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MAP3K11 is a tumor suppressor targeted by the oncomiR miR-125b in early B cells

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MicroRNAs (miRNAs) are a class of small, non-coding RNAs that posttranscriptionally regulate gene expression and thereby control most, if not all, biological processes. Aberrant miRNA expression has been linked to a variety of human diseases including cancer, but the underlying molecular mechanism often remains unclear. Here we have screened a miRNA expression library in a growth factor-dependent mouse pre-B-cell system to identify miRNAs with oncogenic activity. We show that miR-125b is sufficient to render pre-B cells growth factor independent and demonstrate that continuous expression of miR-125b is necessary to keep these cells in a transformed state. Mechanistically, we find that the expression of miR-125b protects against apoptosis induced by growth factor withdrawal, and that it blocks the differentiation of pre-B to immature B cells. In consequence, miR-125b-transformed cells maintain expression of their pre-B-cell receptor that provides signals for continuous proliferation and survival even in the absence of growth factor. Employing microarray analysis, we identified numerous targets of miR-125b, but only reconstitution of *MAP3K11*, a critical regulator of mitogen- and stress-activated kinase signaling, interferes with the cellular fitness of the transformed cells. Together, this indicates that MAP3K11 might function as an important tumor suppressor neutralized by oncomiR-125b in B-cell leukemia.

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MicroRNAs (miRNAs) are a class of small, non-coding RNAs that posttranscriptionally regulate the stability and translation of mRNAs.[1] As such, miRNAs have an important role in almost all biological processes, but have also been linked to a variety of human diseases such as blood cancer.[2–4] For example, miR-125b, the highly conserved orthologue of *Caenorhabditis elegans* lin-4, has been described to exert diverse physiological functions in mammalian hematopoiesis, among them the control of hematopoietic stem cell homeostasis and output, plasma cell differentiation, T-cell activation and macrophage function.[5–11] Contrary to these physiological functions, however, abnormally increased expression of miR-125b is associated with a diverse set of hematological malignancies. Elevated levels of miR-125b have been found in oncoprotein breakpoint cluster region-Abelson tyrosine kinase (BCR-ABL)-positive precursor B-cell acute lymphoblastic leukemia (ALL) as well as in TEL-AML1 ALL.[12,13] Likewise, miR-125b has been shown to be dramatically increased in patients with precursor B-cell ALL that harbor a t(11;14)(q24;q32) chromosomal translocation, which brings the *miR-125b* gene into close proximity of the immunoglobulin heavy chain (HC)

enhancer.[14–16] A mouse model that mimics this translocation recapitulates the disease, indicating that the abnormally high levels of miR-125b are indeed causal for this malignancy.[12] Furthermore, deregulated expression of miR-125b was reported in chronic myeloid leukemia, acute promyeloblastic leukemia, multiple myeloma, acute megakaryoblastic leukemia associated with Down syndrome, as well as in acute myeloid leukemia or in patients with t(2;11)(p21;q23)-positive myelodysplasia.[17–20] In line with these data, *in vivo* transplantation experiments with cells expressing elevated levels of miR-125b have been shown to perturb normal hematopoiesis and eventually promote leukemia in mice.[6,8,21] Target genes that have been identified and postulated to have a role for the oncogenic function of miR-125b include pro-apoptotic factors such as *Bak1* (BCL2-antagonist/killer 1), *Bmf* (Bcl2-modifying factor) and *Trp53inp1* (transformation related protein 53 inducible nuclear protein 1), anti-proliferative *Abtb1* (ankyrin repeat and BTB (POZ) domain containing 1), tumor-suppressor genes *Irf4* (interferon regulatory factor 4), *TNFAIP3* (tumor necrosis factor-α-induced protein 3) and *CBFB* (core-binding factor, β-subunit), as well as *Arid3a*

(AT-rich interactive domain-containing protein 3A).[9,12,17,22–26] However, the precise molecular mechanism underlying the transforming activity of miR-125b remains unclear.

Here we screened a miRNA expression library using a well-defined interleukin-7 (IL-7)-dependent pre-B-cell model system and observed that miR-125b is sufficient to provoke an acute pre-B-cell lymphoblastic leukemia (pre-B ALL)-like phenotype, rendering B-cell precursors growth factor independent, cell death resistant and refractory to differentiation signals. Nonetheless, these transformed cells still require pre-BCR signals for survival, supporting the strategy to target pre-B ALL with spleen tyrosine kinase (SYK) and/or Bruton's tyrosine kinase (BTK) inhibitors. Surprisingly, miR-125b-transformed cells depend on the repression of only one of its many targets, that is, MAP3K11 (also referred to as mixed lineage kinase 3), a regulator of mitogen- and stress-activated kinase signaling. Together, our findings identify MAP3K11 as a critical target underlying miR-125b-driven transformation of pre-B cells and provide a rational to explore the consequences of enforced MAP3K11 activity in other types of blood cancers that associate with miR-125b overexpression.

Results

MiR-125b acts as an oncomiR in B-cell precursors.
To gain insight into disease-promoting miRNAs in the hematopoietic system, we established an unbiased screen exploiting a B-cell precursor model deficient for the adaptor proteins SLP-65 (Src homology domain-containing leukocyte protein of 65 kDa) and LAT (linker for activation of T cells).[27] These cells are blocked at the pre-B-cell stage and proliferate continuously in the presence of IL-7. Upon growth factor withdrawal, they undergo cell cycle arrest and differentiate into BCR-positive cells, but subsequently die due to the lack of appropriate survival signals. In this setting, oncogenes such as Ras^{V12} or c-Myc promote ongoing proliferation and survival even in the absence of IL-7, resulting in the transformation and abnormal expansion of precursor B cells and thus recapitulating, to a certain degree, the molecular phenotype of acute pre-B ALL. Consequently, this system provides a suitable tool for the identification of pro-leukemic activities in coding as well as in non-coding genes (Supplementary Figure S1).

Anticipating that cancer-associated miRNAs induce transformation of pre-B cells, we individually infected the SLP-65/LAT-deficient 1676 pre-B cell line with a retroviral library comprising 101 pri-miRNAs out of 67 families (Figure 1a and Supplementary Table 1). Upon IL-7 withdrawal, only two miRNA families reproducibly demonstrated the ability to transform 1676 pre-B cells: MiR-26 (manuscript in preparation) and miR-125, comprising miR-125a and b (Figures 1b and c for miR-125b and data not shown). MiR-125a and b are weakly expressed throughout early B-cell development (Supplementary Figure S2 and Kuchen *et al.*[28]), but deregulated expression of family member miR-125b has been associated with pre-B cell and other types of leukemia in men and in mice.[12,14–16,21] We therefore focused on miR-125b for the remainder of the study.

Having established an *in vitro* transformation model, we were wondering whether the transformed B-cell precursors depend on ongoing miR-125b expression. To this end, we generated a doxycycline-inducible 1676 cell line in which miRNA expression is directly linked to dsRed as a fluorescent marker (Supplementary Figure S3). Transformation was induced by constant doxycycline supply to drive miR-125b expression in the absence of IL-7. In established transformants, doxycycline washout resulted in loss of dsRed fluorescence and miR-125b expression, followed by rapid cell death, confirming oncogene addiction (Figure 1d). These data clearly demonstrate that enhanced miR-125b expression results in transformation of pre-B cells, and that its sustained function is necessary to maintain the transformed state.

