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Reduced expression of SRC family kinases decreases PI3K activity in *NBS1*$^{-/-}$ lymphoblasts

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ABSTRACT

SRC family kinases (SFKs) are involved in the activation of phosphatidylinositol-3-kinase (PI3K). In addition, the activity of this lipid kinase can be regulated by the DNA repair protein NBS1. Here, we describe a disturbed expression of some members of the non-receptor tyrosine kinase family in lymphoblastoid cell lines generated from cells of Nijmegen breakage syndrome (NBS) patients. Especially, only minor amounts of the kinases LCK and HCK are expressed in the *NBS1*$^{-/-}$ cell lines as compared to the consanguineous *NBS1*$^{+/-}$ cells. We demonstrate that SFK activity is important for a proper activation of PI3K in these cells and that it is reduced in *NBS1*$^{-/-}$ cells. We provide evidence that the observed reduced PI3K activity in NBS lymphoblasts is caused by an impaired expression of the SFKs LCK and/or HCK. Thus, our data establish a new function for the NBS1 protein as a regulator of PI3K activity via SFK members.

NBS1—the gene mutated in persons suffering from NBS—controls important genomic maintenance mechanisms like DNA repair, cell cycle arrest, and apoptosis. Enhanced radiosensitivity of NBS patients and also of cell lines generated from the patients' cells is a hallmark of this human genomic instability syndrome, which has been explained by impaired signaling of radiation-induced DNA double-strand breaks (DSB) and enhanced radiation-induced apoptosis [1–4]. Biochemically, the NBS1 protein builds a trimeric complex with the proteins MRE11 and RAD50, the so-called MRN complex. This complex is necessary for the full activation of the kinase ATM which is a key protein for mediating downstream effects after DSB induction [5].

Recent reports demonstrate a role for NBS1 in processes besides DNA repair. It has been shown that NBS1 positively influences the activity of the pro-survival PI3K/AKT pathway and that its overexpression leads to cell transformation [6,7]. Additionally, overexpression of NBS1 is a marker for aggressive head and neck cancer, possibly mediated by an increased activation of PI3K [8]. Furthermore, we showed recently that the impaired activation of the PI3K/AKT pathway is relevant for enhanced radiation-induced apoptosis in NBS patient cell lines [3].

PI3K signaling through class IA PI3K in mammalian cells is essential for the response to several extracellular signals, like growth factors, hormones, or antigens. These lipid kinases consist of one regulatory and one catalytic subunit with a molecular mass of 85, 55, or 50 kDa and 110 kDa, respectively. The regulatory subunit enables the recruitment of the heterodimer to the membrane, by binding of its SH2 domain to P-Tyr (phosphorylated tyrosine) residues of receptor complexes. Upon receptor activation, PI3K catalyzes the phosphorylation of membrane-bound phosphatidylinositole-4,5-bisphosphonate (PI(4,5)P_2, short: PIP_2) to phosphatidylinositole-3,4,5-triphosphonate (PI(3,4,5)P_3, short: PIP_3). PIP3, a so-called second messenger, activates several effector proteins like AKT, leading to changes in gene transcription, proliferation, or survival of stress stimuli [9–11]. Activation of the PI3K/AKT pathway after stress stimuli, e.g., exposure to ionizing radiation or to H_2O_2, can shift the balance between survival and cell death towards cellular resistance [12]. PI3K signaling is turned off by dephosphorylation of the second messenger PIP_3 to PIP_2 by phosphatases of the SHIP family or by PTEN [10,11].

A proper regulation of the PI3K activity is crucial for both, cellular survival and for the avoidance of uncontrolled growth. Key proteins that are involved in the regulation of PI3K activity are members of the family of SRC kinases. These kinases induce PI3K activity upon receptor activation or after exposure of the cells to H_2O_2 [11,13–16]. Motivated by initial microarray experiments, we started to analyze the role of SFK for PI3K activation in cell lines derived from NBS patients' cells in comparison to cell lines generated from cells of consanguineous, healthy donors. Since PI3K/AKT activation is disturbed in these cells as published earlier [3], we were interested in the molecular cause for this phenotype. Here, we provide evidence for a new link between NBS1 and PI3K signaling via SFK.

