

# Development of novel all-in-one inducible lentiviral vectors for potent gene silencing and overexpression

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# Abstract

Studying gene function usually involves loss-of-function strategies such as gene expression downregulation or gene deletion. However, precise control of the timing and level of gene silencing is crucial to minimise toxicity and off-target effects in transduced cells. Additionally, generating multi-gene knockout models or combining gene silencing with transgene overexpression can be labour-intensive and time-consuming. In the present study, we report the development of a series of vectors incorporating shRNA sequences within the *miR30-E* backbone, driven by the inducible Tet-On promoter  $P_{TRE3G}$  for investigating temporal regulation of gene silencing. The tandem expression of a reporter gene allows for efficient monitoring of transduction and selection of transduced clones. These vectors achieved over 90% downregulation of the expression of large transcripts, maintained stable genome integration over time and exhibited consistent inducibility *in vitro* and *in vivo*. The resulting vectors displayed tight regulation, minimal background expression and robust inducibility. Additionally, they were adapted for gain-of-function studies, achieving up to  $10^4$ -fold induced transgene overexpression. Overall, these dual-colour, all-in-one vectors provide a versatile and efficient platform for studying gene function with precise temporal control and reliable monitoring through stable reporter expression.

## Introduction

The most widely used approach for defining a gene's function is to manipulate its expression. Both gene silencing through RNA interference (RNAi) and genome editing technologies based on nucleases have provided the opportunity to control gene expression with great specificity. Despite the latter tools having recently revolutionized the field of biological research, achieving temporal, dosage, and on/off gene manipulation is still difficult to attain compared to RNAi-mediated knockdown, in particular when is virally delivered into cells [1–4]. Indeed, viral delivery of synthetic short hairpin RNAs (shRNAs) in gene expression cassettes has allowed stable and heritable gene silencing. In particular, lentiviral vectors are capable of infecting a wide variety of dividing and non-dividing cells and integrating into the host genome with long-term expression of the transgene [5].

The widely used shRNA expression systems rely on *RNA Pol III* promoters such as the *H1* or *U6*, which direct high levels of shRNA expression mediating highly potent gene silencing. However, these promoters provide exceedingly high levels of shRNA that can elicit off-target silencing and non-specific effects such as interferon response and cellular toxicity [6–8]. Thus, a second generation of RNAi-systems has been designed where shRNA is embedded into endogenous microRNA backbones (*miRshRNA*). This facilitates processing through the miRNA pathway and overcomes cellular toxicity while eliciting potent knockdowns [9, 10]. In particular, human *miRNA30* has been extensively used in these systems [11–14] where processing of the larger pre-miRNA transcript to its functional *miRNA* has been well characterized *in vitro* and *in vivo* [15]. Furthermore, the expression of *miRshRNAs* is driven by *Pol II* promoters, which allow transcription of a reporter gene linked to the *miRshRNA* in a polycistronic sequence that, if properly processed, allows monitoring of shRNA expression [10, 14].

Inducible *miRshRNA* systems have also been developed and optimized in the last decade [13, 14] which offer many advantages over the constitutive systems, particularly for studying essential genes. Controlling the timing and the levels of shRNA expression is central for many experimental settings, since constitutively expressed shRNAs targeting essential genes promote a toxic effect in the transduced cells. Moreover, the ability to regulate shRNA expression makes it possible to investigate time- and dose-dependent effects which can be completely reversed. Also, inducible systems allow for strict isogenic controls within an experiment, since the ability to control expression of the gene of interest in a population of cells can minimize experimental variations due to clonal heterogeneity.

In a Tet-On system, baseline transcription can be kept to background levels when using an optimized reverse tetracycline responsive transactivator (rtTA). Thus, several efforts have been made towards the optimization of the rtTA protein in order to improve dynamic range, by reducing residual DNA binding in the non-induced state and enhancing doxycycline (dox) sensitivity and transcriptional activity in the induced state [16–18]. The rtTA3 protein has been developed to meet these criteria [17], showing good efficacy in the different inducible systems [13, 14]. Other approaches have focused on the manipulation of the tetracycline responsive elements (TRE) and downstream promoter (usually a minimal CMV promoter) to minimize background expression while maintaining high levels of induced expression [19, 20]. In this regard, a synthetic inducible promoter (commercialized by Takara Bio Inc. as  $P_{TRE3G}$ ) was modified for optimal performance, lacking binding sites for endogenous mammalian transcription factors thus, being virtually silent in the absence of induction [19].

