUMB will enhance or maintain the signaling form these receptors

Imatinib is an inhibitor of BCR-ABL, nowadays the front-line drug for CML, together with other related molecules, as dasatinib or nilotinib. One of the most important caveats for the treatment of CML is the acquired resistance to imatinib observed in a substantial proportion of patients due to prolonged drug exposure, which makes them refractory to this treatment and causes patient relapse [39]. We describe that dnNUMB-K562 cells show partial resistance to the cells death induced by imatinib and related molecules (dasatinib or nilotinib). This resistance is consistent with the unabated activity of BCR-ABL kinase of KdnNUMB cells in the presence of imatinib as assessed by CRKL phosphorylation. In agreement with this result, it has been recently shown in primary CML cells that NOTCH signaling leads to BCR-ABL hyperactivation [40]. It has also been reported that imatinib induces erythroid differentiation in K562 cells [31] and NOTCH activity stimulates erythroid differentiation in hematopoietic cells [32, 33]. We found that dnNUMB expression not only conferred partial resistance to imatinib-induced cell death, but also enhanced imatinib-induced differentiation.

Further work will be required to unveil the mechanism for ~~this~~ ~~interaction~~ the interaction between NUMB and BCR-ABL. In summary, our results suggest that low NUMB levels or activity contribute to proliferation of CML cells through mechanisms independent of NOTCH and p53. This finding support the hypothesis that activation or stabilization of NUMB may act as a new therapeutic target in CML.

MATERIALS AND METHODS

Cell Culture: All K56-derived cell lines were culturing in non-tissue culture plates using RPMI 1640 medium supplemented with 10% fetal calf serum and gentamycin (60 µg/ml) at 37ºC under normoxia conditions. HeLa cells were grown in DMEM with 10% fetal calf serum and gentamycin (60 µg/ml). Kbcl2 and KbclX cell lines were previously described [28, 29]. Cells were passaged every 3 days. Cell proliferation was assayed with a Nucleocounter (Chemometec). When indicated, cells were treated with imatinib, dasatinib or nilotinib (from LC Laboratories) or DATP (Sigma-Aldrich).

Cell cycle and erythroid differentiation analysis. Cell cycle distributions were assayed by ethanol fixation overnight and propidium iodide (PI) staining of DNA followed by fluorescence-activated cell sorting using Guava EasyCyte Flow Cytometer (Guava Technologies), following the manufacture's instructions. Viable cell counts were determined with the same cytometer using the ViaCount assay kit. The fraction of hemoglobin-producing cells was scored by the benzidine assay essentially as described [41]

Immunoblot: Nuclear and cytoplasmic extracts were obtained using lysis buffer (1% NP40, 0.2% SDS, 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 10 mM NaF and protease inhibitors) for 20 minutes at 4°C. Lysates were cleared by centrifugation after 4 pulses ON/OFF of 30sc in the Bioruptor. 50 µg of lysates were subjected to immunoblot as described [42]. The antibodies used were anti-MYC tag (mouse monoclonal from Santa Cruz Biotech, 9E10), anti-PARP (rabbit polyclonal, from Santa Cruz Biotech, H-250), anti-cleaved NOTCH1 (NICD, Val1744) (rabbit polyclonal from Cell Signalling), anti-BCLX (rabbit monoclonal from Cell Signalling), anti-BCL2 (mouse monoclonal from BD Transduction Laboratories), anti γ-globin (rabbit polyclonal from Santa Cruz Biotech), anti anti-β-actin (goat polyclonal from Sigma, I-19) and anti-α-tubulin (mouse monoclonal from Sigma, T6074). All antibodies were from Santa Cruz Biotech and were diluted 1:1000.The blots

were developed with secondary antibodies conjugated to IRDye680 and IRDye800 (Li-Cor Biosciences) and visualized in an Odyssey scanner.

RNA extraction and expression analysis Total RNA was isolated using Trizol or RNeasy kit (Qiagen). For reverse transcription and polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 1 µg of total RNA using SuperScriptTM II RNase reverse transcriptase (Invitrogen) and random primers. Quantitative RT-PCR was performed with a SYBR Green PCR kit (BioRad). The expression levels were normalized to the expression of human actin mRNA r human ribosomal protein S14 (RPS14)**.** The primers used in the PCR reactions are described in Table 1.

^{3}H-Thymidine incorporation: $5x10^{4}$ cells per well were cultured during 72h in a 96 well plate. Cultures were pulsed with 0.3uCi of 3H-thymidine (3H-TdR) for the final 15 hours of culture, harvested, and counted

BrdU labeling. BrdU labeling was performed by incubating K562 cells at 37°C in medium containing 100 µg/ml BrdU (Sigma). After 24 hours, cells were collected, washed with PBS and incubated with Lysis Solution FACs (BD Bioescience). Then, they were fixed with 1% paraformaldehyde (Panreac) and NP-40 (Sigma) for 30 minutes at 4ºC. Cells were incubated with Dnase I (Sigma) for 30 minutes and stained with BrdU-FITC for 45 minutes, followed by flow cytometry analysis.