MiR-125b does not affect the proliferation of B-cell precursors but protects from apoptosis induced by IL-7 withdrawal. To decipher how miR-125b promotes pre-B-cell transformation, we first tested its effect on cellular proliferation by ethynyl-2'-deoxyuridine (EdU) incorporation both in the presence and in the absence of IL-7. When cultured with IL-7, we did not observe any differences between miR-125b and the controls, indicating that elevated miR-125b levels do not affect proliferation (Figure 2a, left panel). Even upon IL-7 withdrawal, expression of miR-125b only resulted in a small increase in proliferation compared with control cells (Figure 2a, right panel).

This raised the question whether miR-125b confers increased cell death resistance under such conditions. Indeed, after 3 days of culture without IL-7, cells expressing miR-125b were significantly protected from apoptosis compared with controls (Figure 2b). However, when treated with Nutlin-3, a selective Mdm2 (mouse double-minute 2 homolog) antagonist that activates the p53 pathway, we observed comparable apoptosis rates (Figure 2c). Similarly, treatment with the topoisomerase inhibitor Etoposide, an inducer of DNA double-strand breaks, did not reveal any pro-survival effect of miR-125b (data not shown), suggesting selective protection from apoptosis on cytokine deprivation.

MiR-125b arrests differentiation at a pre-BCR-positive stage. The inhibition of differentiation is often considered as a first step in malignant transformation.[29] Thus, we were wondering whether miR-125b arrests 1676 cells at the pre-B stage, defined by the expression of the pre-BCR, or whether it allows the survival of more differentiated immature B cells. To investigate this, we analyzed parental 1676 cells cultured with or without IL-7 for 48 h and cells transformed by miR-125b with respect to their pre-BCR and mature BCR expression, respectively. In the presence of IL-7, 1676 cells expressed significant amounts of the pre-BCR (lambda5[+], muHC[lo]; Figure 3a, second row), but lacked expression of the mature BCR when compared with a pro-/pre-B-cell line unable to assemble a receptor. Differentiation induced by IL-7 withdrawal was accompanied by a downregulation of pre-BCR expression indicated by loss of lambda5 and by an increased expression of the BCR (kappa[+], muHC[high]; Figure 3a, third row). Interestingly, cells transformed by miR-125b and cultured without IL-7 for extended time displayed an intermediate phenotype. Although most cells displayed only
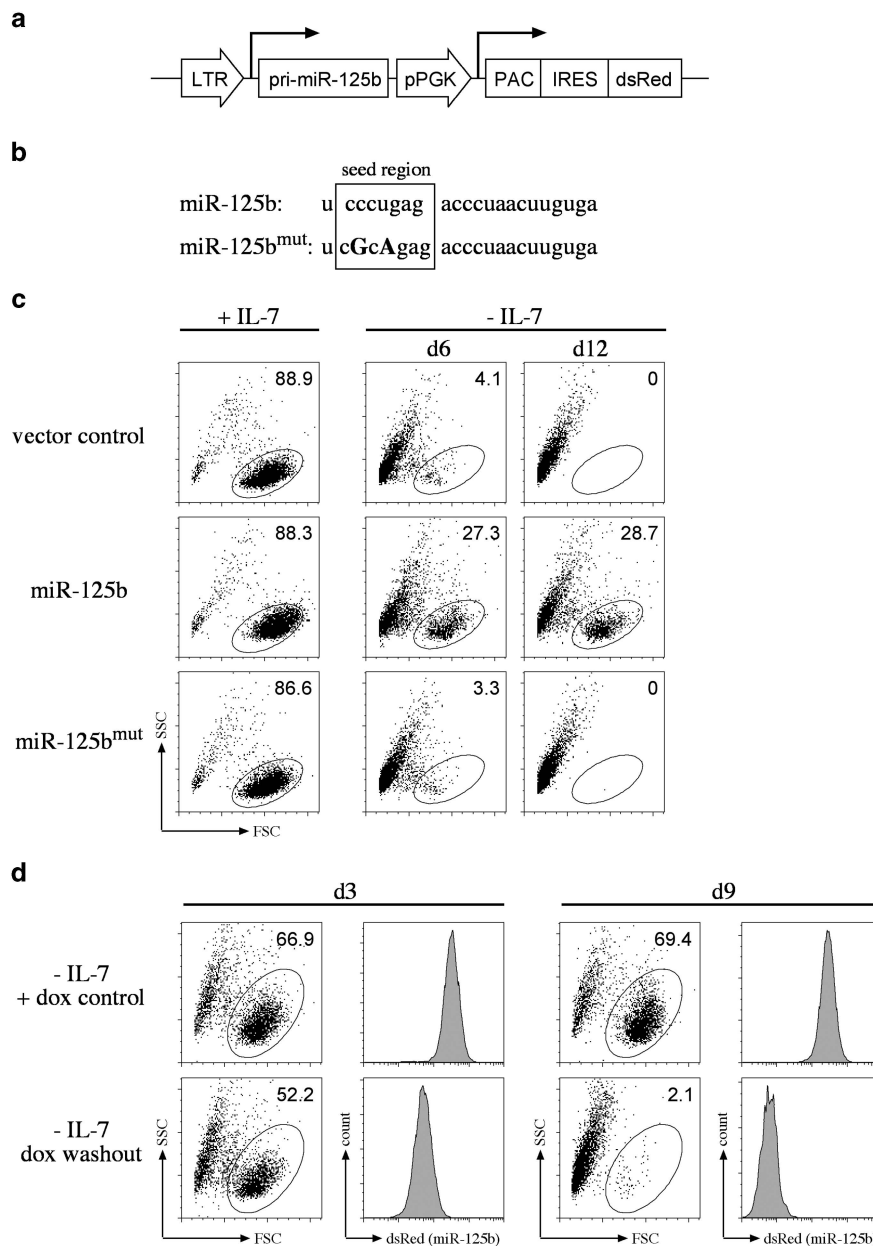
Figure 1 MiR-125b transforms B-cell precursors *in vitro*. (**a**) Schematic drawing of the microRNA expression system. The primary miR containing the stem–loop with about 50 bp of flanking region on both sides is expressed from the viral LTR, whereas genes for puromycine resistance and dsRed are transcribed from the PGK promoter. (**b**) Compared with miR-125b, two nucleotides within the seed region of the control miR-125b^{mut} are mutated. (**c**) Pre-B cells selected for the expression of the respective constructs were cultured in the presence of IL-7 or without IL-7 for 6 and 12 days before analysis by flow cytometry. (**d**) Pre-B cells transformed by induced expression of miR-125b were cultured without IL-7 and either with or without doxycyclin (dox). After 3 and 9 days, respectively, cells were analyzed by flow cytometry for the living population as well as for dsRed expression. Numbers indicate the percentage of cells in the respective gates

the pre-BCR on the surface, a minor population (8.9% and 13.9%, respectively) was also positive for the mature BCR (Figure 3a, fourth and fifth row). This shows that transformed cells arrest at the pre-B stage and suggested that aberrant miR-125b expression might interfere with the pre-B to immature B-cell differentiation. Indeed, when 1676 pre-B cells expressing miR-125b were cultured without growth factor for 3 days, the percentage of cells that had made the pre-B to immature B transition was significantly reduced compared with controls (Figure 3b). Of note, transformed

cells arrested at the pre-B-cell stage strongly depended on the proliferative and pro-survival signals from the pre-BCR, as their treatment with the Syk kinase inhibitor R406 resulted in a rapid growth arrest accompanied by a reduction in cell size and subsequent cell death (Figure 3c and data not shown).