However, many inducible shRNA systems still require the delivery of two vectors to introduce the transactivator protein and the inducible promoter controlling the transgene expression. These two-vector systems usually involve a time-consuming multi-step clone selection process, precluding studies in cell types with limited lifespan in culture, such as stem cells. Moreover, another key problem associated with inducible vectors is the heterogeneity in transgene expression levels among cells due to different integration sites [21] or the variable inducibility due to different rtTA expression levels. To circumvent these issues, all-in-one dual colour inducible systems like the pINDUCER lentiviral system [13] and the retroviral TRMPVIR [14] have been developed, which couple fluorescent reporters to both the inducible shRNA expression and the constitutive rtTA expression. These systems broaden the applicability of inducible shRNA vectors and allow the identification not only of the transduced cells but more specifically of cells that express the functional shRNA construct upon induction, thus enabling the isolation of cells with higher rtTA expression and shRNA inducibility. However, these systems still present some limitations, like intrinsic leakiness and unstable reporter expression [22, 23].

In this work, we present a novel lentiviral vector system for inducible gene silencing that combines the best features available in the literature to overcome the above-mentioned limitations of existing vectors. We have developed a series of vectors where the *miR30-EshRNA* sequence is placed inside an *EF1 $\alpha$*  intron, upstream of the reporter gene, and expression is driven by a very tight minimal synthetic promoter. The pri-*miR30-EshRNA* is spliced out from the transcript and follows a stable *miR30-E* processing while the translation of the reporter takes place at the same time. The vectors incorporate an

additional cassette with rtTA3 and a second reporter gene for monitoring infection efficiency. These novel all-in-one dual colour vectors are tight and demonstrate efficient inducibility that can be reliably monitored through stable reporter expression both *in vitro* and *in vivo*. In light of the exceptional performance of the Tet-On *miR30*-EshRNA lentivectors, these were adapted for the inducible transgene overexpression, which can be valuable for gain-of-function studies. Once more, these inducible vectors demonstrate stable genome integration over time, constant levels of inducibility and negligible leakiness both *in vitro* and *in vivo*.

## Materials and methods

### Vector construction and *miR30*shRNA cloning

This and all the subsequent lentiviral vectors are HIV1pseudotyped constructs containing Rev response element (RRE), central polypurine tract (cPPT/CTS) and Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). The pLV.EF1 $\alpha$ -*miR30*shRNA-*Gfp* lentiviral vector was based on the pLV.EF1 $\alpha$ -pre*miRNA30a*-rPuro (Biosettia Inc.) vector. It contains the EF1 $\alpha$  promoter and intron with the *miR30a* precursor sequence inside the intron. The enhanced *Gfp* sequence was amplified by polymerase chain reaction (PCR) from an in-house vector and cloned using *Xba*I and *Sal*I restriction sites (RS) through standard molecular cloning techniques thus, replacing rPuro. The *miR30*shRNA sequence was amplified from pGIPZ™ (Horizon Discovery Ltd) by PCR flanked by *Bam*HI and *Nhe*I RS and then cloned into an intermediate vector (pECFP-C1; Takara Bio Inc.). shRNA sequences were cloned inside the *miR30* backbone through *Xho*I and *Eco*RI RS. shRNA sequences were designed according to the rules for *miR30*shRNA structure and 110 bp phosphorylated oligos with 5'-*Xho*I and *Eco*RI – 3' overhangs were purchased from Merck. For annealing, forward and reverse oligos (**Table S1**) were resuspended in water to 1 mM and 2  $\mu$ l of each oligo were added to 48  $\mu$ l of homemade annealing buffer (100 mM potassium acetate, 30 mM HEPES pH7.4, 2 mM magnesium acetate) and incubated at 95°C for 4 min followed by 70°C for 10 min and allowed to cool down at room temperature (RT) for 30 min. Annealed oligos were diluted 1:10 in water prior to ligation. The *miR30*shRNA sequence in the intermediate vector was then cloned in the pLV.EF1 $\alpha$ -*miR30*shRNA-*Gfp* vector using *Bam*HI and *Nhe*I RS.

