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Name of the opponent\*: Jonas Larsson

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# Exploring the Potential of CRISPRa and CRISPRi to Systematically Dissect Fate Options in Hematopoiesis

## SUMMARY

Advances in our understanding of hematopoietic stem cells (HSCs) have enabled the establishment of differentiation pathways throughout the hematopoietic hierarchy. To further explore the molecular cues governing cell fate options, there is a need to manipulate gene function in a controllable manner. Gene manipulation systems based on CRISPR activation (CRISPRa) or interference (CRISPRi) could be used to achieve this requirement. Here, we have generated doxycycline-inducible CRISPRa and CRISPRi mouse models. To functionally validate these systems, we aimed to induce or repress the chosen genes *C/EBP $\beta$*  and *Notch1*, respectively. An initial requirement to this end was to obtain functional high titer lentiviruses that could be used to deliver guide RNAs (gRNAs) to cells from the mouse lines, and work was conducted to optimize the production of lentiviruses. Furthermore, we demonstrate the ability to induce the CRISPRa system. However, initial experiments show difficulties in *C/EBP $\beta$*  mediated transdifferentiation of B cells from the CRISPRa mouse model. Thus, further detailed validations are necessary to address the potential problems.

## INTRODUCTION

Hematopoietic stem cells (HSCs) in the adult human bone marrow (BM) are responsible for the replenishment of blood cells throughout life. Every day, blood cells from the different lineages are produced when extrinsic signals instruct uncommitted HSCs and progenitor cells to develop into more differentiated progeny (Boulais & Frenette, 2015). The knowledge about the developmental pathways of the differentiated cells has increased significantly and thereby, enabled the establishment of road maps of cellular differentiation (Iwasaki & Akashi, 2007). However, less is known about the underlying molecular cues of these processes. Thus, there is a desire to acquire more knowledge about the finely tuned transcriptional networks of defined hematopoietic progenitor cells that control the hierarchical differentiation of cells into specific lineages, which could give insights into both developmental hematopoiesis and the etiology of malignancies (Lin, et al., 2015).

To explore the molecular cues involved in cell fate options, there is a requirement to manipulate gene function in a controllable manner (Dominguez, et al., 2016). There are several techniques that have been extensively used for gene manipulation, including RNA interference to downregulate gene expression, introduction of cDNA for expression of genes and the generation of transgenic animal models (Gilbert, et al., 2014). However, such approaches

unfortunately have limitations, including uncontrolled expression levels, off-target effects or long execution times. The use of gene manipulation systems based on CRISPR activation or interference could possibly circumvent these drawbacks (Gilbert, et al., 2014). Therefore, we aim to develop gene manipulation systems, based on CRISPR activation or interference, which could be used to induce or repress any gene of interest and in the end would allow us to examine the molecular cues governing cell fate options in hematopoiesis.

The CRISPR (clustered regularly interspaced short palindromic repeats) Type II system is an adaptive immune system of bacteria and archaea, used as protection against insertion of foreign DNA into the genome. The CRISPR system functions by a CRISPR-associated endonuclease, Cas9, which is directed to the target DNA sequence by a guide RNA (gRNA) (Garneau, et al., 2010). The gRNA consists of an RNA scaffold sequence, required for the binding of Cas9, together with a sequence of approximately 20 nucleotides that determines the DNA target. Hence, the genomic targeting of Cas9 can be altered by changing the target sequence of the gRNA. The target sequence must furthermore be located directly upstream of a short sequence termed a Protospacer Adjacent Motif (PAM), allowing the CRISPR-Cas9 to recognize self from non-self DNA sequences (Dominguez, et al., 2016). Upon binding of the Cas9-gRNA complex to its genomic target, a double-strand break (DSB) is introduced, and the underlying sequence can subsequently be modified by the DNA repair machinery of the cells in which the events occur (Garneau, et al., 2010). More recently, modifications of the CRISPR system have extended its application beyond genome editing. For instance, the cleaving mechanism of CRISPR-Cas9 can be removed by introducing specific point mutations, resulting in a nuclease-deficient Cas9 (dCas9), which in turn cannot cleave DNA but retains the ability to bind DNA based on the gRNA targeting sequence (Qi, et al., 2013). Recently, a CRISPR activation (CRISPRa) system was established. This was reported to result in strong activation of the target gene using the repeating peptide array SunTag fused to dCas9, which in turn recruits several copies of single-chain variable fragment (ScFv) antibodies fused to the transcriptional activator VP64 to the dCas9 endonuclease (Tanenbaum, et al., 2014). Likewise, in the CRISPR interference (CRISPRi) system, the transcriptional repressor domain Krüppel-associated box (KRAB) can be fused to the carboxyl terminus of dCas9. Thereby transcriptional repression of the desired target gene is achieved by targeting the gRNA to the promoter region of an endogenous gene (Dominguez, et al., 2016).

A requirement for all CRISPR systems is the delivery of targeting gRNA (Dominguez, et al., 2016). As a gene delivery system, lentiviruses provide several advantageous properties including the ability to transduce both actively dividing and non-dividing cells (Sakuma, et al., 2012). Since lentiviral vectors are based on the pathogenic HIV-1, the components required

for production of a complete lentivirus are divided across multiple plasmids for safety reasons. In second generation lentiviral systems, the components are split across three plasmids: the lentiviral transfer plasmid, the packaging plasmid and the envelope plasmid, where the transfer plasmid encodes the target sequence to be expressed (Zufferey, et al., 1997). In addition, a fluorescent reporter can be included in the transfer plasmid for monitoring purposes (Sakuma, et al., 2012). The single packaging plasmid encodes the genes *Gag*, *Pol*, *Rev*, and *Tat*, which are needed for the production of a fully functional lentiviral particle (Zufferey, et al., 1997). To ensure broad tissue tropism and thereby provide robust transduction of multiple cell types, vesicular stomatitis virus envelope glycoprotein G (VSV-G) is commonly used as envelope protein (Sakuma, et al., 2012). Taken together, these characteristics makes lentiviral vectors a tractable system for introduction of the gRNA sequences.

We have generated inducible CRISPRa and CRISPRi mouse models, in which the expression is controlled by a tetracycline operon upon addition of doxycycline. In previous studies, a lentiviral vector system has been used for transduction, where one of the vectors contains the gene for dCas9-SunTag or dCas9-KRAB while the other contains the gRNA for the target gene (Tanenbaum, et al., 2014; Dominguez, et al., 2016). The disadvantage of this kind of system is the requirement of co-transduction of the cells of interest, which severely compromise its efficiency. Our mouse models, in which every cell expresses the dCas9 fusion gene, would circumvent this drawback by allowing for transduction of only the vector containing the gRNA. To validate the generated CRISPR gene manipulation systems, we aimed to induce or repress two selected genes; the transcription factors *C/EBP $\beta$*  and *Notch1*, respectively. These transcription factors were chosen based on their well-established functions, which can be validated using murine hematopoietic cells (Xie, et al., 2004; Rapino, et al., 2013; Schmitt & Zúñiga-Pflücker, 2002). The myeloid transcription factor *C/EBP $\beta$*  has been shown to be able to transdifferentiate murine B cell progenitors into macrophages in a rapid and efficient manner. The transcription network of these B cells is remodeled into that of macrophages in a sequential manner, downregulating late lymphoid markers while at the same time upregulating markers characteristic for myeloid cells (Xie, et al., 2004; Rapino, et al., 2013). Regarding the transcription factor *Notch1*, it has been demonstrated that OP9DL stromal cells, which ectopically express the Notch ligand Delta-like-1, promote differentiation of early hematopoietic progenitor cells into T cells by providing the responsible signals for this process (Schmitt & Zúñiga-Pflücker, 2002). Based on this, inhibition of *Notch1* signaling should compromise T cell generation. Here, to validate our established mouse models, we hypothesized that while B cell progenitors from the CRISPRa mouse model can be transdifferentiated into myeloid cells upon introduction of gRNA targeting *C/EBP $\beta$* , granulocyte-monocyte-lymphoid progenitor (GMLP)

cells from the CRISPRi mouse model should be blocked from differentiating into T cells when gRNA targeting *Notch1* is expressed.