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Materials and methods

Cell lines and cell culture. Two pairs of consanguineous EBV-immortalized lymphoblastoid cell lines (P111, P112; P306, P247) derived from two families with one line of an *NBS* patient in each pair, which is homozygous for the common *NBS1* 657del5 mutation (*NBS1*$^{-/-}$, P112, P247) were used [17]. The consanguineous corresponding cell line is heterozygous for the *NBS1* locus (*NBS1*$^{+/-}$, P111, P306). The cells were kindly provided by Prof. Martin Digweed, Charité, Institute of Human Genetics, Berlin. The cells were propagated in RPMI 1640 medium (PAA), supplemented with 15% FCS (PAA) and penicillin/streptomycin (10 IU/ml; Invitrogen).

Quantitative real time PCR. RNA was extracted using "Trizol" (Invitrogen) according to the manufacturer's protocol. The "Quantitect Reverse Transcription Kit" was used for reverse transcription. PCR cycling was done with the "LightCycler" (Roche) using the "LightCycler Fast Start DNA Master SYBR Green I" kit. Transcript amounts were quantified relative to transcript amounts of the housekeeping gene *B2M* (primer supplied by Roche). The following primer pairs were used (forward, reverse): *SRC*: GAGGAGCCCATT TACATCGT, TGAGAAAGTCCAGCAAACTCC; *HCK*: AGCTCCTGAAGCC ATCAACT, ACTTCAGGGTTTGACATCCC; *LYN*: GGCTCCAGAAGCAATC AACT, TCACGTCGGCATTAGTTCTC; *FYN*: ATGCAAGATTGCTGAC TTCG, GGGAACTTTGCACCTTGTCT; *FRK*: GGGAAGGTCTGTGGAAC AAT, GCAAACAGCATAAAGCTGGA; *FGR*: CCTACATGGAACGCATGA AC, GGAACTTGGAACCTTGGC; *BLK*: GAGCAGATTGTCACTCCCAA, A GCACAAGGCCTCAGACAC; *LCK*: TTACCCAGGGATGACCAACC, TGTGCAGAGTCCATATGTGCA; *YES1*: CTAGTAACAAAGGGCCGAGTG, ATCCTGTATCCTCGCTCCAC.

Protein analysis. Whole cell extracts were prepared by resuspending cells in lysis buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% desoxycholat, 5 mM EDTA supplemented with standard protease inhibitors (Sigma)). The suspension was incubated on ice for 10 min and subsequently centrifuged at 14,000*g* at 4 °C for 10 min. The supernatant was used for Western blot analysis accomplished according to standard procedures using ECL detection (Amersham). Equal gel loading was demonstrated by Actin β detection. The antibodies against LCK, SRC, AKT1, and P-Ser473-AKT1 were purchased from Cell signaling Technology, the antibodies against Actin β and HCK and secondary antibodies were from Santa Cruz.

Pharmacological PI3K or SFK inhibition. Inhibition was carried out 2 h prior cell harvesting with either 1 μM wortmannin (Sigma) for inhibition of PI3K or 5 μM PP2 (Sigma) for SFK inhibition, by adding inhibitors to the culture medium. For the time of treatment, the cells were kept under standard culture conditions.