Lentiviral vectors. The HIV packaging (pCMVDR8.91) and VSV-G (pMD.G) plasmids have been described elsewhere [43, 44]. The HIV packaging pCMVDR8.91 encodes gag, pol, tat and rev genes. The pMD.G plasmid encodes the Vesicular Stomatitis Virus (VSV) G protein (VSVg). The lentiviral vector plasmid SEWP (HRSIN-CSGW) [25] encodes the emerald green fluorescent protein (eGFP) under the control of the spleen focus forming virus (SFFV) promoter and contains the Woodchuck post-regulatory element (WPRE). The lentiviral vector SPTBNWP was generated by replacing the eGFP cDNA in the SE vector for a 519 bp fragment containing the PTB domain of numb gene with a Myc tag. To do this we generated a PCR fragment from the p-EF-Numb plasmid using the following primers: Forward: 5'CCGGATCCCACCATGGAACAAAAACTTATTTCTGAAGAAGATCTGAACAAACTACGGC AAAGCTTCAGG-3' and Reverse: 5'-CGCGCGGCCGCCTACTACTACTTCTCCCGC TTCTGTTTACGCTC-3' and cloned it into pGEM-T easy vector (Promega Corp.). The 519 bp fragment containing the PTB domain of numb and the myc tag was excised from pGEM-T by BamHI-NotI digestion and inserted into the lentiviral vector backbone. For, vector production, 293T cells were transfected with the three plasmids described above (pCMVDR8.91, pMD.G and SEWP or SPTBNWP) using Lipofectamine 2000 (GibcoBRL) as

previously described [45]. Two days after transfection, supernatants containing vector particles were harvested and filtered through 0.22um and frozen at -80^{o}C until use. Lentiviral particles were concentrated by ultrafiltration at 2000×g and 4°C, using 100 kDa centrifugal filter devices (Amicon Ultra-15, Millipore, Billerica, MA)

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FIGURE LEGENDS

Figure 1 Construction of KdnNUMB. (A) Schematic drawing of the NUMB protein and the region used as dnNUMB in this study. The Phosphotyrosine-Binding domain (PTB) and Prolin Rich Region (PRR) domains are as described [23]. (B) Schematic drawing of the lentiviral constructs used in this work. LTR, long terminal repeat; Ψ, retroviral packaging signal; SFFV, spleen forming focus virus LTR promoter; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. (C) Immunoblot showing dnNUMB protein expression in K562 transduced with dnNUMB lentivirus, using an anti-Myc tag antibody. Actin levels were determined as a protein loading control of the samples. (D) mRNA levels of the indicated NOTCH target genes, determined by RT-qPCR in KdnNUMB and KGFP cells. The data are mean values from two independent RNA preparations. Error bars are SEM.

Figure 2 Proliferation rate of KdnNUMB Higher proliferation ratio of KdnNUMB vs. control cell line KGFP analyzed by (A) Viable cells counting at different times in culture (mean values of three independent experiments, error bars are SEM); (B) ^{3}H-Thymidine incorporation after 72h in culture (mean values of two experiments with SEM) and (C) Incorporation of BrdU for 24 hours. (D) dnNUMB overexpression tested by Immunoblot in several dnNUMB-transfected stable clones carrying PCEFL KdnNUMB. (E) BrdU uptake after 24 hours of two different clones, measured by intracellular flow cytometry. K562 cells in the absence of BrdU (thin solid line), control K562 cells (thick solid line) or K562-dnNb cells (broken line). Data are representative of three independent experiments.

Figure 3. KdnNUMB resistance to Imatinib. (A) Viable cell counting along 72 h of culture after addition of 0.5 μM of Imatinib, showing a partial resistance to the antiproliferative effect of the drug. (B) Fraction of cells in S-phase of the cell cycle in KGFP and KdnNUMB treated with 50 nM dasatinib, 50 nM nilotinib or with 0.5 μM Imatinib respectively for 24 hours. (C) Reduced imatinib-induced apoptosis assessed by Immunoblot of the cleavage of PARP 24 hours after addition of 0.5 μM imatinib. The arrows mark the position of uncleaved (116 kDa) and cleaved PARP (89 kDa). (D) DNA content of KGFP and KdnNUMB cells treated with 1 μM imatinib for 24 h. Cells were stained with propidium iodide and analyzed by flow cytometry. (E) Expression of BCLX and BCL2 analyzed by immunoblot in KGFP cells and KdnNUMB. HeLa and KBcl2 cells were included as controls for the expression of BCLX and BCL2, respectively. Cells were treated for 24 h with 1 μM imatinib. Actin levels were determined as a control for protein loading. The lane “M” contains the molecular weight markers.