To generate the *miR30*-EshRNA vectors, the *miR30*shLuc was modified by PCR to obtain the exact *miR30* backbone described in [23]. The amplicon was sub-cloned in the intermediate vector pECFP-C1 using *Bam*HI and *Nhe*I RS to allow cloning of new shRNA oligos with *Xho*I and *Eco*RI RS. Of note, when using the *mir30*-E backbone, because the *Eco*RI RS position was changed, custom oligos must be 125 bp, instead of 110 bp. Forward and reverse oligos for *miR30*-EshRNA are listed in **Table S2**. The *miR30*-EshRNA sequence in the intermediate vector was then cloned in the final vector using *Bam*HI and *Nhe*I RS.

The inducible pLV.P<sub>TRE3G</sub>-*miR30*shRNA-*Gfp* vectors were generated by adding and replacing the necessary components to the constitutive pLV.EF1 $\alpha$ -*miR30*shRNA-*Gfp* vector. The *mCherry* reporter was amplified by PCR from the pmCherry vector (Takara Bio Inc.) adding *Apa*I and *Sal*I RS. The amplicon was

inserted into the pRRL.SFFV-d20Gfp-T2A-mBfp vector (a gift from Andrew Scharenberg; Addgene plasmid #31485) replacing the *mBfp* sequence using *Apal* and *Sall* RS. *rtTA3* was amplified from the pINDUCER21 vector (ORF-EG, a gift from Stephen Elledge & Thomas Westbrook; Addgene plasmid #46948) [13] adding *XhoI* and *BmgBI* RS and a portion of the T2A sequence in the 3' end, then replacing the d20Gfp-T2A sequence using *XhoI* and *BmgBI* RS. This generated the pRRL.SFFV-*rtTA3*-T2A-mCherry vector. Following this, the SFFV-*rtTA3*-T2A-mCherry cassette was amplified by PCR using the High Fidelity PCR Master (Roche) by which *MluI* and *Sall* RS were added. The amplicon was then cloned in the constitutive vector pLV.EF1 $\alpha$ -miR30shRNA-Gfp using *MluI* and *Sall* RS generating the pLV.EF1 $\alpha$ -miR30shRNA-Gfp-SFFV-*rtTA3*-T2A-mCherry vector. To replace the EF1 $\alpha$  promoter, the inducible promoter  $P_{TRE3G}$  was obtained from the pTRE3G-mCherry (Takara Bio Inc.) vector. The following sequential cloning steps were performed: a 744 bp sequence of the EF1 $\alpha$  intron containing a 3' end *BamHI* RS was amplified to insert a 5'-*Sall* RS and sub-cloned in front of the  $P_{TRE3G}$  using *Sall* and *BamHI* RS. Then, the  $P_{TRE3G}$ -EF1 $\alpha$  intron sequence was amplified, introducing a 5'-*Clal* RS and the amplicon was then inserted in the pLV.EF1 $\alpha$ -miR30shRNA-Gfp-SFFV-*rtTA3*-T2A-mCherry vector using *Clal* and *BamHI* RS thus, removing the EF1 $\alpha$  promoter and the original 744 bp intron portion. This originated the inducible vector pLV. $P_{TRE3G}$ -miR30shRNA-Gfp-SFFV-*rtTA3*-mCherry. To replace the SFFV promoter, human PGK and UBC promoters were first sub-cloned into the intermediate vector pRRL.SFFV-*rtTA3*-T2A-mCherry. The PGK promoter sequence was amplified from an in-house vector adding a 5'-*NheI* and *MluI* and 3' end *XhoI* RS. The UBC promoter was amplified from the pLVUT-tTR-KRAB vector (a gift from Patrick Aebischer & Didier Trono; Addgene plasmid # 11651) [29] with a high-fidelity polymerase, adding the same restriction sites. The respective amplicons were inserted in the pRRL.SFFV-*rtTA3*-T2A-mCherry vector, replacing the SFFV promoter with *NheI* and *XhoI* RS, then finally cloned in the inducible vector with *MluI* (upstream the promoter) and *NdeI* (inside the *rtTA3*) RS. As for the constitutive promoter, any miR30shRNA tested was cloned using *BamHI* and *NheI* RS.