In vitro PI3K activity. PI3K activity was determined as described with slight modifications [18]. Two hours before harvesting, approximately 60×10^6 exponentially growing cells were concentrated by centrifugation and resuspended in 10 ml fresh medium and treated with the inhibitor PP2 or mock-treated. Cell extracts were prepared in lysis buffer (20 mM Tris/HCl, pH 7.5; 137 mM NaCl; 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.3% Triton X-100), and immunoprecipitation was performed by using antibodies against p85α (Upstate, as a positive control) or against P-Tyr (clone 4G10, Upstate) with an equal amount of total protein (1.5 mg). Immunocomplexes were immobilized on protein-G coated agarose beads and washed twice in lysis buffer, once in lysis buffer containing 0.5 M LiCl and twice in reaction buffer (20 mM Tris/HCl, pH 7.5; 100 mM NaCl; 0.5 mM EGTA). Subsequently, the beads were resuspended in 50 μl of reaction buffer and 5 μg phosphatidylinositol (Sigma) was added from a sonified stock solution. Then, 5 μl of a solution of 200 mM $MgCl_2$ and 200 μM ATP containing 20 μCi of [γ-^{32}P]ATP (Hartmann Analytik) were added to the reaction mixture. The tubes were incubated at room temperature for 45 min before the reaction was stopped by adding 150 μl of stop solution ($CHCl_3$/CH_3OH/HCl concd, 100:200:2) and 120 μl of $CHCl_3$. The organic phase was separated by centrifugation and washed once with CH3OH/HCl, 1 N (1:1). The samples were concentrated by over night drying. The dried reaction product was redissolved in 10 μl CH_3Cl/MeOH (1:1) and separated by thin-layer chromatography on a silica gel plate (Merck) coated with 1% K-oxalate, 2 mM EDTA in H_2O/CH_3OH (3:2). The plates were developed in $CHCl_3$/CH_3OH/H_2O/NH_4OH (45:35:8.5:1.5) and phosphorylated lipids were visualized by autoradiography.

Results

Recently, we described reduced PI3K/AKT activity in NBS lymphoblastoid cell lines. Remarkably, immunoprecipitates of cell extracts of *NBS1*$^{-/-}$ cells or *NBS1*$^{+/-}$ cells generated with antibodies against the 100 or 85 kDa subunits of the PI3K did not differ in their PI3K activity [3]. To further investigate the mechanism of impaired PI3K activity in these lymphoblastoid cell lines, we determined the PI3K activity of anti-P-Tyr immunoprecipitates. Therefore, P-Tyr-proteins were purified by immunoprecipitation and the enzymatic activity was assayed as described in Materials and methods (Fig. 1A). Clearly, P-Tyr-associated PI3K activity was detected in extracts of *NBS1*$^{+/-}$ cells, but only a reduced amount was measured in the extracts of *NBS1*$^{-/-}$ cells. Mock immunoprecipitations without antibody were used to determine the background of non-P-Tyr-associated activity that could not be removed, even after extensive washing of the beads used for immunoprecipitation. To assess a role for SFK for PI3K activity, immunoprecipitates were also prepared after pre-incubation of the cells with the SFK inhibitor PP2. In these samples, a reduced PI3K activity was observed, indicating the necessity of SFK activity for PI3K signaling.

Additionally, the activity of the PI3K/AKT pathway and the dependency on SFKs was monitored by analyzing the phosphorylation status of AKT. Clearly, the amount of phosphorylated AKT was reduced to a nearly undetectable level in extracts of the *NBS1*$^{-/-}$ cells compared to extracts of *NBS1*$^{+/-}$ cells. Notably, the dependency of AKT phosphorylation on PI3K activity was proven by the absence of P-Ser473-AKT in extracts of cells that were treated with the PI3K inhibitor wortmannin (Fig. 1B, left). Furthermore, we studied the importance of SFK activity for AKT phosphorylation by using the SFK inhibitor PP2. Upon pre-incubation of the cells with the inhibitor, P-Ser473-AKT was no longer detectable in the cellular extracts, indicating that an intact SFK activity is important for proper function of the PI3K/AKT pathway (Fig. 1B, right).

We performed SFK expression profiling by quantitative real time PCR since PI3K/AKT signaling is dependent on SFKs and since we detected reduced *LCK* expression in *NBS1*$^{-/-}$ cells by initial microarray experiments [19]. As shown in Fig. 2A, all members of this kinase family plus the *FYN*-related kinase *FRK* kinase were expressed in detectable amounts using the method described in Materials and methods. Differences in the kinase expression by a factor of 2 or higher or 0.5 or lower were considered to reflect differences in gene expression (Table 1). Two kinases were expressed in similar amounts in *NBS1*$^{-/-}$ and *NBS1*$^{+/-}$ cells in the cell lines of both cell pairs: *SRC* and *LYN*. Four kinases—*FRK*, *FGR*, *YES*, and *BLK*—showed different expression patterns in the two cell pairs. The expressions of the kinases *FRK*, *FGR*, and *YES* were up-regulated in the *NBS1*$^{-/-}$ P112 cells as compared to the *NBS1*$^{+/-}$ P111 cells, but no difference in expression was detected between the cell lines of the other pair (P247/P306). *BLK* is expressed to a higher amount in *NBS1*$^{-/-}$ P247 cells as compared to the *NBS1*$^{+/-}$ P306 cells, but no difference in expression could be observed between the P112 and P111 cells. Expression of *FYN* is higher in both *NBS1*$^{-/-}$ cell lines as