Figure 4. Activity of KdnNUMB over BCR ABL and NOTCH pathway. (A) Immunoblot showing higher levels of phospho-BCR-ABL and his phosphorylated substrate P-CRKL in KdnNUMB cells. Actin levels were determined as a protein loading control of the samples. (B) mRNA levels assessed by real time PCR of different NOTCH target genes 48 hours after addition of 0.5 μM imatinib.. Mean data of two experiments with SEM bar errors. (C) mRNA expression of indicated erythroid specific genes in KGFP and KdnNUMB cells untreated or

treated for 24 h with 1 μM imatinib. mRNA was determined by RT-qPCR. Data are relative to the basal expression in KFGP cells. β-globin mRNA expression is shown in a different scale. (D) Immunoblot showing the expression of γ-globin protein in KGFP and KdnNUMB cells. Actin levels were determined as a protein loading control. (E) Fraction of hemoglobin-producing cells in KGFP and KdnNUMB cells treated for 36 h with 0,5 μM imatinib. The hemoglobinized cells were scored by the benzidine cytochemical test.

Figure 5. **dnNUMB-mediated resistance to imatinib does not depend on Notch signaling**. (A) Notch cleavage inhibition by DATP en K562 cells. Cells were treated with 5 μM DAPT and, when indicated, with 1 μM imatinib for 24 h. Cell lysates were prepared and the protein levels of cleaved intracellular Notch an alpha-tubulin were assessed by western blot. NICD, NOTCH Intracellular Domain. (B) Cell proliferation of KGFP and KdnNUMB cells treated for 48 h with 5 μM and 10 μM DAPT, assessed by cell counting. Data are mean values of two independent experiments, error bars are S.E.M. (C) Cell proliferation of KGFP and KdnNUMB treated with 10 μM DAPT and 1 μM imatinib as indicated. The cell countings were normalized to the proliferation of untreated cells.

Figure 6. Expression of Notch target genes in KGFP and KdnNUMB. (A) Cells were treated for 24 h with 1 μM imatinib and/or 10 μM DAPT as indicated. The mRNA expression of the indicated genes was determined by RT-qPCR and normalized against the expression of β-actin mRNA. Data are mean values of two independent experiments. (B) Immunoblot showing BCLX levels in KGFP and KdnNUMB treated for 24 h with 1 μM imatinib and 10 μM DAPT as indicated. Actin was determined as a protein loading control.

Table 1. List of primer used in the RT-qPCR assays in this work. All primers are form human genes and have a TM of 57ºC

Gene	Forward primer	Reverse primer
β-Actin	AAAATCTGGCACCACACCTTC	TAGCACAGCCTGGATAGCAA
BCL2L1/BCLXL	ACATCCCAGCTCCACATCAC	AAGAGTGAGCCCAGCAGAAC
CCND1	CCCTCGGTGTCCTACTTCAA	AGGAAGCGGTCCAGGTAGTT
CDK2	GGAGAACTTCCAAAAGGTGGA	GAGATCTCTCGGATGGCAGT
EPOR	CTGGTCGGAGCCTGTGTC	GAGCACGGTCAGCAGCAC
β-globin	TTGCCACACTGAGTGAGCTG	GCCACCACTTTCTGATAGGC
γ-globin	GCTCTGAATCATGGGCAGTG	TGAACTGCACTGTGACAAGC
HES1	CGGACATTCTGGAAATGACA	GTGCGCACCTCGGTATTAAC
MYC	AAGACTCCAGCGCCTTCTCT	GTTTTCCAACTCCGGGATCT
RPS14	TCACCGCCCTACACATCAAACT	CTGCGAGTGCTGTCAGAGG
TCF3	CCAGACCAAACTGCTCATCC	GGAGCTGAAAGCACCATCTG