## RESULTS

### Establishment of a Doxycycline-inducible CRISPR SunTag/KRAB Mouse Model

To generate CRISPRa and CRISPRi systems (Figure 1A), we fused either the transcriptional repressor KRAB or the repeating peptide array SunTag to the carboxyl terminus of dCas9. The CRISPRa construct is a large cassette containing dCas9 fused to a repeated peptide array (dCas9-GCN peptide), a 2A self-cleaving peptide sequence and a single-chain fragment variable antibody (scFV) fused to a VP64 activation domain, while the CRISPRi construct consists of dCas9 fused to KRAB. Further, to generate an inducible model for these CRISPR SunTag/KRAB fusion proteins, the KH2 embryonic stem cell (ESC) system (Beard, et al., 2006) was used, in which the expression of the M2 reverse tetracycline transactivator (M2-rtTA) is driven constitutively by the *Rosa26* promoter. The fusion gene for SunTag or KRAB was placed within the 3'UTR of the *Col1a1* locus under the control of a tetracycline operon (TetOP), which in turn enables expression of the fusion gene upon addition of doxycycline (Dox) (Figure 1B).

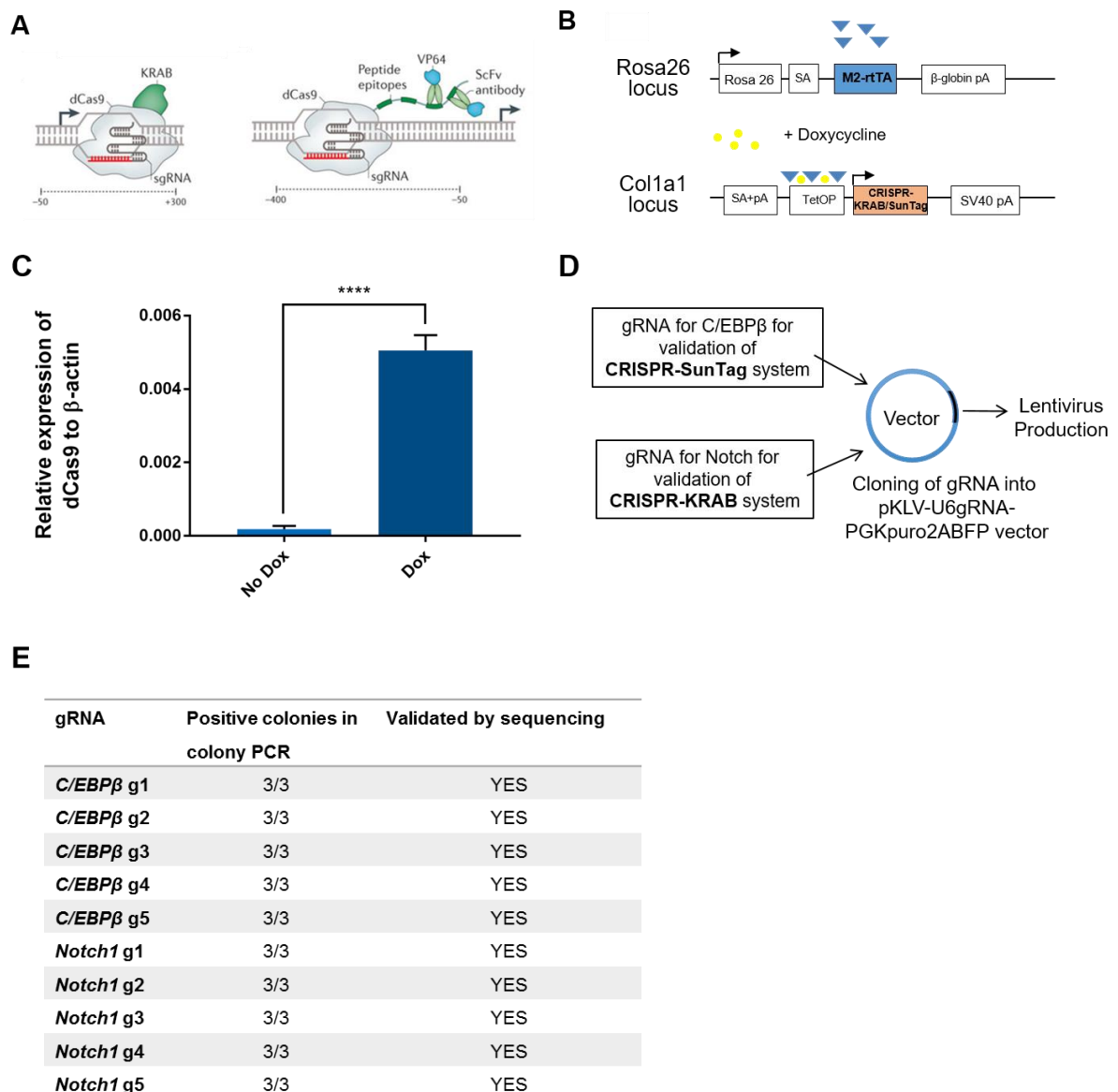
### Induction Test of the CRISPRa System

To begin to evaluate the functionality of the inducible CRISPR SunTag mouse model, we first analyzed mRNA expression of dCas9 upon doxycycline-induction. We isolated c-kit expressing hematopoietic stem and progenitor cells (HSPCs) from the bone marrow (BM) of a heterozygous SunTag mouse. These cells were cultured with or without Dox for 48 hours and subjected to quantitative real time PCR (qRT-PCR). While no expression of dCas9 could be detected in the cells cultured without Dox, clear expression of dCas9 was detected in cells that were exposed to Dox (Figure 1C). After verifying the induction ability of the CRISPRa system, we next aimed to optimize the production of lentiviruses for delivery of gRNA to cells expressing the CRISPRa or CRISPRi system (Figure 1D). Therefore, we first cloned gRNA targeting the selected genes *C/EBP $\beta$*  and *Notch1* into the vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP, which will be used for the production of lentiviruses.

### Cloning of gRNAs

To generate double strand guide inserts for cloning of gRNA for *C/EBP $\beta$*  and *Notch1* into the expression vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP, oligonucleotides were used. The annealed oligonucleotides contain single strand 5' and 3' overhangs that are compatible with the BbsI digested expression vector. First, the oligonucleotides were phosphorylated and

annealed. Second, digestion of the expression vector was performed using BbsI and T7 DNA ligase. Thereafter, the *Escherichia coli* strain JM109 was used for transformation. Each transformation was cultivated on Luria Bertani Agar plates. To select for colonies containing the vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP with the gRNA insert, we performed colony PCR on three clones for each gRNA (Figure 1E). If the gRNA was successfully inserted, the result was a product of approximately 80 bp. We observed one band of the expected size for each of the three colonies for all gRNA inserts. The selected colonies, one colony for each insert, were then cultivated for plasmid DNA purification. To validate the insertion of the different gRNAs in the expression vector, samples of plasmid DNA were subjected to Sanger sequencing. All insertions were validated as correct.