Identification of target genes regulated by miR-125b. In order to identify target genes of miR-125b, we performed a transcriptome analysis of 1676 cells expressing miR-125b *versus* control cells, both in the presence as well as in the
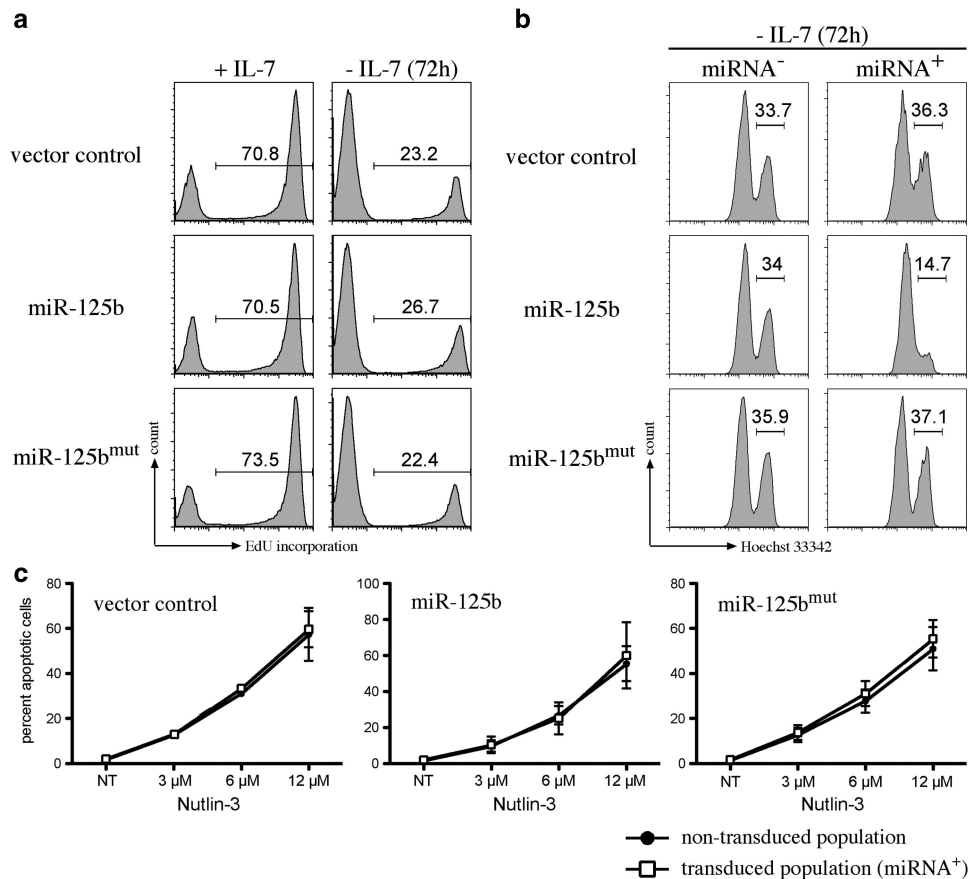
Figure 2 Effects of miR-125b on pre-B-cell proliferation and apoptosis. (**a**) Pre-B cells selected for the expression of the indicated constructs were cultured with IL-7 or without IL-7 for 3 days and pulsed with EdU to measure DNA synthesis. (**b**) Transduced pre-B cells were cultured without IL-7 for 3 days before apoptotic analysis by flow cytometry. The left column represents the non-transduced populations within the same samples, whereas plots in the right column show the populations expressing the respective constructs. Numbers indicate the percentage of cells in the respective gates. (**c**) Transduced pre-B cells (white square) and non-transduced cells within the same sample (black circle) were cultured in IL-7 and treated with increasing concentrations of Nutlin-3 for 12 h before measuring apoptosis; NT, non-treated

absence of IL-7. When cultured with IL-7, expression of miR-125b resulted in more than 1.5-fold downregulation of 198 genes, compared with 553 genes downregulated in the absence of IL-7 ($P<0.1$). One hundred and four genes were found under both conditions, and hence constitute possible direct or indirect miR-125b targets (Figure 4a and Supplementary Tables 2 and 3). Filtering for conserved miR-125b-binding sites in the 3′- untranslated region (UTR) predicted by at least one of the PicTar, miRanda and PITA algorithms gave rise to 113 genes in total, 44 and 105 of them being identified in the presence and in the absence of IL-7, respectively, with an overlap of 36 genes (Figure 4a and Supplementary Table 4).[30–32]

A manual analysis of this putative targets for an association with cancer as well as for signaling proteins, transcription factors and regulators of proliferation and survival narrowed the list down to 23 genes, most of which had not been investigated yet in the context of miR-125b overexpression, but also containing previously reported targets such as *Arid3a*, *Abtb1* and *Trp53inp1*, validating our screening approach (Supplementary Table 5).[12,24,26] To test whether miR-125b indeed represses these genes, we co-expressed corresponding 3′-UTR reporter constructs with vectors encoding miR-125b, miR-125b[mut] or an empty control in 1676 pre-B cells. Flow

cytometric analysis revealed that out of the 23 constructs, 12 showed little or no suppression when expressed with miR-125b, despite harboring conserved binding sites (Supplementary Figure S4). The remaining constructs, among them several described target genes, were downregulated by >20% through miR-125b, but not when expressed with controls (Figure 4b).

MAP3K11 is the target of miR-125b facilitating survival of transformed precursor B cells. To investigate their individual contribution to the oncogenic activity of miR-125b, we reconstituted three independent clones of miR-125b-transformed cells with cDNA expression constructs encoding the 11 target gene candidates and monitored green fluorescent protein (GFP) as an expression marker in a competitive growth assay over time (Figure 5a).[33] The expression constructs lacked a 3′-UTR and were therefore uncoupled from miR-125b-mediated regulation. We hypothesized that reconstitution of a target whose repression is involved in maintenance of the transformed state should counteract miR-125b function and reduce the percentage of GFP+ cells.