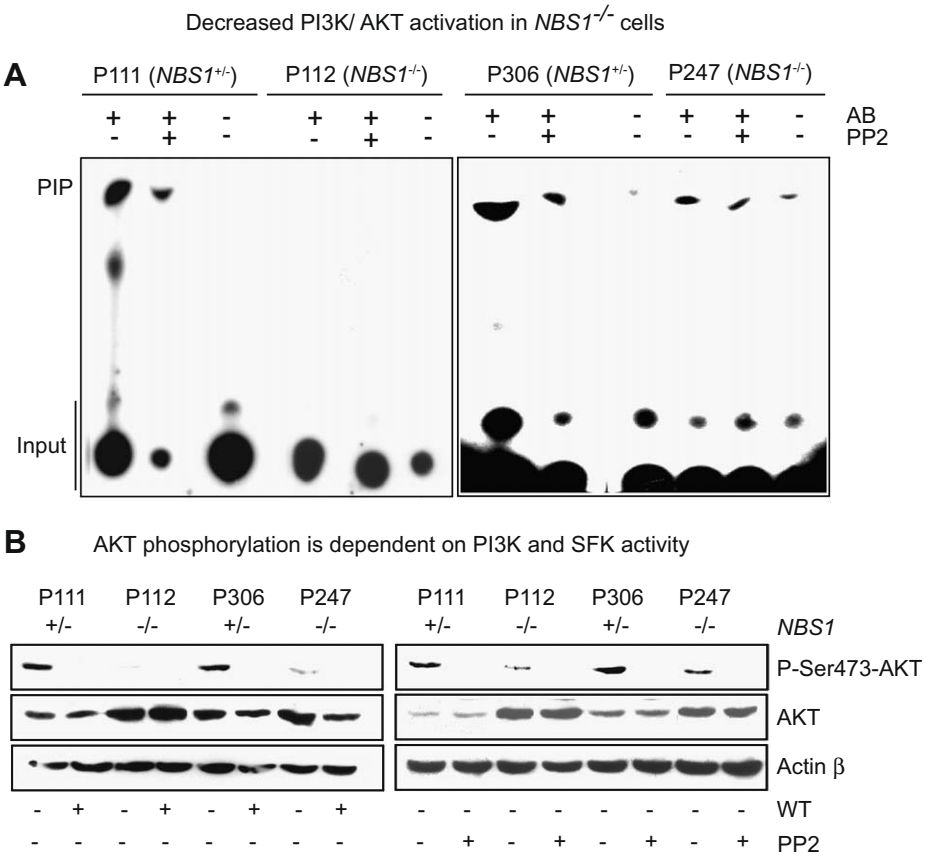

Fig. 1. P-Tyr-associated PI3K activity is decreased in extracts of $NBS1^{-/-}$ cells. Furthermore, PI3K activity could be reduced by cell treatment with the SFK inhibitor PP2. As background control, PI3K activity was also determined in mock immunoprecipitates, i.e., without adding the anti-P-Tyr antibody (AB, −) to the cell extracts. A representative result for two independent experiments is shown. PIP: phosphatidylinositole-phosphonate, input: remaining [γ-^{32}P]ATP (A). AKT phosphorylation as an *in vivo* indicator for PI3K activity. AKT phosphorylation (Ser473) is decreased in $NBS1^{-/-}$ cells. By adding the PI3K inhibitor wortmannin (WT, +) to the cells, phosphorylation was decreased to an undetectable level. Remarkably, treatment of the cells with the SFK inhibitor PP2 had the same effect. Blots shown are representative for two independent experiments (B).

compared to the corresponding $NBS1^{+/-}$ cell lines. *LCK* and *HCK* are the two kinases which are expressed in lower amounts in the $NBS1^{-/-}$ cells of both cell pairs as compared to the $NBS1^{+/-}$ cells. Additionally, Western blot analysis was performed to examine LCK and HCK expression on the protein level. As a control, protein expression of the name-giving member for the SFK family, SRC, was analyzed (Fig. 2B). Reduced transcript amounts of *LCK* and *HCK* were indeed reflected and even stressed by a severe lack of LCK and HCK protein expression in the $NBS1^{-/-}$ cells of both cell pairs. Notably, SRC is equally expressed in all cell lines, independent of the *NBS1* status.