Each inducible OE vector was generated from its respective inducible miR30shRNA vector by replacing the whole EF1 $\alpha$  intron for a multiple cloning site (MCS) followed by a sequence that encodes the P2A self-cleaving peptide. For this, the miR30shRNA-Gfp sequence was replaced for MCS-P2A-Gfp obtained from an in-house vector using *Clal* and *MluI* RS. Control vectors were created by replacing the MCS-P2A-Gfp sequence with the Gfp sequence alone using *BamHI* and *MluI* RS. The Gfp sequence was amplified from an in-house vector, introducing 5'-*BamHI* RS and a consensus kozak sequence. Luciferase overexpression vectors were generated by cloning the *Luc* sequence using *NheI* and *BamHI* RS. The *Luc* sequence was amplified from a pGL2 vector (Promega) with a high-fidelity polymerase, introducing a 5'-*NheI* RS and a consensus kozak sequence and a 3' end *BamHI* RS without stop codon.

## Production of lentiviruses

Lenti-X HEK293T cells [30] were seeded in tissue culture dishes at  $5 \times 10^4$  cells/cm<sup>2</sup> 16-18h before transfection in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% inactivated foetal bovine serum (FBS), 1% GlutaMAX™ (Thermo Fisher Scientific) and 1% Penicillin-Streptomycin (P/S). Culture medium was carefully changed 2 h before transfection to Opti-MEM™

(Thermo Fisher Scientific) with 2% FBS, adding enough medium to cover the monolayer. Transfection was performed using the calcium phosphate method. For the plasmid DNA mix the amount of transfer vector was calculated based on a molar ratio 2:1:1 for the transfer:packaging (pCMVdR8.74):envelope (pMD2.G) vectors and  $\sim 0.3 \mu\text{g}$  total DNA/cm<sup>2</sup>. For a 150 cm<sup>2</sup> culture dish, the DNA mix consisted in 8  $\mu\text{g}$  of pMD2.G, 16  $\mu\text{g}$  pCMVdR8.74, variable amount of transfer vector depending on molar concentration and nuclease-free water up to 1130  $\mu\text{l}$ . Then, 120  $\mu\text{l}$  of CaCl<sub>2</sub> (2.5 M) was added to the DNA mix dropwise and mixed gently by flicking. Finally, 2× HEPES buffered saline (Merck) was added dropwise while vortexing in mild speed. The mixture was incubated for 10 min at RT and then added to the cells drop by drop while gently swirling the dish. Cells were incubated at 37°C and after 7–8 h the culture medium was carefully replaced with pre-warmed collection media (Opti-MEM supplemented with 2% FBS, 1% P/S and 12.5 mM of HEPES). On the following day cells were checked under a fluorescent microscope to assess the quality of the transfection. Note that at this point, cells transfected with inducible vectors show both GFP and mCherry expression since Tet-On regulation is only effective upon viral transduction. The virus supernatant was collected  $\sim 48$  h after the medium change, centrifuged for 5 min at 1000× *g* to pellet detached cells and debris, filtered through a 0.45  $\mu\text{m}$  filter and loaded into thin-wall polypropylene conical tubes (Beckman Coulter). Ultracentrifugation was performed in a SW32Ti rotor (Optima XPN-80 Ultracentrifuge, Beckman Coulter), at 4°C and 90000× *g* for 2.5 h. The centrifuged media was carefully discarded and empty tubes were placed inverted on a filter paper to remove excess liquid. Walls of the tube were carefully wiped with a sterilized cotton swab or aspirated without touching the pellet. Between 50 to 100  $\mu\text{l}$  of complete Roswell Park Memorial Institute-1640 (RPMI) medium supplemented with 10% FBS and 1% P/S was added to the viral pellet with gently pipetting up and down several times. Tubes were left sealed with parafilm for 2 h at 4°C and occasionally flicked. Resuspended virus media were transferred to a microcentrifuge tube and centrifuged for 1 min at 1000× *g* at 4°C to pellet any major debris. The concentrated virus was aliquoted and stored immediately at -80°C. Virus titres were determined by serial dilutions in HeLa cells. Transduction efficiencies were analysed by flow cytometry in a BD LSRFortessa™, measuring the percentage of GFP or mCherry positive cells for the constitutive and inducible vectors, respectively.