Surprisingly, most target genes behaved similar to the control when expressed in miR-125b-transformed pre-B cells
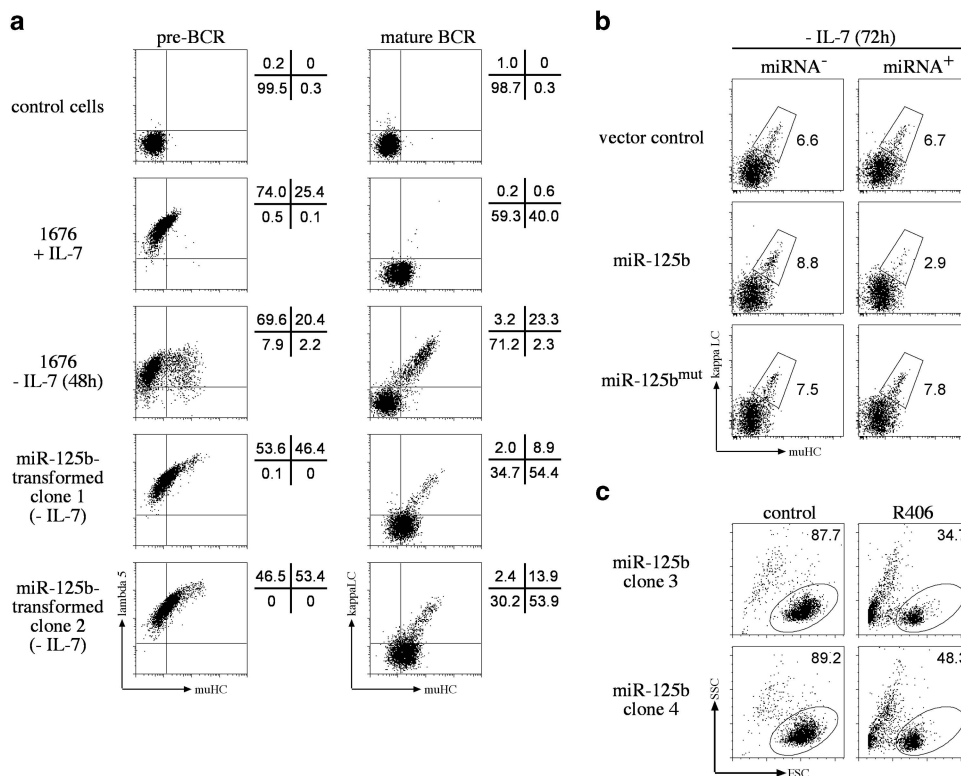
Figure 3 MiR-125b arrests differentiation at a pre-BCR-positive stage. (**a**) Non-transformed pre-B cells and cells transformed by miR-125b were analyzed for surface expression of the pre-BCR and BCR, respectively. Receptor-deficient TKO cells lacking *Rag2* (recombination activating gene 2), *Igll1* and *SLP-65* genes were used as a control. (**b**) Pre-B cells transduced as indicated were cultured without IL-7 for 3 days. The percentage of cells expressing the BCR was analyzed by flow cytometry. Numbers indicate the percentage of cells in the respective quadrants and regions. (**c**) Two independent clones of miR-125b-transformed pre-B cells cultured without IL-7 were treated with the Syk inhibitor R406 (2 µM) for 2 days. The percentage of living, proliferating cells as defined by FSC-SSC characteristics was analyzed by flow cytometry

(Figure 5a), suggesting that their repression is dispensable for transformation. Only cells expressing MAP3K11, a kinase that has been shown to regulate c-Jun N-terminal kinase (Jnk), p38 and extracellular signal-regulated kinase (Erk)-1/2 signaling, consistently got lost in competition with non-transduced cells over time (Figures 5a and b). Notably, this function was strictly dependent on the enzymatic activity of MAP3K11, as reconstitution of a kinase-dead mutant (MAP3K11 KD) was not counterselected (Supplementary Figure S5).[34]

On protein level, expression of miR-125b resulted in a strong reduction of endogenous MAP3K11 to about 25% of the normal amounts in 1676 cells (Figures 5c and d). Moreover, disruption of its putative binding site within the MAP3K11 3′-UTR abolished the suppressive effect of miR-125b in a reporter assay (Supplementary Figure S6), confirming MAP3K11 as a bona fide target of miR-125b in B-cell precursors.

The miR-125b-mediated repression of MAP3K11 appears to provide a competitive advantage to transformed cells, which is at least partially due to the inhibition of a pro-apoptotic function of MAP3K11 (Supplementary Figures S7A and B). Knockdown of MAP3K11 in pre-B cells, however, failed to block apoptosis or differentiation of pre-B to immature B cells on IL-7 withdrawal (Supplementary Figure S8 and Figures 6a and b), indicating that a repression of MAP3K11

alone is not sufficient to recapitulate the phenotype of miR-125b.

Given that MAP3K11 feeds into downstream kinases Erk1/2 as well as the stress kinases p38 and Jnk, we tested whether the miR-125b-mediated reduction in MAP3K11 levels affects activation of these signaling pathways.[35] To this end, we applied western blot analysis to assess the phosphorylation of the respective kinases in control cells or on miR-125b overexpression, both in the presence of IL-7 and on IL-7 withdrawal for 24 h. Although we failed to detect Erk1/2 phosphorylation under these conditions (Supplementary Figure S9), steady-state p38 and Jnk phosphorylation levels were comparable to the respective controls (Figure 6c). Moreover, phosphorylation and thus activation of all three kinases was comparably induced by a strong stimuli, indicating that the tested signaling pathways were not perturbed in the context of miR-125b-mediated repression of MAP3K11 (Figure 6d).

Reconstitution of MAP3K11 in transformed cells limits leukemic outgrowth *in vivo*. Based on our *in vitro* reconstitution experiments, we wondered whether MAP3K11 also exerts a tumor-suppressive role *in vivo*. To investigate this, miR-125b-transformed cells expressing dsRed were transduced in a second round with an empty control or with vectors encoding MAP3K11 or MAP3K11 KD,
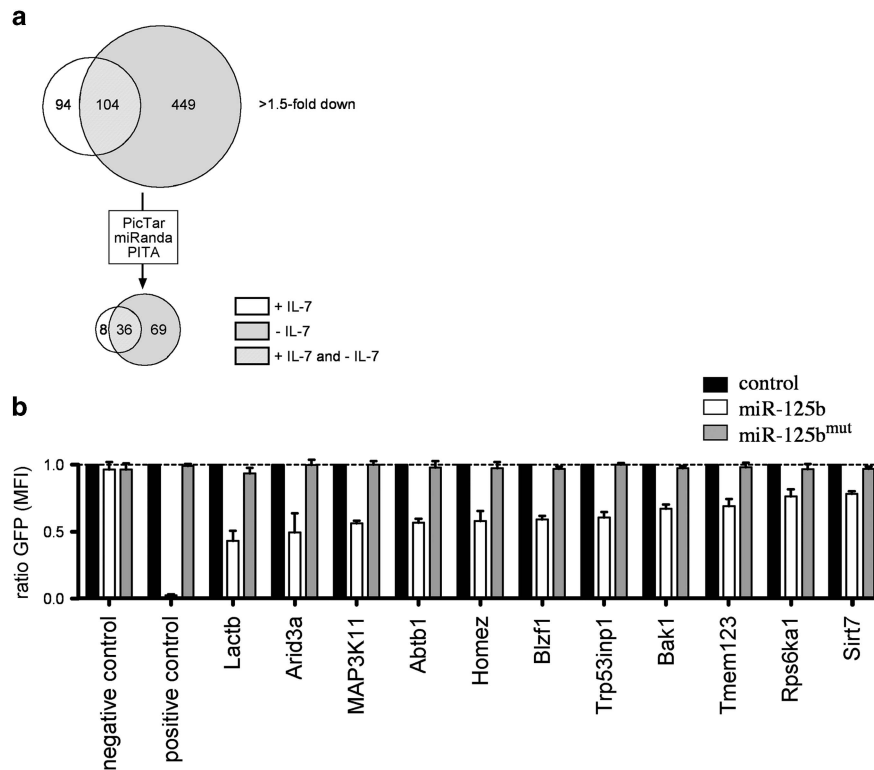
Figure 4 Identification of target genes regulated by miR-125b. (**a**) The transcriptome of pre-B cells expressing miR-125b was compared with the transcriptome of control cells in two conditions, either cultured with IL-7 or after IL-7 withdrawal for 24 h. Genes that were downregulated more than 1.5-fold ($P < 0.1$) were further filtered based on predictions of Pictar, miRanda or PITA algorithms, to generate a list of putative miR-125b target genes. (**b**) Pre-B cells selected for the expression of the respective 3′-UTR GFP reporter were transduced with vectors encoding miR-125b (white), miR-125b[mut] (grey) or an empty control (black) and analyzed for GFP mean fluorescence intensity (MFI) after 3 days by flow cytometry. A reporter with two perfectly matched miR-125b target sequences was used as a positive control, a reporter without a UTR was used as a negative control. Depicted is the ratio of GFP MFI comparing the transduced and the non-transduced population within a sample. Values for each reporter were normalized to cells expressing the vector control (set as 1). Data represent at least three independent experiments