**Figure 1. Generation of a Doxycycline-inducible CRISPR SunTag/KRAB Mouse Model**

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## Optimization of Transfection Efficiency of HEK 293T Packaging Cells for Lentiviral Production

As we have selected to use the lentiviral system as a gene delivery vehicle, it is important to produce high titer lentiviruses for a successful application. Although there are different protocols available for lentivirus production, the change in more than one variable makes it difficult to directly compare these based on the published information. To begin with, we optimized the variables for transfection of human embryonic kidney (HEK) 293T cells for lentiviral production. We investigated the use of antibiotics in the culture media and also if the amount of transfer plasmid used for lentiviral production affected the transfection efficiency. HEK 293T cells were transfected in Opti-MEM with or without Penicillin-Streptomycin antibiotics using the vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP containing gRNA for *C/EBP $\beta$* . Increasing amount of the transfer plasmid, 2.5  $\mu$ g, 3.75  $\mu$ g, 5  $\mu$ g, 6.25  $\mu$ g or 7.5  $\mu$ g, was used for transfection in a 6-well plate format (all data not shown). Performing flow cytometry analysis 24 hours post transfection, we found that the presence of antibiotics in culture media during transfection has a severe negative effect on cell viability (Figure 2). The absence of antibiotics resulted in an increased percentage of live cells, from 46% to 77%. Therefore it can be speculated that these cells are dying and no longer able to produce functional lentivirus, which in turn might affect the viral titer obtained. However, the addition of antibiotics did not seem to affect the transfection efficiency of the HEK293T producer cells, as

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(A) Illustrations of the CRISPR interference (CRISPRi) and activation (CRISPRa) system adapted from Shalem et al., 2015. The dCas9-KRAB fusion protein is created by fusing the transcriptional repressor domain KRAB to the carboxyl terminus of dCas9 to achieve transcriptional repression. The dCas9-SunTag fusion protein is created by fusing the repeating peptide array SunTag to the carboxyl terminus of dCas9 to achieve transcriptional activation by recruiting several copies of the transcriptional activator VP64. The gRNA directs dCas9 to the genomic target of interest.

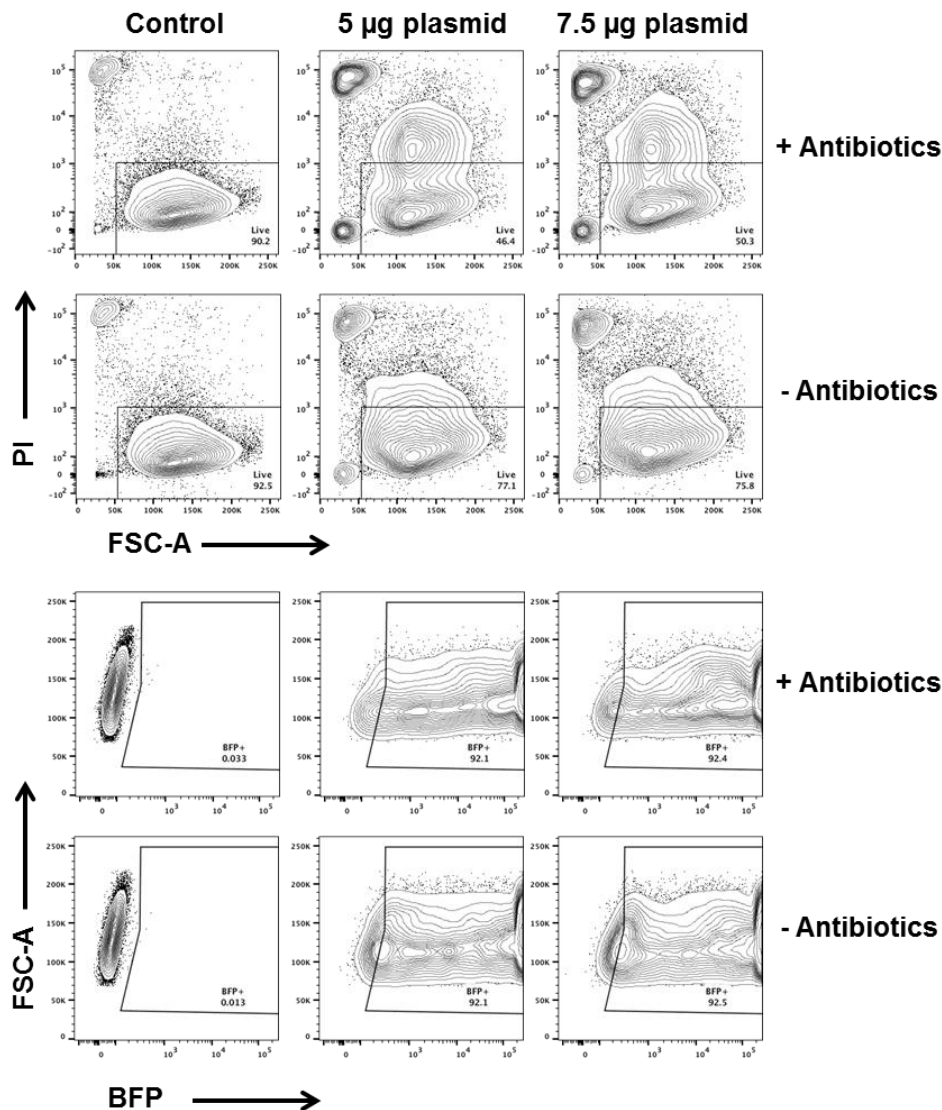
(B) Schematic representation of the inducible CRISPR SunTag/KRAB mouse model where the *Rosa26* promoter drives the expression of the M2 reverse tetracycline transactivator. The fusion gene for SunTag or KRAB is positioned within the 3'UTR of the *Co1a1* locus under the control of a tetracycline operon (TetOP). SA; splice acceptor, pA; polyadenylation signal.

(C) qRT-PCR analysis of dCas9 expression relative to  $\beta$ -actin expression in c-kit<sup>+</sup> cells from the inducible CRISPR SunTag mouse model, cultured with or without Dox for 48 hours. An unpaired student's t-test was used to assess the statistical significance of the dCas9 expression. Data are presented as mean  $\pm$  SD (n=3). \*\*\*\* p < 0.0001.

(D) The experimental approach to generate lentiviruses containing gRNA for *C/EBP $\beta$*  or *Notch1* for validation of the CRISPRa and CRISPRi system, respectively.

(E) Colony PCR was performed to select for colonies containing the expression vector with the gRNA inserts. All inserts were validated by Sanger Sequencing.

the % of cells expressing the reporter Blue Fluorescent Protein (BFP), was not altered significantly. In addition, the amount of transfer plasmid in the tested range 2.5 – 7.5  $\mu\text{g}$  did not affect either the viability of HEK 293T cells or the transfection efficiency. Therefore, we decided to use 5  $\mu\text{g}$  of lentiviral transfer plasmid along with the other lentiviral component encoding plasmids for production in the absence of antibiotics in the culture media.