Discussion

The presented data propose that the *NBS1* status influences PI3K activity by an impact on the expression of SFKs. In general, several mechanisms are described to control PI3K activity, e.g., by phosphorylation of the subunits of the lipid kinase. Tyrosine phosphorylation is discussed to be involved in the positive regulation of PI3K activity [20–22]. On the other hand, it has been demonstrated that serine phosphorylation of the regulatory p85α subunit of PI3K down-regulates the lipid kinase activity [23]. Furthermore, phosphorylation of Tyr-688, a Tyr residue located in the SH2 domain in the C-terminal region of the protein, has no influence on the PI3K activity, but changes the binding properties of the subunit to some phosphoproteins. This phosphorylation event is catalyzed by LCK as well as by other kinases [24].

In addition, members of the SFK family are described to increase PI3K activity [11,13–16]. These small non-receptor kinases are associated with a number of surface receptors and positively regulate receptor signaling by phosphorylation of Tyr residues of their target proteins [25]. The phosphorylation events contribute to PI3K activation via the following model. The regulatory subunits of PI3K contain two SH2 domains which enable the binding of the lipid kinase to phosphorylated Tyr residues of activated receptors. By recruitment of the PI3K heterodimer to the membrane-associated receptors, via the binding of the regulatory subunit to Tyr-phosphorylated proteins, the catalytic subunit gets in close contact to its membrane-associated substrate PIP_2, and PIP_2 is phosphorylated to PIP_3 [11]. Thus, SFKs mediate PI3K activation by controlling the recruitment of PI3K to its substrate PIP_2 via generating recognition sites for the SH2 domains of the regulatory subunits of PI3K by phosphorylation of Tyr residues.

Considering this model, we conclude that the observed reduced PI3K activity of $NBS1^{-/-}$ cells, measured in *vivo* as impaired PI3K-dependent AKT phosphorylation, is the result of the reduced expression of the SFK members LCK and/or HCK (Fig. 3). By inhibiting SFK activity by PP2, AKT-Ser473 phosphorylation was almost completely blocked in $NBS1^{+/-}$ cells, demonstrating the necessity of these kinases for PI3K activity. We propose that due to a lack of HCK and/or LCK expression in the $NBS1^{-/-}$ cells, the recruitment of the PI3K to the membrane is blocked as the phosphorylation of certain Tyr residues that serve as docking sites for the regulatory subunit of PI3K is disturbed. This hypothesis is substantiated by the measurement of PI3K activity as phosphorylation of PI *in vitro* of immunoprecipitates generated with an anti-P-Tyr antibody. Here, precipitates of cell extracts of $NBS1^{-/-}$ cells show a reduced PI3K activity as compared to that of $NBS1^{+/-}$ cells.