respectively, each expressing GFP as a marker (Supplementary Figure S10). Double-transduced GFP⁺dsRed⁺ cells were recovered by cell sorting and injected into $Rag1^{-/-}$ recipient mice, promoting a pre-B ALL-like disease characterized by the presence of precursor B cells in the periphery. Detailed analysis of the bone marrow and splenic cells after disease onset revealed that cells expressing the empty control vector or the MAP3K11 KD mutant, in addition to miR-125b, expanded at comparable rates (Figures 7a and b, data not shown). In contrast, a significant portion of transformed cells sorted for expression of MAP3K11 (GFP⁺dsRed⁺) had lost GFP fluorescence, suggesting counter selection against reconstitution of MAP3K11 (Figures 7a and b). Accordingly, spleen weight and size as a quantitative measure of leukemic burden was reduced in these mice, albeit not to a statistically significant level, when compared with the relevant controls (Figure 7c and Supplementary Figure S11). Together, this corroborates our observations made *in vitro* and indicates that repression of MAP3K11 by miR-125b promotes leukemogenesis. Conversely, MAP3K11 limits pre-B-cell leukemia, establishing its function as a tumor suppressor in the context of miR-125b overexpression.

Discussion

Here an unbiased screen revealed that aberrant expression of miR-125b enabled growth factor-independent survival and proliferation of immortalized but factor-dependent precursor B cells, and thus induced their transformation (Figure 1). Our observations are in accordance with previous findings demonstrating the oncogenic potential of miR-125b in mice and in human hematopoietic malignancies.[6,8,12,14–16,21] It is feasible to assume that cells derived from mice lacking SLP-65 and LAT, such as the 1676 line used throughout this study, are in an immortalized but pre-leukemic state and can serve as a model to study the early stages of pre-B ALL. This allows to reveal the oncogenic potential of candidate genes associated with malignant transformation in humans, such as miR-125b. Consistent with the need of a 'primed' genetic background, or 'second hits', to elicit its full transforming potential, we repeatedly failed to transform primary bone marrow-derived pre-B cells by miR-125b overexpression alone, recapitulating previous findings.[26] Accordingly, mice with a transgenic overexpression of miR-125b in early B cells develop leukemia only after long latency, indicating the requirement for additional pro-tumorigenic events.[12,23] Nevertheless, despite the need for immortalization, our data convincingly demonstrate that miR-125b is not only required for the process itself, but is also indispensable for the maintenance of the transformation (Figure 1). This oncomiR addiction has been described in the context of a miR-21-driven lymphoma mouse model, but more recently in a miR-125a-induced model of myeloproliferative neoplasm as well.[36,37]
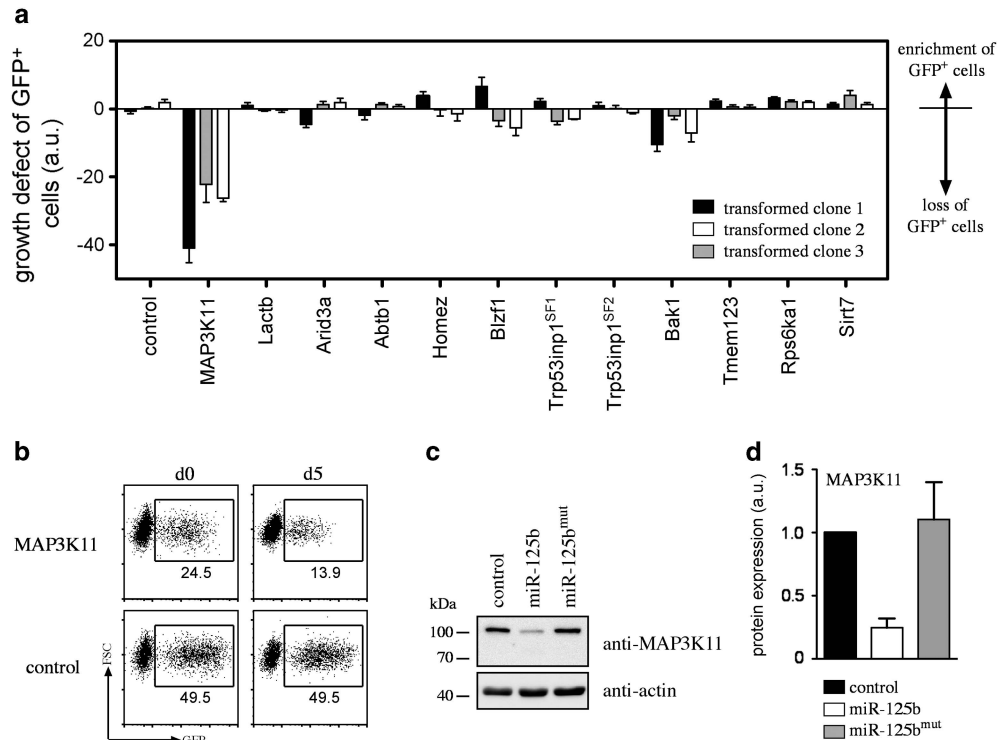
Figure 5 MAP3K11 is a target of miR-125b that is implicated in the proliferation of transformed precursor B cells *in vitro*. (**a**) Effect of reconstituted expression of putative miR-125b targets on the proliferation of transformed B-cell precursors. Three independent clones of miR-125b-transformed pre-B cells cultured without IL-7 were transduced with the indicated constructs and analyzed for the expression of GFP at d0 and d5 by flow cytometry. Changes in the percentage of GFP^{+} cells over time allows calculation of the growth defect of GFP^{+} cells compared with non-transduced GFP^{−} cells. Negative values indicate a loss of GFP^{+} cells. Data represent four independent experiments. (**b**) Representative flow cytometric data for MAP3K11 and the control vector as described in **a**. (**c**) Pre-B cells selected for the expression of the indicated constructs were lysed and subjected to SDS-PAGE and western blotting. Blots were analysed for the expression of MAP3K11 and actin as a loading control. (**d**) Quantification of three independent western blottings as shown in **c**. SF1 and SF2, spliceform 1 and 2, respectively