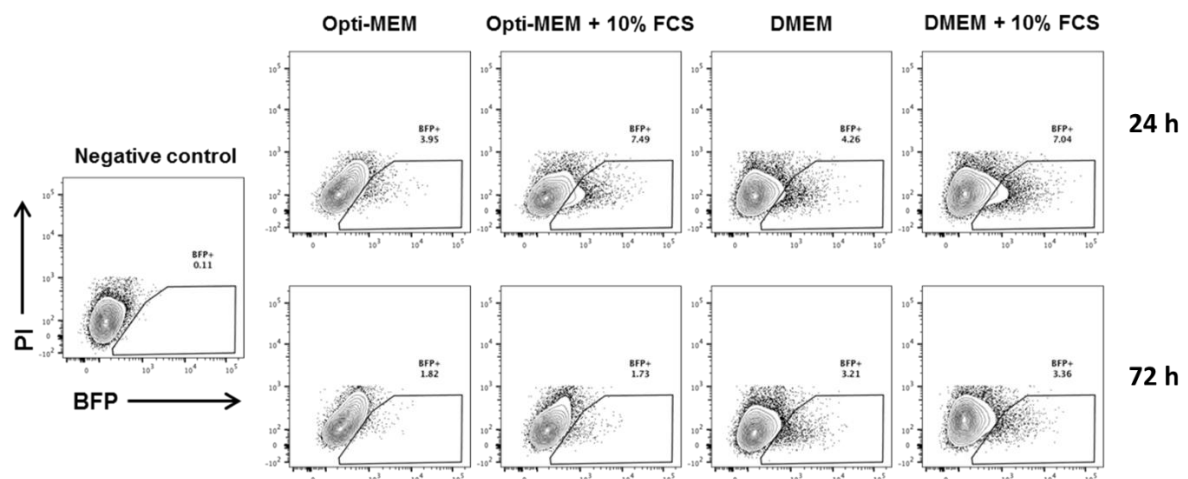
**Figure 2. Optimization of Transfection of HEK 293T Packaging Cells for Lentiviral Production**

Analysis of HEK 293T cells transfected in Opti-MEM with or without Penicillin-Streptomycin using the lentivirus production plasmid system consisting of the packaging vector psPAX2 and the envelope vector pMD2.G together with the transducing vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP containing gRNA for *C/EBP $\beta$*  24 hours post transfection. No transfer plasmid, 2.5  $\mu\text{g}$ , 3.75  $\mu\text{g}$ , 5  $\mu\text{g}$ , 6.25  $\mu\text{g}$  or 7.5  $\mu\text{g}$  of the transfer plasmid was used for transfection (all data not shown).



## Optimization of Lentivirus Production

Next, we optimized the lentiviral production process by assessing the effect of other variables. We evaluated lentivirus production using different culture media composition such as Opti-MEM or Dulbecco's modified Eagle's medium (DMEM) along with the presence or absence of 10% fetal calf serum (FCS) to investigate if this affects the viral production efficiency. We used the lentivirus production plasmid system consisting of the packaging vector psPAX2 and the envelope vector pMD2.G together with the lentiviral vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP containing gRNA for *C/EBP $\beta$* . Furthermore, to investigate if/how the viral titer is affected by freezing and thawing, we performed transduction of leukemic cells with fresh or frozen viral supernatants harvested at 24 and 72 hours post transfection (Figure 3). However, no significant difference was observed in the percentage of transduced cells, suggesting that in our hands the transduction efficiency is not affected by freezing the virus at  $-80^{\circ}\text{C}$  and thawing it once prior to usage (data not shown). By contrast, the transduction efficiency was affected by the transfection culture media used during the lentiviral production. We found that the highest titer virus was harvested at 24 hours post transfection and consistently produced in Opti-MEM supplemented with 10% FCS (Figure 3). Additionally, many protocols for production of lentiviruses use cell confluency to decide the starting seeding amount of the packaging cells. This measurement can be subjective from individual to individual and therefore we decided to assess the absolute number of cells that could be optimal for virus production. For this, we seeded 250 000, 500 000 and 750 000 cells to assess the effect on the viral production efficiency for a 6-well plate format (data not shown). Based on these findings, we selected to use 500 000 HEK 293T packaging cells for the lentivirus production.



**Figure 3. Lentivirus Transduction Tests Using Lentiviruses Produced in Culture Media With or Without Serum**

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## **Lentiviral Transduction Test of Leukemic Cells Using Two Lentivirus Production Plasmid Systems**

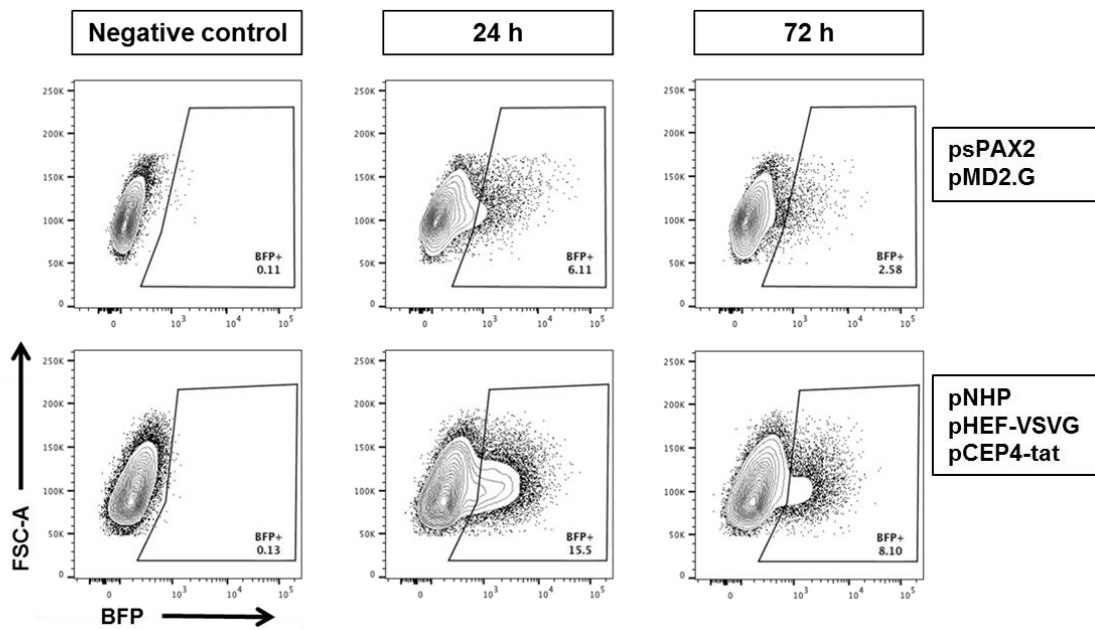
Although we managed to optimize different parameters for virus production, we failed to produce lentiviruses with a high titer. Therefore, we decided to compare lentivirus production with another widely used production plasmid system. Transduction of leukemic cells was performed using lentiviruses produced with two production plasmid systems along with the transducing vector pKLV-U6gRNA-PGKpuro2ABFP containing gRNA for *C/EBP $\beta$* : the previously used system or the plasmid system consisting of the packaging vector pNHP, the envelope vector pHEF-VSVG and the transactivator of transcription vector pCEP4-tat (Coleman, et al., 2003) (Figure 4). Both of these lentivirus production plasmid systems are second generation systems. While the third generation systems are safer due to the splitting of the packaging system into different plasmids, these systems can be more cumbersome to use. (Sakuma, et al., 2012) Based on previous experiments, we produced lentivirus in Opti-MEM supplemented with 10% FCS in the absence of antibiotics. Non-transduced leukemic cells were used as negative controls. The lentiviruses used for transduction were harvested at 24 or 72 hours post transfection. Flow cytometry analysis of transduced leukemic cells showed that a higher percentage of transduced cells were obtained using the second lentivirus production plasmid system. These results also suggest that lentivirus produced using this second production plasmid system should be harvested at 24 hours post transfection to obtain a higher titer.