Figure 1

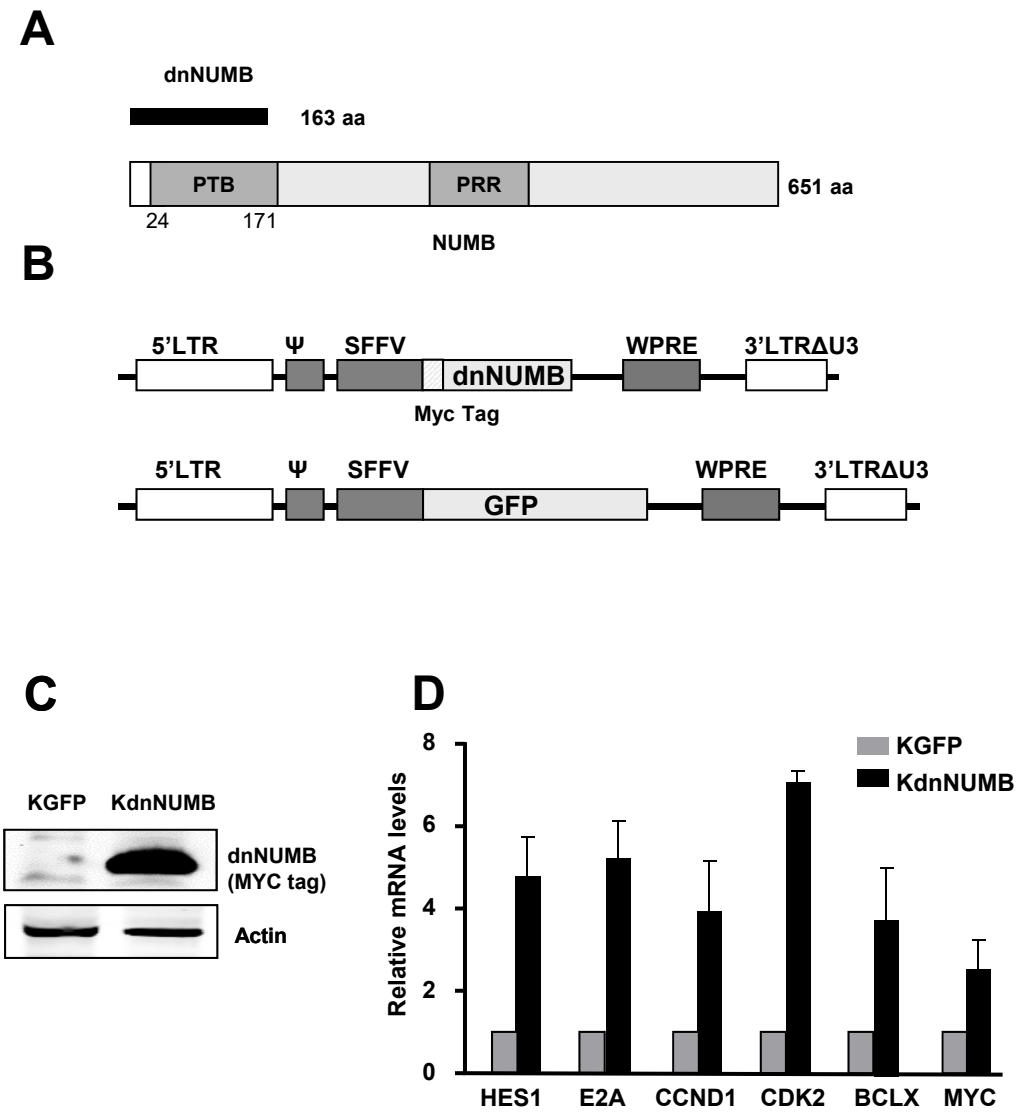

A
dnNUMB
163 aa
PTB
PRR
651 aa
24
171
NUMB
B
5'LTR
Ψ
SFFV
dnNUMB
WPRE
3'LTRΔU3
Myc Tag
5'LTR
Ψ
SFFV
GFP
WPRE
3'LTRΔU3
C
KGFP
KdnNUMB
dnNUMB
(MYC tag)
Actin
D
KGFP
KdnNUMB
Relative mRNA levels
8
6
4
2
0
HES1
E2A
CCND1
CDK2
BCLX
MYC