Our data suggest that one aspect of miR-125b's transforming activity is the suppression of apoptosis induced by IL-7 withdrawal, whereas apoptotic function *per se* is not compromised (Figure 2). These observations are in line with data showing an anti-apoptotic effect of miR-125b, especially in the absence of growth factors or low-serum conditions, in several *in vitro* systems.[24,26,37,38] However, in contrast to others, we find only a limited impact of miR-125b on pre-B-cell proliferation, suggesting that this oncomiR has the dual potential to affect proliferative as well as apoptotic signaling pathways, depending on the priming events or cellular context.[26] The noted discrepancy, however, may be reconciled by the fact that in contrast to primary wild-type B-cell precursors, 1676 cells proliferate already at a high rate that can simply not be enhanced further by miR-125b. Upon IL-7 withdrawal, however, pre-B-cell proliferation rates dropped significantly to allow maturation, but in the context of miR-125b overexpression a subset of cells uncoupled from the differentiation program. In consequence, miR-125b^{+} cells continued to express the pre-BCR, which provides additional signals promoting cell survival and sustained proliferation. These cells eventually give rise to the transformed population, which still expresses and depends on the pre-BCR. This makes them highly sensitive to the anti-proliferative actions of SYK and BTK inhibitors (Figure 3 and data not shown), unmasking a possible Achilles heel exploitable for the treatment of miR-125b-overexpressing pre-B ALL.

To better understand the transforming activity of miR-125b we employed microarray analysis (Figure 4), confirming several putative targets described in previous studies, such as *Arid3a*, *Abtb1*, *Trp53inp1* and *Bak1*.[12,24,26] In addition, we have identified some novel genes whose expression is repressed in response to ectopic expression of miR-125b, for example, *Lactb* (lactamase, β), *Homez* (homeobox and leucine zipper encoding), *Blzf1* (basic leucine zipper nuclear factor 1) and *MAP3K11*. Surprisingly, reconstitution of most of these genes in miR-125b-transformed 1676 cells did not compromise their competitive fitness. Several possible scenarios may explain this unexpected result. First, some of these genes may be simply irrelevant for the transformation mediated by miR-125b, at least in B-cell precursors, but may be critical to execute one or the other physiological function of miR-125b. Alternatively, they may have a role in the transformation, as proposed by others, but are part of a redundant pathway. An example for this may be the apoptotic cell death and p53 networks, which have been shown to be targeted by miR-125b on different levels.[7,21,25,39,40] Accordingly, our data reveal miR-125b-mediated suppression of pro-apoptotic genes *Bak1* and *Trp53inp1*, as well as slightly reduced expression, albeit either not statistically significant or not within our cutoff criteria, in pro-apoptotic *Bmf* and *Bbc3* (BCL2-binding component 3; Puma). Moreover, *TNFAIP3*, which has been shown to regulate apoptosis under low-serum conditions, was strongly decreased.[25] It is tempting to
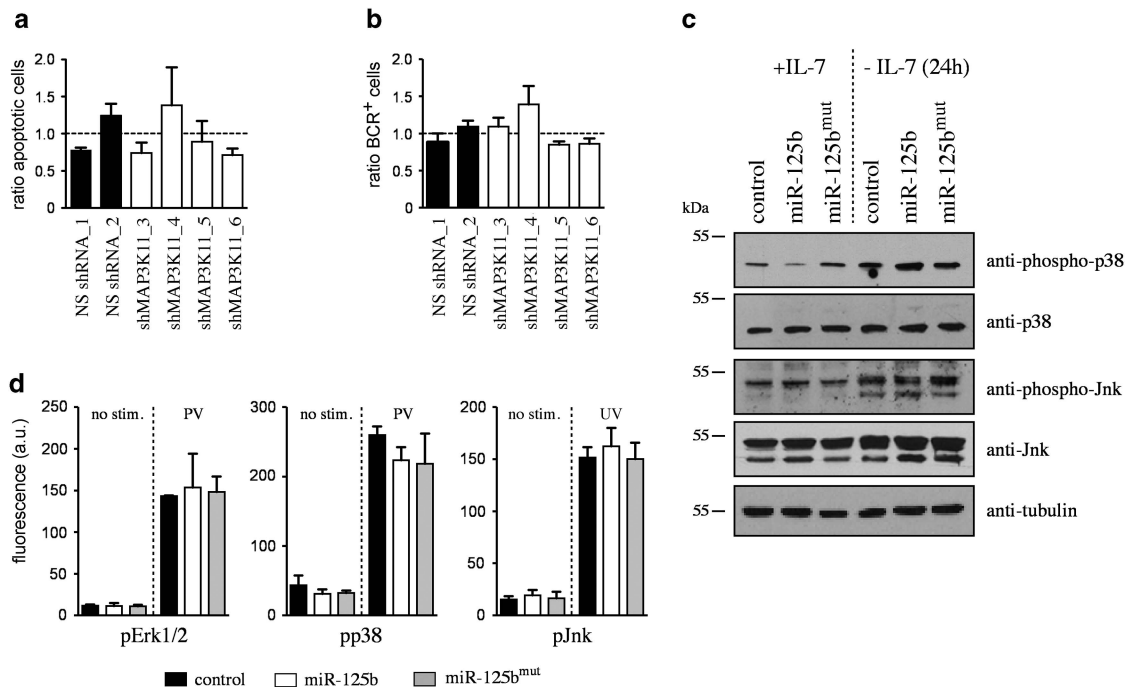
Figure 6 MiR-125b expression does not perturb signaling pathways regulated by MAP3K11. (**a** and **b**) Effect of MAP3K11 knockdown on apoptosis and differentiation of pre-B cells on growth-factor withdrawal. The 1676 cells selected for the expression of the respective constructs were cultured without IL-7 for 60 h before apoptotic analysis (**a**) or evaluating the percentage of BCR-positive cells (**b**) by flow cytometry. Bar graphs depict the ratio of apoptotic (**a**) or BCR⁺ (**b**) cells comparing the transduced *versus* the non-transduced population within the same sample. Values are expressed as mean ± S.D. (**c**) Western blot analysis for the impact of miR-125b expression on MAP3K11-regulated signaling pathways. The 1676 cells selected for expression of miR-125b or controls were cultured in the presence of IL-7 or without IL-7 for 24 h, lysed and subjected to SDS-PAGE and western blotting. Blots were analysed for the expression of phospho-p38, phospho-Jnk1/2 and the respective non-phosphorylated forms. Tubulin served as a loading control. (**d**) Intracellular flow cytometric analysis for the induced phosphorylation of signaling proteins Erk1/2, p38 and Jnk1/2. Pre-B cells selected for the expression of the respective constructs were left untreated or were stimulated with pervanadate (PV) or UV irradiation as indicated and analysed for phosphorylation of signaling proteins by intracellular flow cytometry. Values were normalized to stainings with an isotype control IgG antibody and are expressed as mean ± S.D.

speculate that miR-125b targets the apoptotic machinery at different levels, which may be sufficient to suppress cell death even if one of them, such as *Bak1* or *Trp53inp1*, is reconstituted (Figure 5). Likewise, *Arid3a*, *Homez* or *Abtb1* may contribute to the transforming activity, but it is possible that miR-125b simultaneously targets the upstream network required for their activation and function. If so, reconstitution of the targets *per se* would fail to counteract leukemic growth.