#### Transduction and generation of stable cell lines

K562 and OCI-AML3 cells (DSMZ, Germany) were plated at  $5 \times 10^5$  cells/mL in culture wells with complete RPMI and Iscove's Modified Dulbecco's Medium (IMDM) with 20% FBS and 1% P/S, respectively. Cells were transduced with MOI 5 or 10 and culture medium was changed 16 h after viral infection and maintained at the concentration of  $5 \times 10^5$  cells/mL and split every 3–4 days. Sorting of mCherry<sup>+</sup> cells was performed on a BD FACSAria™ Fusion, BD Biosciences) with a 16-16-0 precision mode and dead cells and debris were excluded by gating DAPI negative cells. Unless stated otherwise, to induce transcript expression, 0.5  $\mu\text{g}/\text{ml}$  of dox was added in cultures every 48 hours and mCherry and GFP were quantified by FCM.

#### Immunostaining and flow cytometry

For cell surface antibody staining, mouse IgG1 PE isotype control (clone MOPC-21, BD Biosciences) and anti-Notch1 PE (clone MHN1-519, BD Biosciences) antibodies were used for 30 min at 4°C in the dark and analyzed in a BD LSRFortessa™ (BD Biosciences).

### Western blot

Protein concentration of cell lysates was quantified using the DC™ Protein Assay (Bio-Rad Laboratories) according to the manufacturer's instructions. The desired protein quantity (20 µg per well) was mixed with water and 6× Laemmli buffer for a total volume of 24 µl per well. Samples were incubated for 5 min at 95°C and let to cool down at RT for 10 min followed by a quick spin. NuPAGE™ 4–12% Bis-Tris Gel (Thermo Fisher Scientific) was prepared according to the manufacturer's instructions to run with the NuPAGE™ MOPS SDS Running Buffer (Thermo Fisher Scientific) in a XCell SureLock® system (Thermo Fisher Scientific). After assembling, 20 µl of the sample were loaded per well and the gel was run for 2.5–3 h at 100 V. Membrane transfer was performed by wet electroblotting to a PVDF (polyvinylidene difluoride) membrane previously activated in methanol. The gel-membrane sandwich was assembled and run overnight at 30 V and 4°C in NuPAGE™ Transfer Buffer (Thermo Fisher Scientific) with 10% methanol. After the transfer, the membrane was rinsed in tris-buffered saline (TBS) with 0.05% Tween®20 (TBS-T) and blocked for 2–3 h in TBS-T with 5% skimmed milk at RT. After blocking, the membrane was probed with the primary antibody diluted in the blocking solution rotating overnight at 4°C. Anti-Notch1 (3608S; Cell Signaling Technology) and β-Actin (A5441; Merck) primary antibodies were followed by respective horseradish peroxidase (HRP) secondary antibody. Goat anti-rabbit HRP and goat anti-mouse HRP (Agilent Technologies) were diluted 1:5000. For chemiluminescence detection, the membrane was incubated with Immobilon® HRP Substrate (Merck) solution for 3–5 min (for β-Actin detection membrane was incubated with used substrate). The light-producing reaction was captured in a ChemiDoc™ XRS System (Bio-Rad Laboratories).

### Luciferase Assay

The Luciferase Assay System E1500 (Promega, Madison, WI, USA) was used to quantify the expression of firefly luciferase as per manufacturer's instructions. The CLARIOstar® Plus microplate reader was used for the measurement of firefly luciferase luminescence. Values obtained were normalized by subtracting the background value obtained with the lysate of non-transduced cells.