Figure 2

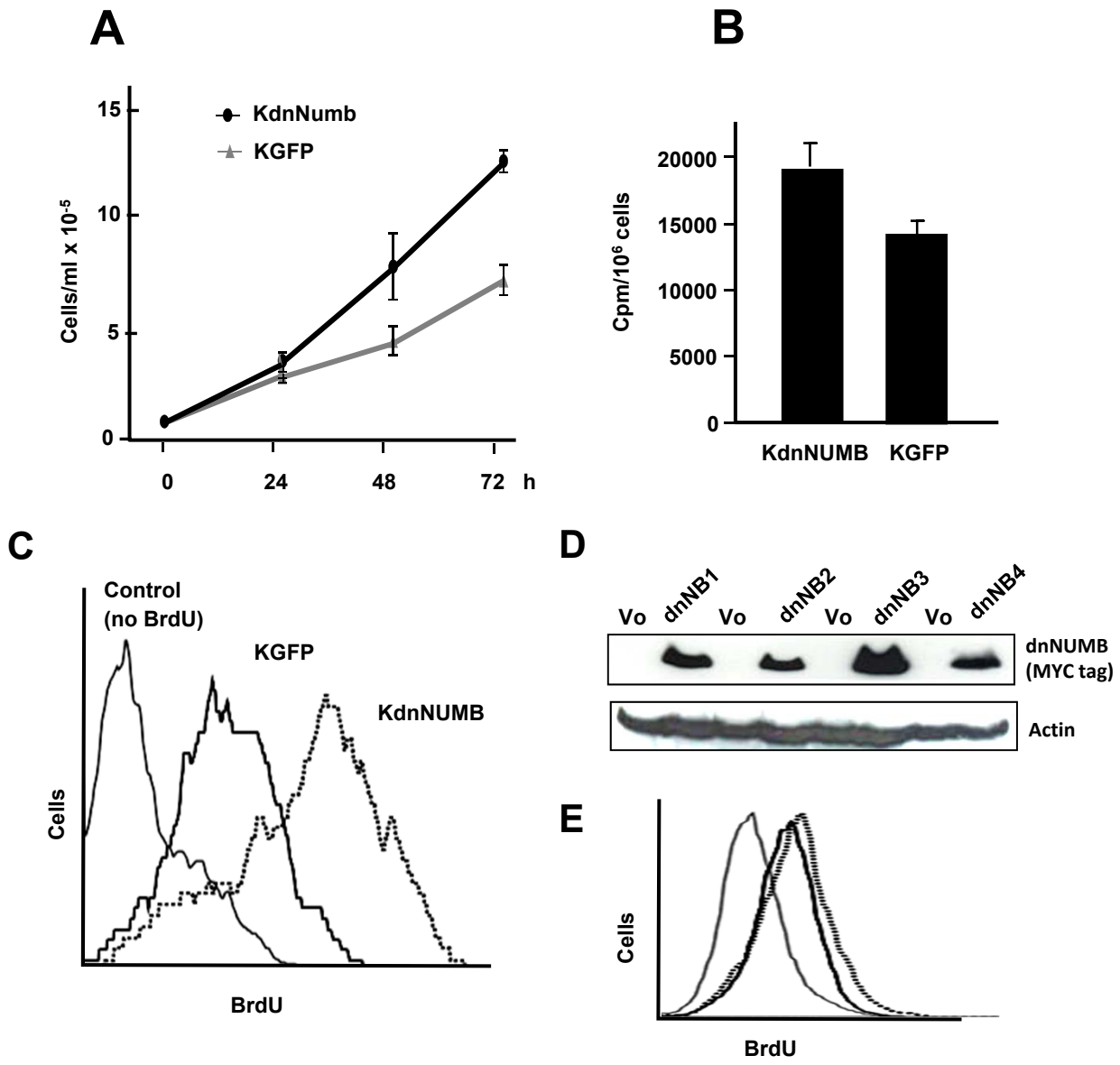

A
KdnNumb
KGFP
Cells/ml x 10-5
15
10
5
0
0
24
48
72 h
B
Cpm/10^6 cells
20000
15000
10000
5000
0
KdnNUMB
KGFP
C
Control
(no BrdU)
KGFP
KdnNUMB
Cells
BrdU
D
Vo
dnNB1
Vo
dnNB2
Vo
dnNB3
Vo
dnNB4
dnNUMB
(MYC tag)
Actin
E
Cells
BrdU