### **Production of Lentiviruses Containing gRNA Targeting *C/EBP $\beta$* or *Notch1***

Next, we produced lentiviruses containing 5 gRNAs for *C/EBP $\beta$*  or *Notch1* using the optimized culture conditions. Lentiviral transduction tests using these viruses were next performed (Figure 5A). Flow cytometry analysis of cells 4 days post transduction showed that the lentivirus containing gRNA for *C/EBP $\beta$*  transduced around 13% of the cells and that the lentivirus containing gRNA for *Notch1* transduced around 10% of the cells. Being able to produce lentiviruses of a reasonable titer, we further continued the validation using the produced lentiviruses to transduce cells from the inducible CRISPR mouse models to explore our hypotheses (Figure 5B).

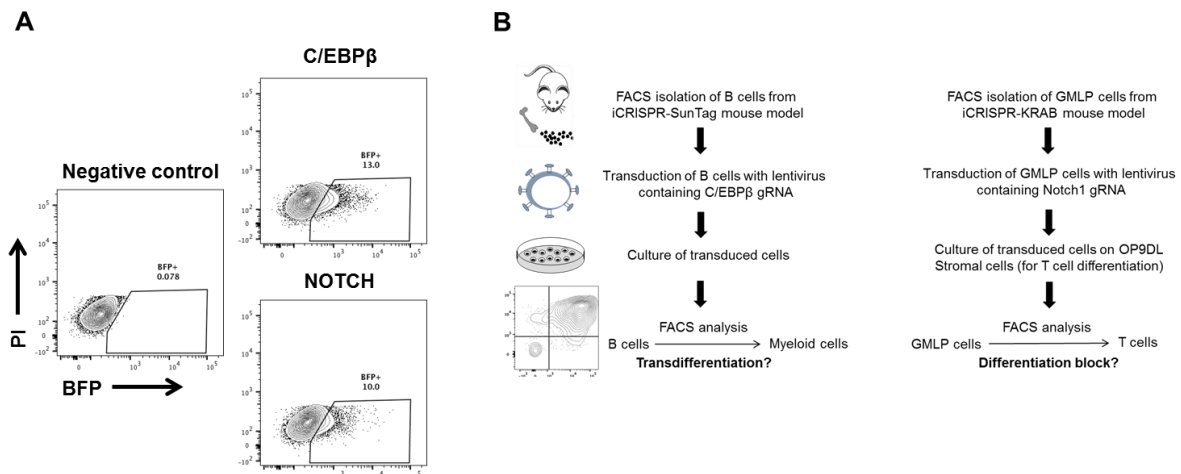
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HEK 293T cells were transfected in Opti-MEM or DMEM with or without 10% fetal calf serum (FCS), using the same lentivirus production plasmid system as previously together with our transducing vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP containing gRNA for *C/EBP $\beta$* . Transduction of leukemic cells was performed with freshly harvested lentivirus, harvested 24 or 72 hours post transfection.



**Figure 4. Lentiviral Transduction Test of Leukemic Cells Using Two Lentivirus Production Plasmid Systems**

Flow cytometry analysis of leukemic cells transduced with lentivirus produced in Opti-MEM supplemented with 10% FCS, harvested at 24 and 72 hours post transfection. Two lentivirus production plasmid systems along with the transducing vector pKLV-U6gRNA-PGKpuro2ABFP containing gRNA for *C/EBP $\beta$*  were used: the packaging vector psPAX2 and the envelope vector pMD2.G or the plasmid system consisting of the packaging vector pNHP, the envelope vector pHEF-VSVG and the transactivator of transcription vector pCEP4-tat.



**Figure 5. Production of Lentivirus Containing gRNA Targeting *C/EBP $\beta$*  and *Notch1***

(A) Representative data (n=5) for transduction of leukemic cells using lentivirus produced in Opti-MEM supplemented with 10% FCS, harvested 24 hours post transfection, using the lentivirus production

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## Lentiviral Transduction of B Cell Progenitors

Finally, we used the 5 gRNAs targeting *C/EBP $\beta$*  to transduce B cell progenitors sorted from the BM of a SunTag mouse, exploring the hypothesis that targeted cells should transdifferentiate into macrophages upon addition of doxycycline and thereby induced expression of *C/EBP $\beta$* . However, performing flow cytometry analysis 4 days post transduction, we observed no transduced cells. We were able to transduce c-kit<sup>+</sup> cells, also isolated from BM of SunTag mouse, using the same lentivirus which suggests that it was not the lentiviruses themselves but the transduction process that was not successful. Therefore, this experiment has to be repeated. We also transduced B cell progenitors extracted from the BM of a SunTag mouse using autoMACS pro separator. These cells were transduced with lentivirus that had been frozen, produced in OptiMEM with 10% FCS and harvested at 24 hours post transfection. The first plasmid system consisting of psPAX2 and pMDG.2 together with our transducing plasmid containing gRNA for *C/EBP $\beta$*  was used. Flow cytometry analysis 5 days after transduction, using the typical marker CD19 for B cells and the typical marker CD11b for myeloid cells, showed that only around 3% of the cells were transduced (Figure 6). The majority of the transduced cells were CD19<sup>+</sup> and CD11b<sup>-</sup>. A small percentage of CD11b expressing cells were present in both transduced and untransduced cells which is most likely due to the performed column isolation, which does not yield a completely pure population of CD19<sup>+</sup> cells. This result suggests that the transduced B cell progenitors have not transdifferentiated into macrophages. Due to time constraints, the validation of the CRISPRi system could not be performed even though lentiviruses containing gRNA targeting *Notch1* were produced.

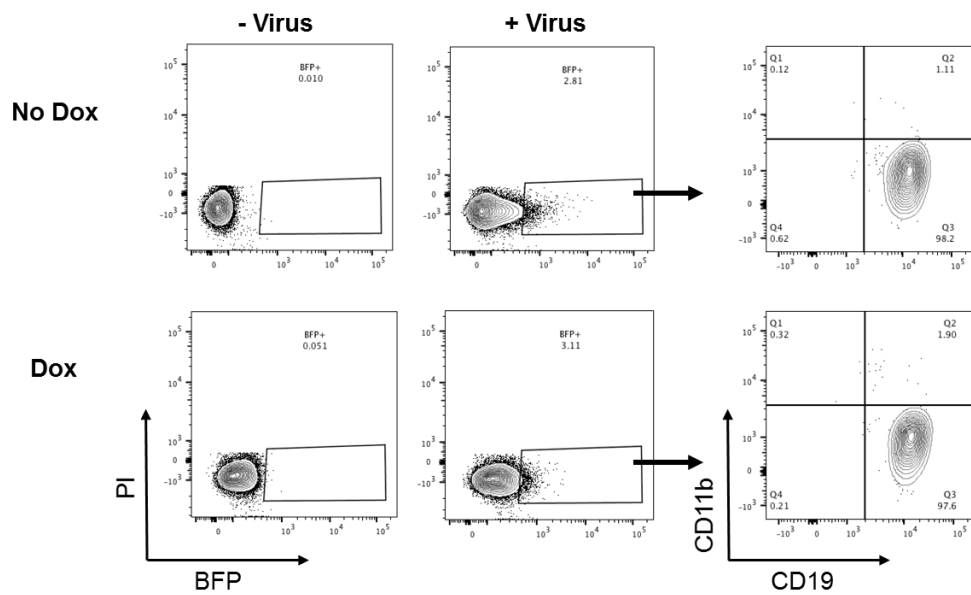
## DISCUSSION

Mature hematopoietic cells of the BM are constantly lost and replenished throughout life. Replacement of these cells occurs from HSCs and their progeny by their ability to regulate fate options such as self-renewal, quiescence and differentiation (Iwasaki & Akashi, 2007). The balance between different fate options is broadly mediated by the coordination of a complex network of extrinsic and intrinsic regulators. Although many insights on the interactions and

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plasmid system consisting of the packaging vector pNHP, the envelope vector pHEF-VSVG and the transactivator of transcription vector pCEP4-tat together with the transducing vector containing 5 different gRNAs for *C/EBP $\beta$*  or *Notch1*.