### *In vivo* transplantation assays

NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice were purchased from The Charles River Laboratories and kept in isolators with aseptic standard operation procedures in the biological research facility of the School of Biosciences at Cardiff University. All mouse husbandry and experiments were carried out in accordance with the UK Home Office regulations and the Animals (Scientific Procedures) Act 1986, and approved by the Cardiff University Animal Welfare Ethical Review Body (AWERB). NSG mice aged 8–12 weeks were intravenously injected with  $0.5 \times 10^6$  OCI-AML3 cells that were transduced with different lentiviruses. Nine to twelve days post-transplant, mice were administered via oral gavage either with

vehicle or doxycycline hyclate (Merck-Sigma) solution (20 mg/Kg). Imaging of -isoflurane-anesthetized animals was undertaken using the Biospace PhotonImager (Biosapcelab) system 5–10 min after ip injection of D-luciferin potassium salt (VivoGlo™ Luciferin; Promega) solution (150 mg/kg). Bioluminescence images were taken from both the ventral and dorsal sides of the mice. The photons emitted were expressed as photons/s/cm<sup>2</sup>/steradian. At the end of each experiment, mice were sacrificed by cervical dislocation and bone marrow cells were harvested from six bones (tibiae, femurs, and ileum) or three bones from the right legs. Recovered cells were immunophenotyped and analyzed by FCM.

## Data Analysis

Statistical analysis was performed with unpaired t-test using the Prism8 software (GraphPad Software). The p-value scale used was: \*p < 0.03, \*\*p < 0.003 and \*\*\*p < 0.0003.

## Results

A stable and efficient lentiviral vector for potent gene silencing through *miR30shRNA*

A constitutive lentiviral vector (pLV.EF1α-*miR30shRNA*-Gfp) was developed that expresses *miR30shRNA* inside the EF1α intron with green fluorescent protein (Gfp) reporter in frame under the control of the human EF1α promoter (Fig. 1A; simply referred as shRNA). This is known to lead to a more stable expression of both Gfp and shRNA, given that splicing allows the separation of the two transcripts, which enter their respective processing pathways and generate *miR* and *mRNA*. Moreover, this avoids competition and maximises the efficiency of both processes, making the GFP reporter a reliable quantification marker of the level of shRNA expression [24]. This is a new feature over the currently available *miRshRNA* vectors.

As efficient silencing of genes that produce large transcripts can be difficult to attain, we sought to test our new lentiviral vector to silence *NOTCH1* receptor as a proof of principle. In addition, this was chosen based on the current knowledge that *NOTCH1* does not appear to have an essential role in cell lines used in this study. A specific shRNA sequence against *NOTCH1* receptor (sh*NOTCH1*) was tested in K562 cells, using shRNA against the non-mammalian target luciferase (sh*Luc*) as control. Cells were transduced with multiplicity of infection (MOI) 10 and cultured for 6 days, after which the level of extracellular Notch1 was quantified by flow cytometry (FCM; Fig. 1B). Based on this, the developed *miR30shRNA* vector promoted a highly potent and significant knockdown of 92 ± 0.8% of the Notch1 protein in K562 cells (Fig. 1C).

Transduced cells were also collected after 9 days of culture for total protein extraction and knockdown validation by western blot (Fig. 1D). Using an antibody that binds to the C-terminus of Notch1 receptor, we observed excellent silencing levels of the immature full-length protein (N1FL) and, importantly 88 ± 6.4% knockdown of the transmembrane form (N1TM), which reflects the quantity of functional receptor available on the cell surface (Fig. 1E). These results are consistent with the above FCM data. Importantly,

the transduction efficiency of this vector was 100% in K562 cells, and the level of transduced cells (GFP<sup>+</sup>) and knockdown efficiency were maintained throughout time in culture (Figs. 1F and 1G), demonstrating the stability of the developed lentiviral vector.

#### Efficient inducible gene silencing with optimised Tet-On *miR30shRNA* lentiviral vectors

To overcome the limitations of existing inducible *miRshRNA* systems, we developed new Tet-On lentiviral vectors that incorporate the *miR30shRNA* and *Gfp* reporter expression (**Figure S1**) under the inducible promoter  $P_{TRE3G}$  [19], with the *miR30shRNA* sequence integrated into the *EF1a* intron, as described above. For the Tet-On system expression of the regulatory protein rtTA3 (3rd generation) [17] is driven by different constitutive promoters: *SFFV* (spleen focus-forming virus), ubiquitous human *PGK* (phosphoglycerate kinase) and human *UBC* (ubiquitin C). rtTA3 is linked to the mCherry reporter, which allows the identification of transduced cells by the self-cleaving peptide T2A (Fig. 1S) simply denoted as *shRNA-SFFV* or *shRNA-PGK* or *shRNA-UBC*).