Figure 3

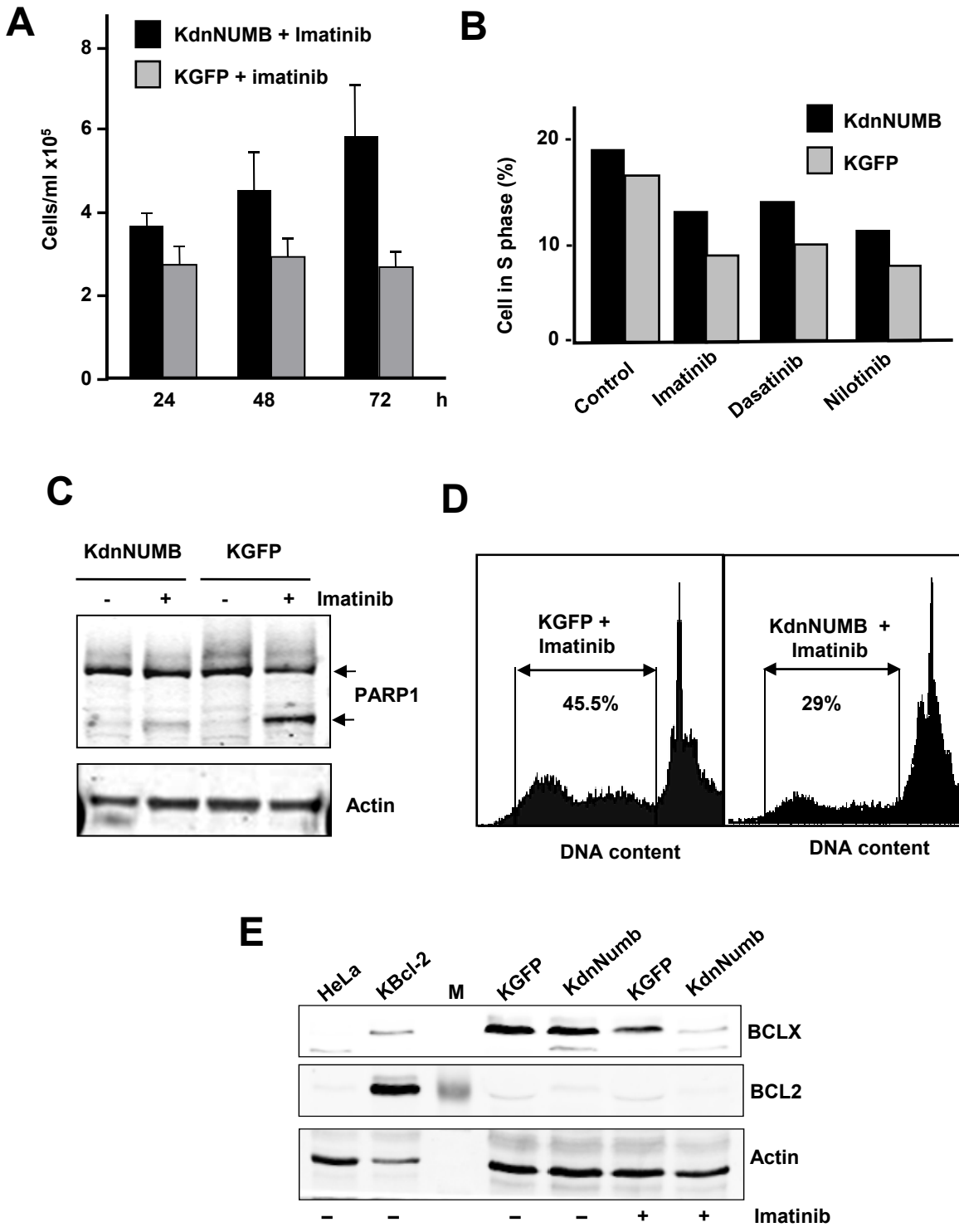

A
KdnNUMB + Imatinib
KGFP + imatinib
Cells/ml x10⁵
0
2
4
6
8
24
48
72
h
B
KdnNUMB
KGFP
Cell in S phase (%)
0
10
20
Control
Imatinib
Dasatinib
Nilotinib
C
KdnNUMB
KGFP
- + - + Imatinib
PARP1
Actin
D
KGFP + Imatinib
45.5%
KdnNUMB + Imatinib
29%
DNA content
DNA content
E
HeLa
KBcl-2
M
KGFP
KdnNumb
KGFP
KdnNumb
BCLX
BCL2
Actin
– – – – + + Imatinib