(B) Schematic representation of the next step in the experimental approach to further validate the CRISPRa and CRISPRi systems.



**Figure 6. Lentiviral Transduction of B Cell Progenitors from the CRISPR SunTag Mouse Model**

B cell progenitors from the CRISPR SunTag mouse model were transduced with lentivirus that had been frozen, produced in OptiMEM with 10% FCS and harvested at 24 hours post transfection. The first plasmid system consisting of psPAX2 and pMDG.2 together with the transducing plasmid containing gRNA for *C/EBP $\beta$*  was used.

fine-tunings of these regulators in normal and diseased hematopoiesis have been established, much still remains to be explored (Seita & Weissman, 2010). Thus, to explore the finely tuned transcriptional networks of defined hematopoietic progenitor cells that control cell differentiation, there is a need to manipulate gene function in a controllable manner. This requirement could be achieved using gene manipulation systems based on CRISPR activation (CRISPRa) or interference (CRISPRi) (Gilbert, et al., 2014). In previous studies, a lentiviral vector system has been used for transduction, where one of the vectors contains the gene for dCas9-SunTag or dCas9-KRAB while the other contains the gRNA for the target gene (Tanenbaum, et al., 2014; Dominguez, et al., 2016). The drawback here is that a double transduction of the cells of interest is required. However, a mouse model where every cell expresses the dCas9 fusion gene would circumvent this drawback by allowing for transduction of only the vector containing the gRNA. Another desired property would be the ability to easily induce the system when expression of the fusion gene is required, and finally this would offer a system where relevant primary cells can be more effectively targeted. Therefore, our laboratory has generated inducible CRISPRa and CRISPRi mouse models and aimed to validate these systems by inducing or repressing the transcription factors *C/EBP $\beta$*  and *Notch1*, respectively. The first step in the validation process was to produce lentiviruses to be used as gene delivery vehicles of gRNAs targeting the genes of interest. For broader successful use

of this system, we decided to use lentiviral delivery of gRNAs. As gene delivery vehicles, lentiviruses are advantageous compared to retroviruses due to their ability to transduce both actively dividing and non-dividing cells (Naldini, et al., 1996). For instance, to study gene function in HSCs, it is not desirable to culture the cells for long periods of time and thereby it would be advantageous to use lentiviruses. Thus, we have optimized the production of lentiviruses by improving conditions to obtain lentivirus of a higher titer.

First, we optimized different variables involved during the transfection of HEK 293T cells for lentiviral production. Second, we evaluated parameters that could affect the lentiviral production process including culture media for production, harvest time point, freezing of the viral supernatant and the number of HEK 293T cells seeded for transfection. Furthermore, in an attempt to increase the viral titer, we compared the production with a different lentivirus production plasmid system. This resulted in the production of lentivirus containing gRNA for *C/EBP $\beta$*  or Notch1 with a transduction efficiency in leukemic cells of 13% or 10 %, respectively. To increase the viral titer, the production protocol could be optimized further by for instance studying the use of different transfection agents for improved transfection efficiency or by concentration.

Previous studies have shown that B cells can be effectively converted to macrophages by enforced expression of the transcription factor *C/EBP $\beta$* , which has been confirmed in our laboratory (Xie, et al., 2004; Rapino, et al., 2013; Ugale, et al., 2014). Therefore, we decided to use same approach for validation of our CRISPRa mouse model. Using our generated lentiviruses carrying gRNA targeting *C/EBP $\beta$* , we were able to transduce B cell progenitors from a heterozygous SunTag mouse. However, no transdifferentiation into macrophages was observed. This might be because heterozygous mice have only one allele of the reverse tetracycline transactivator (rTta) in the *Rosa26* locus and one allele of dCas9-SunTag in the *Col1a1* locus, making expression levels of the CRISPRa components insufficient. Therefore, homozygous mice, which are expected to have higher expression of rTta and dCas9-SunTag in the cells, might improve the ability to induce the target gene. In the future, we will sort the transduced B cell progenitors and subject these to qRT-PCR to analyze the expression of *C/EBP $\beta$*  in the presence and absence of Dox. The CRISPRa construct is a large cassette containing dCas9 fused to a repeated peptide array (dCas9-GCN peptide), a 2A self-cleaving peptide sequence and a single-chain fragment variable antibody (scFV) fused to a VP64 activation domain, which likely makes it more difficult to express than for instance dCas9-KRAB. Although we have demonstrated the efficient induction of dCas9 upon doxycycline treatment of CRISPRa cells, to verify a successful induction of all the components of the construct, we will evaluate their expression levels by qRT-PCR. Finally, while previous studies

have used retroviruses to successfully transdifferentiate B cell progenitors (Xie, et al., 2004; Rapino, et al., 2013; Ugale, et al., 2017), this approach mediates its effect by (over)expressing a cDNA of *C/EBP $\beta$* . By contrast, we are aiming to express *C/EBP $\beta$*  from its endogenous locus. Therefore, one can speculate that locus accessibility might be a prerequisite for CRISPR based approaches. If so, the success of CRISPRa would be contingent upon the chromatin status of the gene of interest, which would be a concern beyond the raised technical concerns. The level of repression of endogenous genes using CRISPR systems based on dCas9 or dCas9-KRAB has been shown to be determined by the gRNA target site, reinforcing the hypothesis that the chromatin structure of the target gene is of importance for the application of these CRISPR systems (Gilbert, et al., 2014).

In summary, we have attempted to optimize protocols for production of lentiviruses, which will be used as gRNA delivery vehicles. This protocol could likely be further improved to increase the viral titer by optimizing additional parameters. Furthermore, we demonstrate the ability to induce dCas9 in our generated CRISPRa mouse model. However, initial experiments showed difficulties in *C/EBP $\beta$*  mediated transdifferentiation of B cell progenitors from the CRISPRa mouse model. Thus, further detailed validations are necessary to address the potential problems.

## **EXPERIMENTAL PROCEDURES**

### **Generation of *Col1a1*-tetOP-CRISPR SunTag/KRAB Mice**

The fusion gene for dCas9 together with SunTag or KRAB was subcloned into the EcoRI site of the pBS31 vector. The vectors were used to target KH2 embryonic stem cells (ESCs) (Beard, et al., 2006). The modified ESCs were then injected into morulae of C57BL/6 mice to generate chimeric mice (Lund University Transgenic Animal Facility). The mice were kept in the Lund University's animal facilities at the Biomedical Center. All experiments involving animals were performed with the consent from the local ethics committee.