To evaluate the stability of the integrated vector (monitored by mCherry expression) as well as the level of inducibility (GFP expression) and gene knockdown over time, cells were transduced with the different vectors and mCherry<sup>+</sup> cells were sorted after 3–6 days of culture. We observed that K562 cells transduced with the *shLuc-SFFV* vector showed a substantial decrease in mCherry expression over 3 weeks of culture when 50% of the population was mCherry<sup>+</sup> (**Figure S2**). This was not observed in K562 cells transduced with either the *shLuc-PGK* (Fig. 2A) or the *shLuc-UBC* vector (Fig. 2E). To validate the stability, inducibility and functionality of the vectors with *PGK* and *UBC* promoters, we assessed Notch1 receptor knockdown in all the conditions. Upon addition of dox, mCherry expression was close to 100% and steady over time, whilst in the absence of dox no GFP leakiness was observed (**Figure S3A**). Overall, steady mCherry and GFP expressions were observed for both inducible vectors independently of the *shRNA* expressed (Figs. 2A, 2B, 2E and 2F), demonstrating that the new Tet-On lentiviral vectors allowed stable vector integration with great inducibility associated with tight control of the *miR30shRNA-Gfp* expression. Importantly, only upon induction with dox, a significant decrease in Notch1 receptor expression was observed in cells transduced with both inducible vectors driving *shNOTCH1*, and this remained constant over time in culture. In contrast with the vectors carrying *shLuc* the expression of Notch1 remained unaltered (**Figures S3B, 2C and 2G**).

To prove the versatility of the developed Tet-On lentiviral vectors, these were also tested in another cell line, OCI-AML3, an acute myeloid leukemia cell line. This was done because OCI-AML3 cells are less permissive to lentiviral infection thus, providing stronger proof of the new lentiviral vectors efficacy. In addition, this cell line can easily engraft in immunodeficient mice thus, offering an essential model to test these vectors *in vivo*. As observed above, these newly developed inducible vectors were also stable in OCI-AML3 cells, with more than 95% of the cell population showing mCherry expression after 18 days of culture (Figs. 2I and 2M). In terms of inducibility, the *shRNA-UBC* lentiviral vector was more efficient in this cell line, as in the presence of dox, GFP expression was consistently expressed by ~ 95% of the population (Fig. 2N), whereas cells transduced with *shRNA-PGK* showed more variable inducibility

ranging from 75 to 95% of GFP<sup>+</sup> cells (Fig. 2J). Nevertheless, Notch1 receptor knockdown efficiencies were consistent and similar between *UBC* and *PGK* lentiviral vectors, showing ~ 75% knockdown which was stable over time in culture (Figs. 2K and 2O). Importantly, cells with stronger GFP expression (higher fluorescence intensity) presented lower Notch1 receptor expression, demonstrating that the levels of GFP reporter in these inducible systems correlated very well with the degree of *miR30*shRNA expression ( $R^2 = 0.86-0.98$ ) (Figs. 2D, 2H, 2L and 2P). This also enabled the isolation of clonal populations with stronger reporter expression which correlates to higher shRNA expression and consequently more potent gene silencing. In summary, the new Tet-On *miR30*shRNA lentiviral vector system allowed stable and robust gene silencing in long-term cultures even in cells that are less permissive to viral vector infection.

#### Effective *in vitro* and *in vivo* gene silencing by the inducible *miR30*-EshRNA lentiviral vectors

The *miR30* backbone has been recently modified to allow optimal *miR*shRNA processing, increasing mature shRNA levels and knockdown efficiency [23]. This new optimized backbone, termed *miR*-E, contains a conserved sequence from endogenous human *miR30* and can be easily cloned from the existing *miR30* sequences. Established potent *miR30*shRNAs demonstrate enhanced silencing when converted into the *miR*-E backbone without causing any toxicity [23]. Thus, to further improve the efficacy of the inducible Tet-On *miR30*shRNA vectors, we introduced the *miR30*-E backbone.