Figure 4

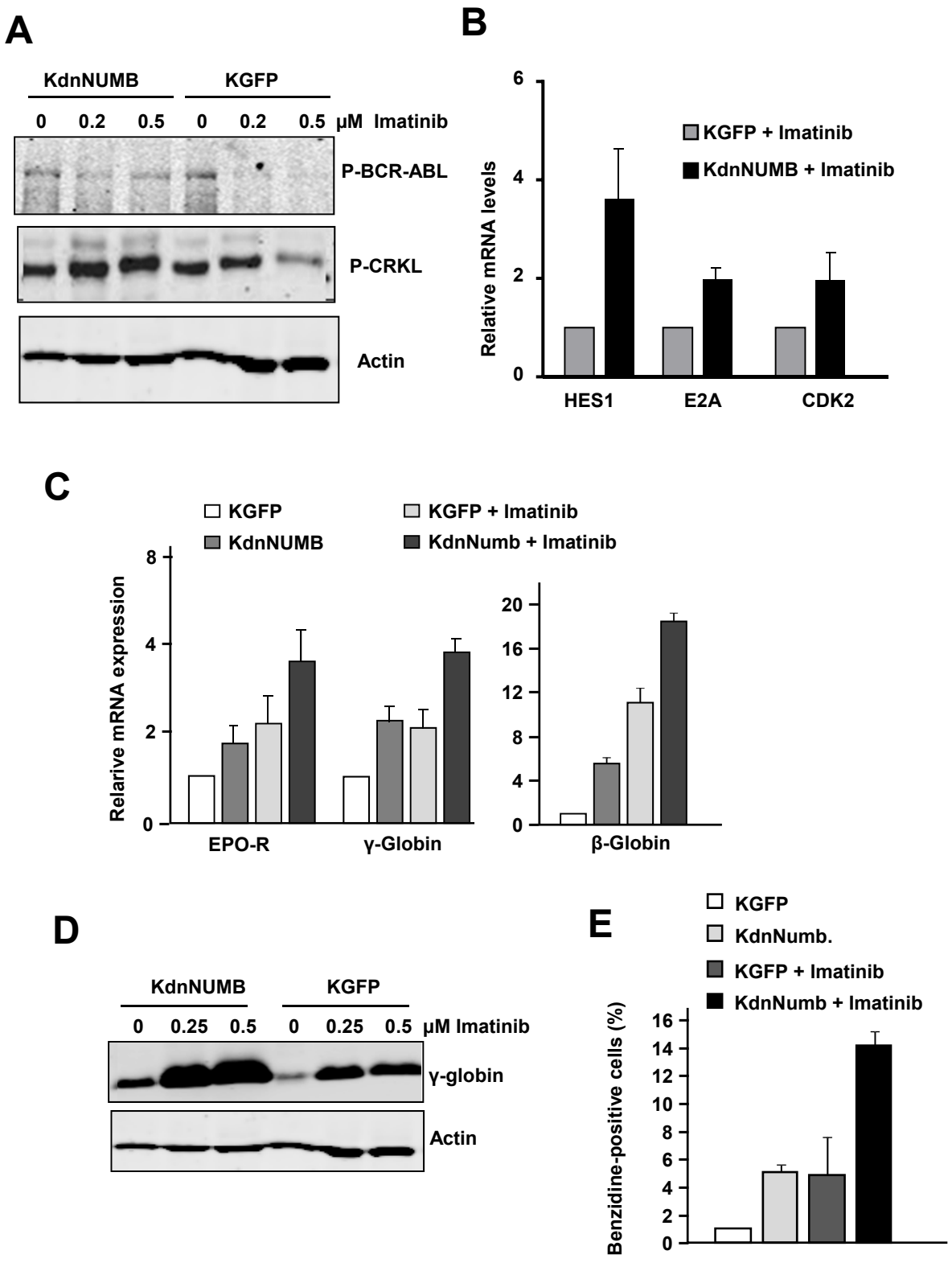
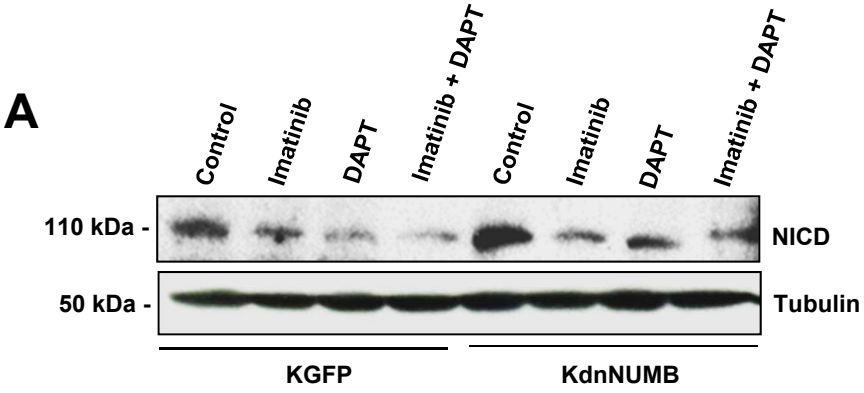
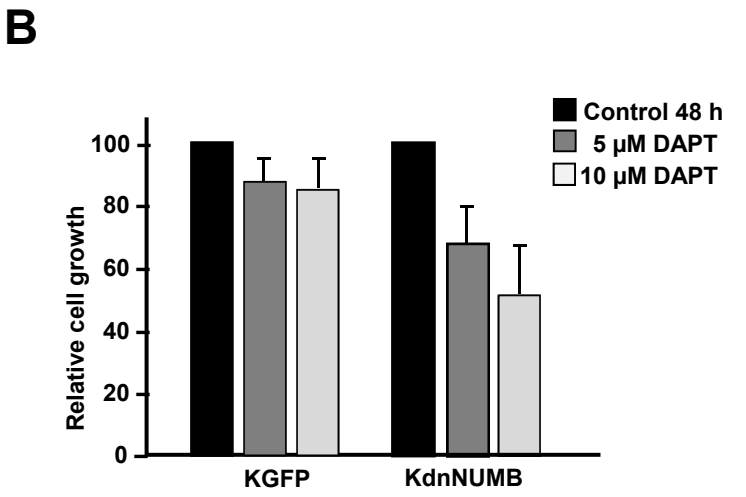

A
KdnNUMB
KGFP
0 0.2 0.5 0 0.2 0.5 μM Imatinib
P-BCR-ABL
P-CRKL
Actin
B
Relative mRNA levels
KGFP + Imatinib
KdnNUMB + Imatinib
HES1
E2A
CDK2
C
KGFP
KdnNUMB
KGFP + Imatinib
KdnNumb + Imatinib
Relarive mRNA expression
EPO-R
γ-Globin
β-Globin
D
KdnNUMB
KGFP
0 0.25 0.5 0 0.25 0.5 μM Imatinib
γ-globin
Actin
E
KGFP
KdnNumb.
KGFP + Imatinib
KdnNumb + Imatinib
Benzidine-positive cells (%)

Figure 5


A
Control
Imatinib
DAPT
Imatinib + DAPT
Control
Imatinib
DAPT
Imatinib + DAPT
110 kDa -
NICD
50 kDa -
Tubulin
KGFP
KdnNUMB


B
Control 48 h
5 μM DAPT
10 μM DAPT
Relative cell growth
100
80
60
40
20
0
KGFP
KdnNUMB