In our hands, the only direct target to interfere with the cellular fitness of miR-125b-transformed cells *in vitro* as well as *in vivo* is MAP3K11 (Figures 5 and 7), indicating that it can function as an important tumor suppressor in this context. At the moment, we can only speculate about the underlying molecular mechanism. MAP3K11 has been shown to regulate activation of Jnk, p38 and Erk in response to mitogens and cytokines.[35] However, in our experiments, basal levels of phosphorylated p38, Jnk or Erk1/2 in pre-B cells were not affected by the expression of miR-125b, neither when cultured with or without growth factor (Figure 6). Furthermore, strong stimuli did not reveal defects in kinase activation in a proof-of-principle experiment, suggesting that the tumor-suppressive effect of MAP3K11 is not due to impaired kinase signaling on miR-125b overexpression. On the other hand, we cannot rule out that such defects may become apparent using more physiological and selective triggers of the individual kinase cascades.

In knockdown experiments, the reduction of MAP3K11 levels achieved also failed to protect against apoptosis induced by growth-factor withdrawal, and likewise pre-B-cell differentiation was not blocked (Figure 6). These results are supported by studies using cells from MAP3K11-deficient mice that show no major cell death defects nor a predisposition to spontaneous tumorigenesis.[41] However, animal studies investigating MAP3K11 deficiency on a tumor-prone background are currently lacking. It is well plausible that a concomitant repression of other miR-125b target genes, in addition to loss of MAP3K11, is ultimately required to induce tumor development. Thus, the tumor-suppressive potential of MAP3K11 may only be revealed when combined with miR-125b overexpression *in vivo*.

It is tempting to speculate that the miR-125b/MAP3K11 axis also has implications beyond leukemia. In mammary carcinoma, for example, miR-125b functions as a tumor suppressor, whereas MAP3K11 has been shown to regulate breast cancer cell migration and confers a malignant phenotype to mammary epithelial cells.[42,43] Thus, a part of the tumor-suppressive function of miR-125b in breast cancer may be mediated by MAP3K11 suppression, thereby controlling tumor cell metastasis and invasiveness. Indeed, such a relationship has recently been described in malignant melanoma, where loss of miR-125b expression promotes proliferation and invasion of cancer cells by upregulation of MAP3K11.[44]

Taken together, our findings define MAP3K11 as a putative tumor suppressor and provide a rational for a possible

a

b

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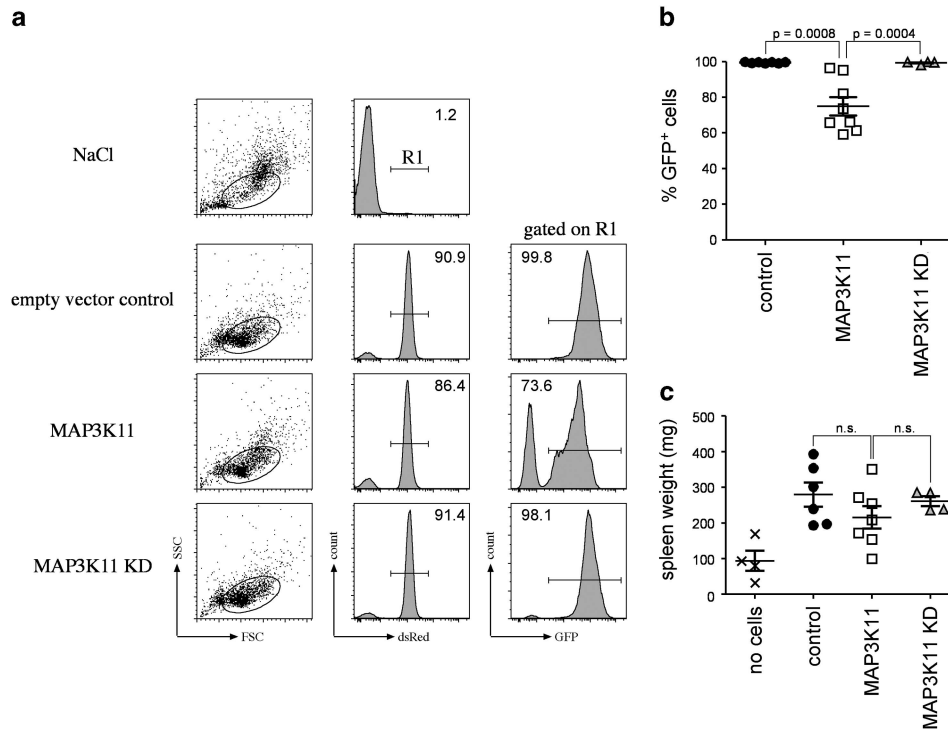
Figure 7 MAP3K11 counteracts leukemic growth of miR-125b-transformed B-cell precursors *in vivo*. (**a**) Bone marrow cells from control *Rag1*^{−/−} mice (NaCl injection) or *Rag1*^{−/−} mice injected with miR-125b-transformed pre-B cells co-expressing the indicated constructs were analyzed by flow cytometry. Based on the depicted FSC-SSC gating, injected cells were first identified based on their expression of dsRed (as a marker for miR-125b; second row) and then further analyzed for the expression of GFP (as a marker for the empty vector control, MAP3K11 and MAP3K11 KD, respectively; third row gated on R1). (**b**) Quantification of data as shown in **a** for all experiments. Values are expressed as mean ± S.D. Groups were compared by unpaired *t*-tests and statistical significance was defined as $P \leqslant 0.05$. Values are expressed as mean ± S.D. (**c**) Spleen weight as a measure of leukemic cell expansion in mice as described in **a**. Groups were compared by unpaired *t*-tests ($P > 0.05$ = not significant, n.s.).

alternative treatment strategy in pre-B ALL, circumventing counter-selection phenomena and resistance phenotypes faced with inhibitors that target pre-BCR signaling directly. Moreover, pharmacological manipulation of MAP3K11 activity may also emerge as a strategy for the treatment of other cancers that associate with aberrant overexpression of miR125b.

Materials and Methods

Cells and cell culture. The pre-B-cell line 1676 and the pre-BCR-negative pro-B/pre-B-cell line TKO were derived from the bone marrow of SLP-65^{−/−}LAT^{−/−} and Igll1^{−/−}Rag2^{−/−}SLP-65^{−/−} mice, respectively.[27,45] Cells were cultured in Iscove's medium containing 7.5% FCS (Biochrom Superior, Biochrom, Berlin, Germany), 100 U/ml penicillin, 100 U/ml streptomycin (PAN) and 50 μM 2-ME. If not stated otherwise, pre-B-cell medium was supplemented with 1.5% supernatant of IL-7-expressing J558L cells.

For retroviral transduction, Plat-E packaging cells[46] were transfected with DNA plasmids using PEI (polyethylenimine, Polysciences, Eppelheim, Germany) at a PEI: DNA ratio of 4:1. The viral supernatant was collected after 36 and 60 h, mixed with polybrene (8 μg/ml final concentration) and was used for spin infection of pre-B cells in a tabletop centrifuge (400 × *g* at 30 °C for 3 h).

Selection with puromycin was done at a concentration of 1 μg/ml for 3–4 days, followed by culture in the absence of selection agent in experimental settings.