### **Quantitative RT-PCR**

50 000 c-kit<sup>+</sup> cells from the BM of SunTag mouse were cultured with or without Dox (1  $\mu$ g/ml) (Sigma-Aldrich) in Opti-MEM (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich) in the presence of stem cell factor (SCF) (10–50 ng/ml), interleukin-3 (IL-3) (5 ng/ml) and granulocyte-colony stimulating factor (G-CSF) (5 ng/ml) for 48 hours. All cytokines were acquired from PeproTech. The cells were harvested and RNA isolation was performed using the Single cell RNA purification kit (Norgen Biotek Corp.) according to the manufacturer's protocol. Reverse transcriptions were performed using

SuperScript III First-strand Synthesis system for RT-PCR (Invitrogen) with random hexamers according to the manufacturer's protocol. Quantitative real-time PCR was performed with EvaGreen (BIO-RAD) on the CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD). The PCR protocol involved one cycle of 95°C (30 s), 40 cycles of 95°C (5 s) and 60°C (15 s). The melting curve analysis was performed at 65-95°C with an increase of 0.5°C per cycle. The expression of  $\beta$ -actin was used as control. The average threshold cycle number (Ct) was used to quantify the relative expression of dCas9:  $2^{-(Ct(dCas9)-Ct(\beta-actin))}$ . For primers used, see Table 1.

### **Cloning of gRNAs**

To generate double strand guide inserts for cloning into the expression vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP, a gift from Kosuke Yusa (Addgene plasmid # 50946), oligonucleotides with the upper and lower strand of the guide sequence was ordered from Integrated DNA Technologies (IDT). When these oligonucleotides are annealed, they contain single strand 5' and 3' overhangs that are compatible with the BbsI digested expression vector. The guide sequences were selected from Weissman lab libraries (Horlbeck, et al., 2016). For oligonucleotides used, see Table 1. The oligonucleotides were diluted in nuclease free H<sub>2</sub>O to a final concentration of 100  $\mu$ M. 10x T4 ligation buffer (NEB) and 10 U of T4 Polynucleotide Kinase 10U/ $\mu$ l (NEB) were used for the reactions. Phosphorylation and annealing of oligonucleotides were performed in T100 Thermal Cycler (BIORAD) at the following conditions: 37°C (30 minutes), 95°C (5 minutes), cooling down to 25°C at 0.1°C/second. Digestion of pKLV-U6gRNA(BbsI)-PGKpuro2ABFP and ligation of the phosphorylated annealed oligonucleotides were performed using 1  $\mu$ l BbsI (NEB) and 1  $\mu$ l T7 DNA ligase (NEB) in T100 Thermal Cycler (BIORAD) during 6 cycles of 37°C (5 minutes) and 22°C (5 minutes). Thereafter, the *Escherichia coli* strain JM109 was used for transformation. The bacterial cells were thawed on ice. The ligation mix was added and the cells were placed on ice for 30 minutes. The cells were then heat shocked at 42°C for 45 seconds followed by incubation on ice for 2 minutes. Thereafter, room temperature SOC (NEB) was added and the cells were incubated shaking at 37°C for 30-60 minutes for the cells to recover and to allow for the selection marker to be expressed. 100  $\mu$ l from each transformation was spread on pre warmed Luria Bertani Agar (Sigma-Aldrich) plates supplemented with ampicillin (50  $\mu$ g/ml). The plates were incubated at 37°C overnight. To select for colonies containing the vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP with the gRNA insert, colony PCR was performed on three clones for each gRNA. If the gRNA was successfully inserted, the result was a product of approximately 80 bp. The PCR protocol involved one cycle of 95°C (30 min), 30 cycles of 95°C (30 s), 58°C (30 s), 72°C (30 s) and one cycle of 72°C (5 min). For primers, see Table 1.



**Table 1.** Primers used for qRT-PCR along with oligonucleotides and primers used for cloning of gRNA.

<b>Primers for qRT-PCR</b>		
<b>Gene</b>	<b>Primer direction</b>	<b>Sequence</b>
<i>β-actin</i>	F	5'-CCACAGCTGAGAGGGAAATC-3'
	R	5'-CTTCTCCAGGGAGGAAGAGG-3'
<i>dCas9</i>	F	5'-CCAGAGGAAGTTCGACAATCTGAC-3'
	R	5'-TTTCCACCAGCTGTCTCTTGATG-3'
<b>Oligonucleotides for cloning of gRNA</b>		
<b>Gene</b>	<b>Primer direction</b>	<b>Sequence</b>
<i>C/EBPβg1</i>	F	5'-CACCGGGGCCATACATAGGGTGGAGT-3'
	R	5'-TAAAACCTCCACCCTATGTATGGCCCC-3'
<i>C/EBPβg2</i>	F	5'-CACCGGGACAGCGGGCAGGATCCGGT-3'
	R	5'-TAAAACCGGATCCTGCCCGCTGTCCC-3'
<i>C/EBPβg3</i>	F	5'-CACCGCCCGCGATGGCCACGGACGGT-3'
	R	5'-TAAAACCGTCCGTGGCCATCGCGGGC-3'
<i>C/EBPβg4</i>	F	5'-CACCGCGCTCCTCCGTGTTCCCGAGT-3'
	R	5'-TAAAACCTCGGGAACACGGAGGAGCGC-3'
<i>C/EBPβg5</i>	F	5'-CACCGCGGCCACGGGAGCGCGAGAGT-3'
	R	5'-TAAAACCTCTCGCGCTCCCGTGGCCGC-3'
<i>Notch1g1</i>	F	5'-CACCGTAGTGCCTCTGCCGCGGGAGT-3'
	R	5'-TAAAACCTCCGCGGCAGAGGCACTAC-3'
<i>Notch1g2</i>	F	5'-CACCGTGGGCCCTACAAGGCACCCGT-3'
	R	5'-TAAAACGGGTGCCTTGTAGGGCCCAC-3'
<i>Notch1g3</i>	F	5'-CACCGAGCGGCGCTGGGCCCTACAGT-3'
	R	5'-TAAAACCTGTAGGGCCCAGCGCCGCTC-3'
<i>Notch1g4</i>	F	5'-CACCGGGCGGCGCTGGGCTCTCAGGT-3'
	R	5'-TAAAACCTGAGAGCCCAGCGCCGCC-3'
<i>Notch1g5</i>	F	5'-CACCGGCCACCCGCCAGGAAAGAGT-3'
	R	5'-TAAAACCTTTTCTGGCGGGTGGGCC-3'
<b>Primers for colony PCR for cloning of gRNA</b>		
<b>Gene</b>	<b>Primer direction</b>	<b>Sequence</b>
<i>C/EBPβg1</i>	F	5'-CACCGGGGCCATACATAGGGTGGAGT-3'
<i>C/EBPβg2</i>	F	5'-CACCGGGACAGCGGGCAGGATCCGGT-3'
<i>C/EBPβg3</i>	F	5'-CACCGCCCGCGATGGCCACGGACGGT-3'
<i>C/EBPβg4</i>	F	5'-CACCGCGCTCCTCCGTGTTCCCGAGT-3'
<i>C/EBPβg5</i>	F	5'-CACCGCGGCCACGGGAGCGCGAGAGT-3'

<i>Notch1g1</i>	F	5'-CACCGTAGTGCCTCTGCCGCGGGAGT-3'
<i>Notch1g2</i>	F	5'-CACCGTGGGCCCTACAAGGCACCCGT-3'
<i>Notch1g3</i>	F	5'-CACCGAGCGGCGCTGGGCCCTACAGT-3'
<i>Notch1g4</i>	F	5'-CACCGGGCGGCGCTGGGCTCTCAGGT-3'
<i>Notch1g5</i>	F	5'-CACCGGCCACCCGCCAGGAAAGAGT-3'
<i>RNA scaffold</i>	R	5'-AAAAGCACCGACTCGGTGCC-3'

The reverse primer 5'-AAAAGCACCGACTCGGTGCC-3', targeting the RNA scaffold in the vector, was used for all samples. The selected colonies harboring the vector pKLV-U6gRNA(BbsI)-PGKpuro2ABFP with the inserted gRNAs, respectively, were then cultivated in LB broth (Sigma-Aldrich) overnight at 37°C. Ampicillin (50 µg/ml) was used for selection. The following day, the bacterial cells were harvested by centrifugation for 4 minutes at 6800 xg at room temperature. Plasmid DNA purification was performed using QIAprep Spin Miniprep Kit (QIAGEN AB) according to the manufacturer's protocol. The concentration and purity of the plasmid DNA was measured using NanoDrop ND-1000 (Thermo scientific). To validate the insertion of the different gRNAs in the expression vector, samples of plasmid DNA were subjected to Sanger sequencing (Eurofins). The reverse primer 5'-AAAAGCACCGACTCGGTGCC-3', targeting the RNA scaffold in the vector, was used as a sequencing primer for all samples.