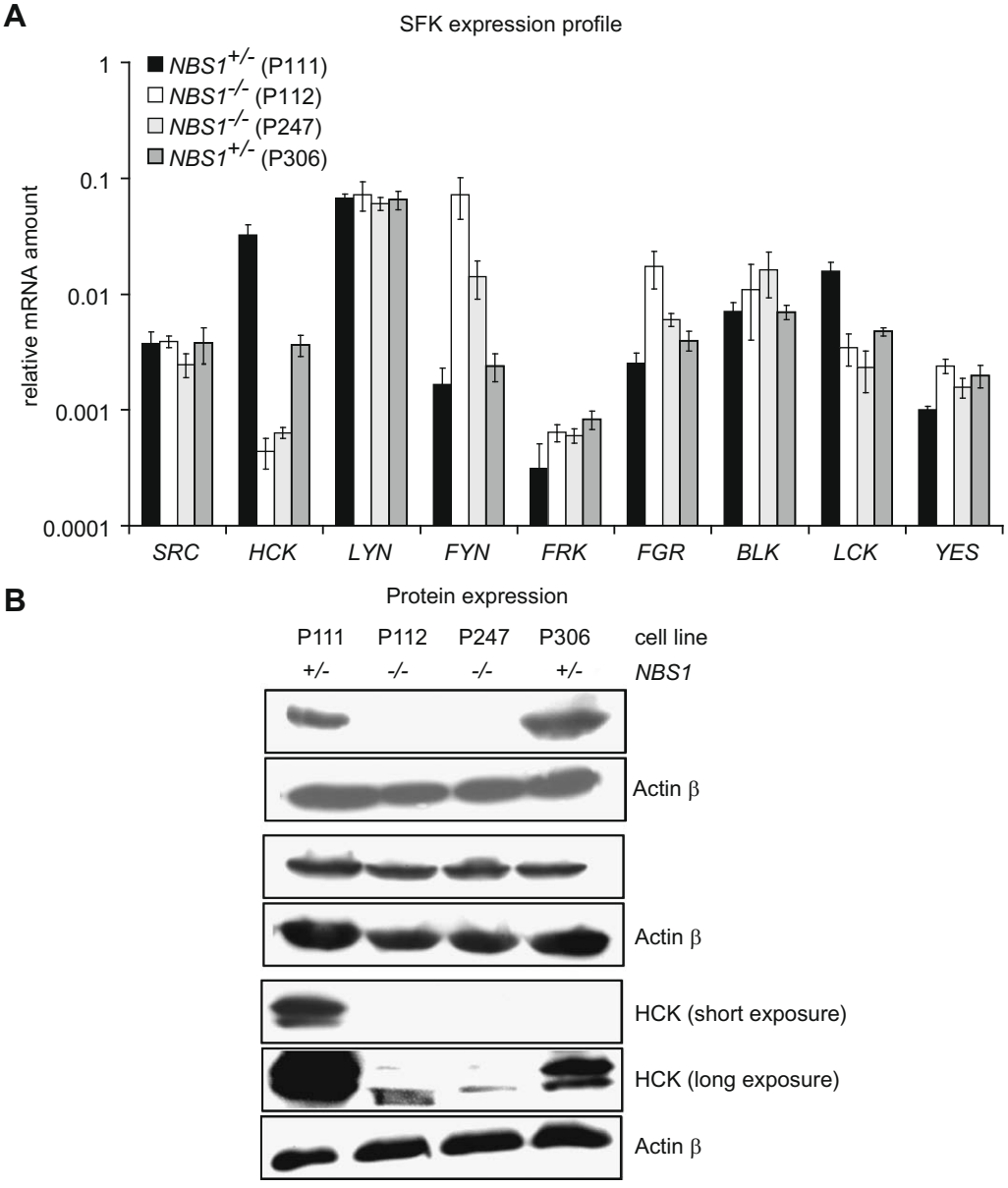

Fig. 2. SFK expression profile. The amount of each SFK transcript and *FRK* for each cell line is plotted relative to the transcript amounts of the *B2M* housekeeping gene. Error bars indicate the standard deviation of three independent experiments (A). LCK, SRC, and HCK protein expression in the four cell lines. Detection of Actin β was used as loading control. Blots shown are representative for at least two independent experiments (B).

Table 1
Expression ratios of SFKs

Gene	Ratio (SD)	
	P112/P111	P247/P306
SRC	1.0 (0.3)	0.65 (0.07)
HCK	0.014 (0.005)	0.17 (0.21)
LYN	1.1 (0.3)	0.93 (1.32)
FYN	44 (24)	5.9 (0.3)
FRK	2.1 (1.4)	0.72 (0.35)
FGR	6.8 (2.8)	1.5 (0.54)
BLK	1.6 (1.0)	2.3 (0.2)
LCK	0.22 (0.08)	0.49 (0.32)
YES	2.4 (0.38)	0.79 (0.23)

The expression of each SFK plus *FRK* is given as the relative transcript amount in *NBS1*$^{-/-}$ cells as compared to *NBS1*$^{+/-}$ cells. Numbers in brackets indicate the standard deviation (SD) of three independent experiments.