Plasmid constructs. For the miRNA expression library, cDNAs encoding the individual pri-miRNAs (amplified by PCR from a human miRNA library, kindly provided by Dr. Gregory Hannon, CSHL, NY[47]) were cloned into a modified LMP vector in exchange for the miR-30-based small hairpin RNA (shRNA) cassette via *Xho*I and *Eco*RI digest and standard ligation procedure.[48] LMP vectors used for

miRNA expression encoded dsRedExpress2 or a tailless CD8 as a marker.[49,50] Expression constructs for protein-coding genes were generated by cloning the respective cDNAs into the MSCV-based vector pMIG. For the 3′-UTR assay, the mir-30-based shRNA cassette in the LMP vector was deleted and the GFP was destabilized by a C-terminal fusion of a PEST domain to generate LMP-desGFP. UTRs of interest were cloned into this LMP-desGFP 3′-end of the destabilized GFP cDNA. Nucleotide-exchange mutants were generated by overlap extension PCR (see Supplementary Information for detailed primer sequences).

For the inducible expression of miR-125b, we used a previously published second-generation tetracycline-regulated reverse transactivator (rtTA) and a novel transcriptional silencer.[51] Based on pWHE644 (Danke *et al.*[51]), we generated a pMIG-derived retroviral construct expressing a tri-cistronic transcript encoding rtTA2[S]-M2, tTS[D]-PP and *Bsd* for blasticidin resistance. Hsa-miR-125b was expressed from a self-inactivating TMP-derived retroviral vector.[48] A tetracycline-responsive promoter regulated expression of a transcript encoding dsRedExpress2 as a fluorescence marker. In analogy to a described strategy, the pri-miR-125b was embedded in an intron of the murine HPRT gene that was placed at the 3′ end of the dsRed cDNA.[52]

Retroviral knockdown constructs were generated by PCR amplification of long oligonucleotides comprising the stem–loop structure (see Supplementary Information for detailed sequences) and cloning of the respective DNA fragments into the miR-30-adapted LMP vector system.[48]

Flow cytometry. Single-cell suspensions were surface stained using anti-IgM (eB121-15F9, eBioscience, Vienna, Austria), anti-κlight chain (RMK-12, Biolegend, Fell, Germany), anti-λ5/CD179b (LM34, BD Pharmingen, San Diego, CA, USA) and streptavidin-APC (Biolegend). Cell proliferation was measured using the EdU Flow Cytometry Cell Proliferation Assay (Life Technologies, Carlsbad, CA, USA) according to the instructions. Pre-B cells were pulsed with 10 μM EdU for 45 min before fixation, permeabilization and labeling of incorporated nucleoside analogs. Apoptotic cells were identified via life/dead cell discrimination (propidium iodide or 7-amino-

actinomycin D) combined with either binding of fluorescence-labeled AnnexinV (eBioscience) or by staining of condensed DNA with Hoechst 33342 (1 μg/ml for 8 min at 37 °C).[53] The fitness of transduced cells compared with non-transduced controls in the same sample was analyzed based on a competitive growth assay.[33] Changes in fluorescent marker expression over time were converted into a growth defect, based on an estimated cell doubling time of 1 day.

For intracellular flow cytometry, cells selected for the expression of the respective constructs were left unstimulated or were stimulated by UV irradiation (60 mJ/cm^2), followed by a recovery phase for 45 min, or with 5 μl/ml freshly prepared pervanadate mix (15 μl sodium orthovanadate 50 mM, 80 μl H$_2$O, 15 μl H$_2$O$_2$ 30%, incubated 5 min on ice) for 3 min. Cells were fixed with 2% paraformaldehyde in PBS for 20 min at room temperature, followed by permeabilization in 90% methanol for 20 min on ice. To improve comparability of the different conditions (control, miR-125b and miR-125b^{mut}), we used fluorescent barcoding as previously described.[54] In short, fixed and permeabilized cells expressing the empty vector miR-125b or miR-125b^{mut} were left unstained or were labeled with an NHS ester of a fluorescent dye (Eterneon 393/523, Jena Bioscience, Jena, Germany) at 30 or 300 ng/ml, respectively. After quenching of unbound dye with 1% BSA in PBS, differently labeled cells were pooled and stained using anti-phospho-p38 (4511), anti-phospho-Jnk (9255) and anti-phospho-Erk1/2 (4370, all from Cell Signaling Technologies, New England Biolabs, Frankfurt, Germany) antibodies, followed by a secondary anti-rabbit antibody. Monoclonal IgG antibodies served as an isotype control. At data analysis, barcoded cells were de-convoluted based on their NHS ester fluorescence.

Data were acquired on an LSR Fortessa (Becton Dickinson, Heidelberg, Germany) or CyAn ADP Analyzer (Beckman Coulter, Krefeld, Germany) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Microarray analysis. The 1676 pre-B cells were selected for expression of the empty control vector or miR-125b, respectively. Total RNA was isolated from cells cultured with IL-7 and after IL-7 withdrawal for 24 h using the RNeasy system (Qiagen, Hilden, Germany). RNA processing, microarray hybridization on an Agilent G3 Mouse 8 × 60K array and data quality control were performed by IMGM (Martinsried, Germany). Microarray data have been deposited under GEO accession number GSE59994. Data were analyzed using the Subio software platform (Subio, Kagoshima, Japan).

RT-PCR analysis. MiR-125b expression levels were measured by RT-PCR as described.[55] In short, small RNAs were isolated from non-transduced and miR-125b-expressing 1676 pre-B cells (mirPremier, Sigma, München, Germany). RNA was reverse-transcribed into cDNA using specific stem-loop RT primers for miR-125b or the control snoRNA-202. For endpoint PCR, cDNA fragments were amplified using specific forward primers and a common reverse primer, respectively (see Supplementary Information for sequences).

Western blot analysis. About 1–2 × 10^6 1676 pre-B cells were lysed in ice-cold RIPA buffer (50 mM Tris HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA pH 8, protease inhibitor cocktail; Sigma), mixed with reducing sample buffer, boiled for 10 min and subjected to SDS-PAGE and western blot analysis. Proteins were detected using anti-MAP3K11 D-11, anti-tubulin (DM1A, both from Santa Cruz Biotechnology, Heidelberg, Germany), anti-phospho-p38 (4511), anti-p38 (8690), anti-phospho-Jnk (4668), anti-Jnk (9252), anti-phospho-Erk1/2 (4370), anti-Erk1/2 (4695) and anti-actin antibodies (4970, all Cell Signaling Technologies, New England Biolabs). Immunoreactive proteins were visualized with HRP-labeled secondary antibodies and the ECL system (Life Technologies) on light-sensitive film or on an LAS-3000 Imager (Fujifilm, Tokyo, Japan).

Adoptive transfer experiments. About 5 × 10^5 miR-125b-transformed 1676 cells sorted for expression of the empty vector control, MAP3K11 or MAP3K11 KD, were injected into the tail vain of female Rag1$^{-/-}$ mice (6–10 weeks old). Mice were killed and analyzed when they showed signs of overt disease, including the presence of pre-B cells in the peripheral blood, general inactivity, lack of food/fluid uptake, hunching and ruffled fur.

Conflict of Interest

The authors declare no conflict of interest.

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