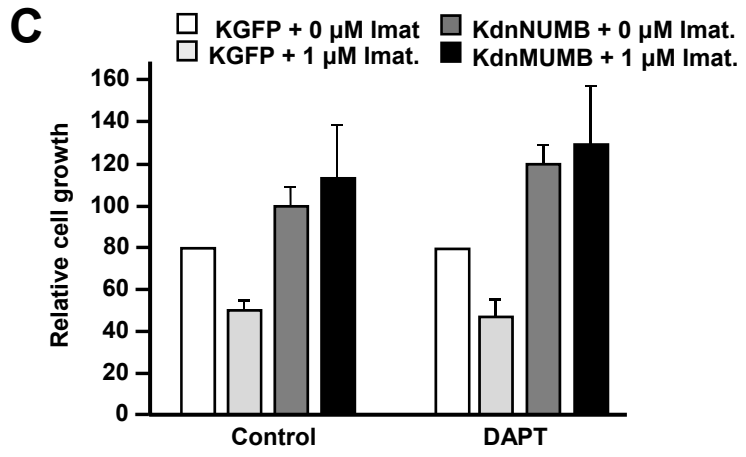
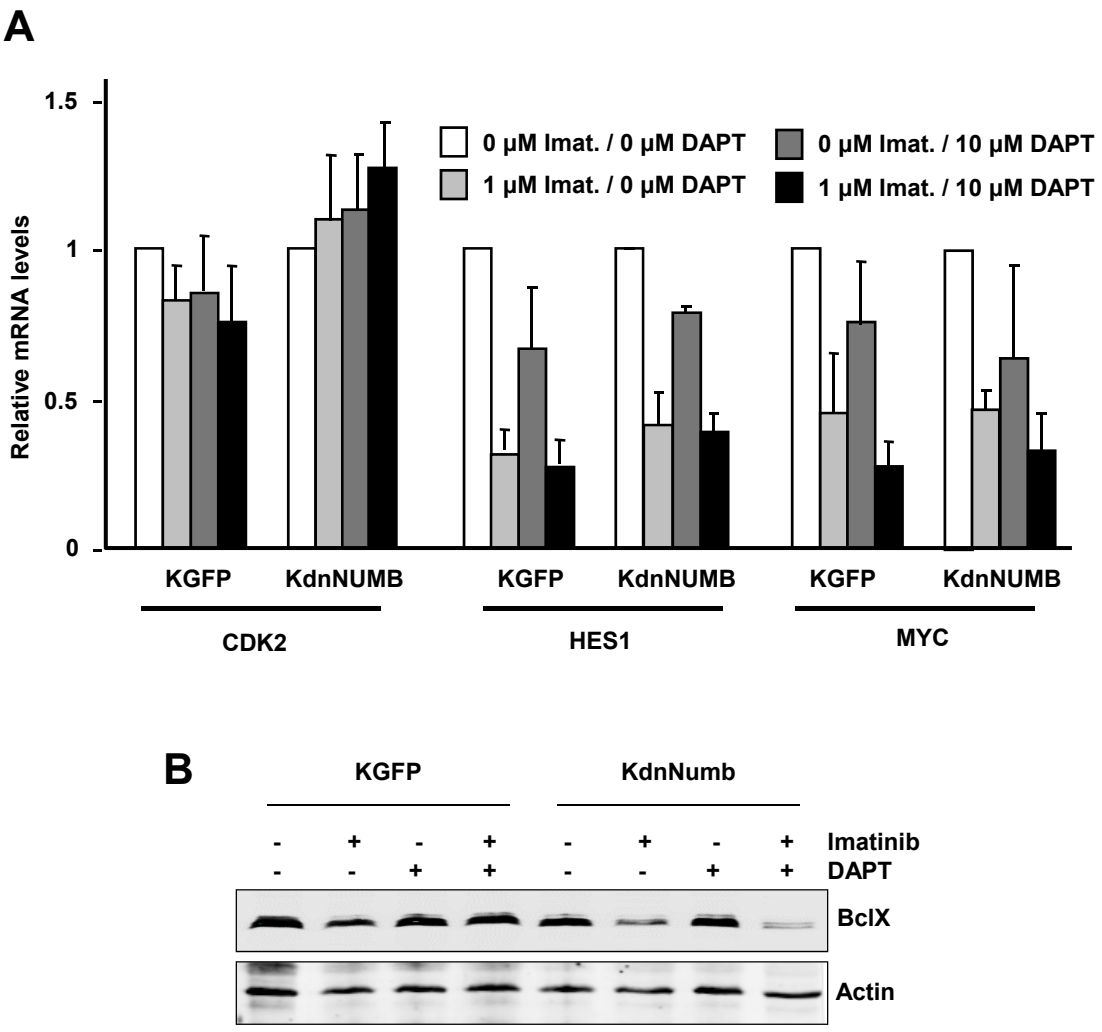

C
KGFP + 0 μM Imat
KGFP + 1 μM Imat.
KdnNUMB + 0 μM Imat.
KdnMUMB + 1 μM Imat.
Relative cell growth
160
140
120
100
80
60
40
20
0
Control
DAPT

Figure 6


A
1.5
1
0.5
0
Relative mRNA levels
0 µM Imat. / 0 µM DAPT
1 µM Imat. / 0 µM DAPT
0 µM Imat. / 10 µM DAPT
1 µM Imat. / 10 µM DAPT
KGFP
KdnNUMB
KGFP
KdnNUMB
KGFP
KdnNUMB
CDK2
HES1
MYC
B
KGFP
KdnNumb
- + - + - + - +
- - + + - - + +
Imatinib
DAPT
BclX
Actin