### Plasmid Preparation

psPAX2 and pMD2.G was a gift from Didier Trono (Addgene plasmid # 12260, # 12259). *E. coli* JM109 harboring the plasmids were cultured in LB broth (Sigma-Aldrich) supplemented with ampicillin (50 µg/ml) shaking at 37°C overnight. Plasmid DNA was purified using the NucleoBond® Xtra Midi kit (MACHEREY-NAGEL) according to the manufacturer's protocol. The concentration and purity of the plasmid DNA was measured using NanoDrop ND-1000 (Thermo scientific). pNHP, pCEP4-tat and pHEF-VSVG were a gift from Sergey Kasparov (Addgene plasmid #22500, #22502, #22501). *E. coli* DH5α harboring the plasmids were cultured in LB broth (Sigma-Aldrich) supplemented with ampicillin (50 µg/ml) shaking at 37°C overnight. Plasmid DNA was purified in the same manner as for psPAX2 and pMD2.G. To verify the purification of the plasmid DNA, control digestions were performed. The expected fragments for pNHP and pCEP4-tat when digesting with HindIII are 0.55, 1.0, and 3.8 kb fragments and 0.25 and 10 kb fragments, respectively. The expected fragments for pHEF-VSVG when digesting with PstI digest are 0.55, 1.3 and 4.0 kb. After incubation for 30 minutes with the restriction enzyme, the samples were loaded on a 1.5% TAE gel. The GeneRuler 1kb Plus DNA ladder (Thermo Scientific) was used. The gel was run at 90 V for 1 hour.

## **Lentiviral Production**

Two second generation lentivirus production plasmid systems along with the transducing vector pKLV-U6gRNA-PGKpuro2ABFP containing gRNA for *C/EBP $\beta$*  or *Notch1* were used: the packaging vector psPAX2 and the envelope vector pMD2.G, or the packaging vector pNHP, the envelope vector pHEF-VSVG and the transactivator of transcription vector pCEP4-tat. The amounts used of the second production plasmid system were scaled down from the original format to a 6-well plate format according to area (Coleman, et al., 2003). The day prior to transfection, 500 000 human embryonic kidney (HEK 293T) cells were seeded in Opti-MEM supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich) and kept in 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The lentivirus production plasmid system together with the transducing vector were then packaged into these HEK 293T cells using 7.5  $\mu$ l Lipofectamine Reagent (Invitrogen) and 3  $\mu$ l PLUS reagent (Invitrogen) to increase the transfection efficiency. The transfected HEK cells were incubated with Opti-MEM (Thermo Fisher Scientific) supplemented with 10% FCS at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The media containing lentivirus was harvested at 24 and/or 72 hours post-transfection. The viral supernatant was aliquoted and stored in -80°C until use.

## **Lentiviral Transduction Tests**

Wells of a non-tissue culture treated 96-well plate (Falcon) were coated with 70  $\mu$ l of 0.04  $\mu$ g/ml Retronectin (Takara) diluted in Phosphate-Buffered Saline (PBS) (Thermo Fisher Scientific). After incubation for 2 hours in room temperature, the wells were blocked with 100  $\mu$ l PBS supplemented with 2% BSA (Sigma-Aldrich) for 30 minutes. The coated wells were then washed with 150  $\mu$ l PBS and the lentiviral vectors were added. The culture plate was then centrifuged for 2 hours at 32°C at 1109 xg. Following the spinoculation, the lentiviral containing solutions were removed and cells in 100  $\mu$ l culture medium were added. Murine leukemic cells from an MLL-ENL model (Ugale, et al., 2014) were used to assess the transduction efficiency of the produced lentiviruses. Leukemic cells and c-kit<sup>+</sup> cells were cultured in Opti-MEM (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich) in the presence of stem cell factor (SCF) (10–50 ng/ml), interleukin-3 (IL-3) (5 ng/ml) and granulocyte-colony stimulating factor (G-CSF) (5 ng/ml). All cytokines were acquired from PeproTech. Lentiviral transduced B cell progenitors were cultured in Opti-MEM supplemented with 10% FCS,  $\beta$ -mercaptoethanol (1:1000), penicillin/streptomycin (Invitrogen), SCF (10 ng/ml), IL-3 (5 ng/ml), interleukin-7 (IL-7) (10 ng/ml), FMS-like tyrosine kinase 3 ligand (Flt3L) (10 ng/ml), macrophage colony-stimulating factor (M-CSF) (10 ng/ml). All cytokines were acquired from PeproTech. As negative controls, non-transduced cells were used. Thereafter, the culture plate was centrifuged for 2 minutes at 32°C at 300 xg and incubated at

37°C in a 5% CO<sub>2</sub> humidified atmosphere. The cells were harvested after 2-6 days for flow cytometry analysis.

### **Flow Cytometry and Cell Isolation**

To perform a positive selection of c-kit<sup>+</sup> cells, BM cells from SunTag mouse were stained with CD117 MicroBeads (Miltenyi Biotec). Thereafter, the cells were run on an autoMACS Pro Separator (Miltenyi Biotec) for positive selection. To extract B cell progenitors, BM cells from a SunTag mouse were stained with biotinylated anti-CD19 (BD Pharmingen, cat. No. 553784, clone 1D3) at 1:200 dilution and run on an autoMACS Pro Separator (Miltenyi Biotec) for positive selection. A double selection was performed to obtain a more pure population of cells. For flow cytometry analysis, lentiviral transduced B cell progenitors were stained with fluorescent-conjugated anti-CD19 and anti-CD11b (BioLegend, cat. No. 101212, clone M1/70) antibodies. Lentiviral transduced c-kit<sup>+</sup> cells were stained against Ly6G (BioLegend, cat. No. 127652, clone 1A8) and CD115 (Biolegend, cat. No. 135517, clone AF598). All antibodies were used at 1:200 dilution. Propidium iodide (PI) (Invitrogen) diluted in PBS supplemented with 0.4% EDTA (Sigma-Aldrich) and 2% FCS at 1:1000 dilution was added prior to analysis. Lentiviral transduced leukemic cells used for lentiviral transduction tests were stained with PI only. Dead cells and debris were excluded by forward scatter (FSC), side scatter (SSC) and PI staining. Cells were sorted on a BD FACSAria III (BD Biosciences) and BD FACSAria IIu. The flow cytometry analyses were performed using BD LSR II (BD Biosciences) or BD LSRFortessa (BD Biosciences) instruments. To analyze the data, the software FlowJo (FlowJo, LLC) was used.

### **Statistical Analysis**

Data were analyzed using GraphPad Prism (GraphPad Software). To assess the statistical significance of dCas9 expression in c-kit<sup>+</sup> cells cultured with or without Dox, an unpaired student's t-test was used. A two-tailed *P*-value is shown. *P* value of <0.0001 is indicated as

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