Furthermore, the PI3K activity could be reduced by adding the SFK inhibitor PP2 to the cell culture before preparing the extracts. Thus, SFK activity is demonstrated to be necessary for a high activity of PI3K.

Summarizing, our data presented here, indicate a putative mechanism how the DNA repair protein NBS1 can influence the activity of PI3K. As mentioned above, an impact of NBS1 on PI3K activity has been observed before, but no molecular explanation could be given for this effect. Recently, an interaction between the NBS1 protein and the catalytic p110α subunit of PI3K has been published to positively influence the lipid kinase activity [6]. Chen et al. demonstrated that the amino acids 653–669 of NBS1 are crucial for PI3K activation [6]. This PI3K activation domain is located on the so-called p70 fragment of NBS1 that is expressed in NBS lymphoblastoid cell lines with the common 657del5 mutation [26] that were used in this study and PI3K activity should therefore not be influenced in these cell lines. However, we propose that reduced PI3K activity in NBS lymphoblastoid cell lines is the result of a reduced expression of LCK and/or HCK due to the *NBS1* mutation. Notably, not all SFK members show a lower expression in NBS cells.

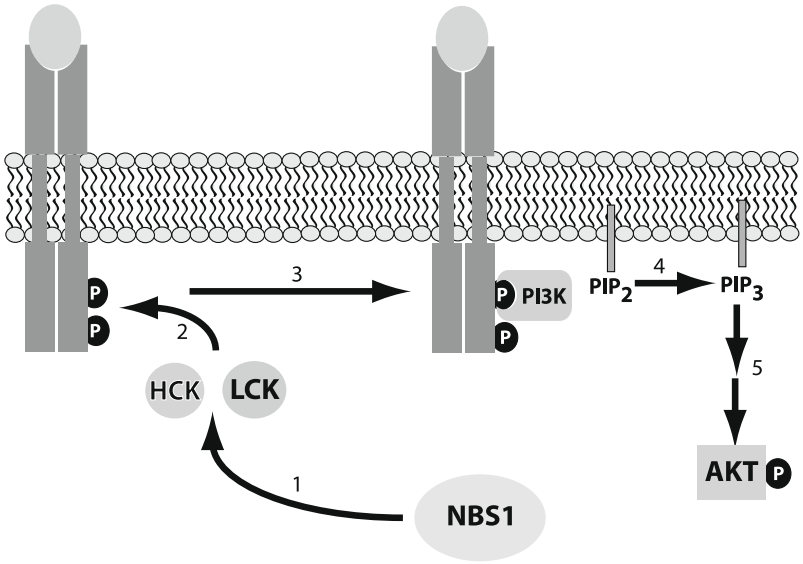

Fig. 3. Influence of NBS1 on PI3K activity. The expression of the SFKs HCK and LCK depends on full functional NBS1 (1). These kinases are important for the generation of phosphorylated Tyr residues, e.g., at receptors (2). Upon Tyr phosphorylation, the PI3K p85/p110 heterodimer is recruited to the membrane (3), and phosphorylates PIP_2 to PIP_3 (4). The second messenger PIP_3 subsequently activates several effector proteins, like AKT (5).

The *FYN* gene is even higher expressed in these cells, but obviously this enhanced expression cannot compensate for the effect of the impaired expression of LCK and/or HCK. At the moment, it is unclear, which molecular mechanism leads to the changed expression patterns of SFK members. Epigenetic effects, mediated by promoter methylation, as have been described to play a role for regulating *LCK* or *HCK* expression [27–29] can be ruled out, since 5-azacytidine treatment (a DNA methyltransferase inhibitor) had no significant impact on the transcription of these genes (data not shown).

In summary, our study revealed a new interesting link between reduced PI3K/AKT activity in NBS lymphoblastoid cell lines and the reduced expression of activators of this important signaling pathway, LCK and HCK, members of the SFKs. Obviously, the so-called DNA repair protein NBS1 fulfills further important functions besides mediating DSB repair mechanisms. Thus, our data contribute to a broader understanding of the complex clinical syndromes of NBS patients that are commonly explained by deregulation of the maintenance of genomic stability.

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