The potency of *miR30*-EshRNAs was initially evaluated using the constitutive lentiviral vector as demonstrated above. We decided to use the less permissive OCI-AML3 cells to viral vector infection to demonstrate potential improvements by using the *miR30*-E backbone. In addition to this, we also compared the knockdown efficiencies of previously validated sh*NOTCH1* with a new shRNA sequence against *NOTCH1* (sh*NOTCH1*-2) that showed modest knockdown efficiency (data not shown). OCI-AML3 were transduced with MOI 10 with either the constitutive *miR30* or *miR30*-E shRNA-expressing vectors and knockdown efficiencies were compared side-by-side after 6 and 12 days of culture. As expected, expression of sh*NOTCH1* in the *miR30*-E backbone (Esh*NOTCH1*) consistently resulted in significantly higher knockdown levels compared to the original sh*NOTCH1*, showing an improvement from  $63 \pm 5.6\%$  to  $78 \pm 3.9\%$  ( $p \leq 0.003$ ). This was even more striking with the Esh*NOTCH1*-2 vector that resulted in a significant increase from  $58 \pm 6.2\%$  to  $86 \pm 0.8\%$  of knockdown ( $p \leq 0.003$ ) (Fig. 3A). Others have shown that the increased shRNA processing associated with the *miR30*-E backbone (not inserted in the *EF1 $\alpha$*  intron for expression and processing) is associated with a significant decrease in the reporter expression [23]. However, we have not observed this with our vector system given the separation of *miR30*shRNA from the reporter during RNA splicing as described above.

We also applied the *miR30*-EshRNA modification to the inducible shRNA-*UBC* lentiviral vector. Inducibility and knockdown efficiencies in OCI-AML3 cells were determined in the presence of dox (Fig. 3B). As verified with the constitutive vector, Esh*NOTCH1*-2-*UBC* showed greater potency with a  $94 \pm 0.6\%$  knockdown efficiency, while Esh*NOTCH1*-*UBC* produced knockdown levels of  $86 \pm 3\%$ , which represents a significant increase of ~ 10% from sh*NOTCH1*-*UBC* as previously shown (Fig. 2O). Similarly to the *miR30*

inducible vectors, *miR30*-E vectors showed great inducibility, with GFP expression ranging from 85 to 95% and no GFP expression in the absence of dox (data not shown).

Furthermore, we sought to evaluate the sensitivity of this inducible system by reducing 10 to 200-fold of the dox concentration that we had used so far. Using 10-fold less dox, 68% of the cells were induced with 91% of knockdown efficiency (Fig. 3C). Even at 25 ng/ml of dox (200-fold less), a very efficient 65% Notch1 knockdown was observed in the induced cells (Fig. 3C), further demonstrating the high sensitivity and performance of this *miR30*-E inducible vector. In summary, *miR30*-EshRNA proved to be highly efficient, allowing improvements of up to 20% in knockdown levels generated by *miR30*shRNAs.

Importantly, we wanted to test whether this all-in-one inducible lentiviral system could also be efficient in silencing Notch1 expression *in vivo*. We used OCI-AML3 cells that are known to be able to engraft in immunodeficient NSG mice. Mice were first transplanted either with OCI-AML3/Esh*Luc-UBC* or OCI-AML3/Esh*NOTCH1-2-UBC* cells and at day 12 post-transplant dox was administered for 4 consecutive days in all groups. Analysis of the bone marrow engrafted (mCherry<sup>+</sup>) cells revealed ~ 70% inducibility (Figs. 3D). Importantly, within the induced cells a significant 90% of Notch1 knockdown was achieved with the Esh*NOTCH1-2-UBC* vector compared to the Esh*Luc-UBC* vector (Figs. 3E), thus demonstrating the *in vivo* gene silencing potential of this newly developed lentiviral system.

#### Optimizing the Tet-on lentiviral vectors for efficient inducible gene overexpression

In light of the exceptional performance of this inducible system, we designed new lentiviral vectors that allow inducible expression of a transgene of interest. The inducible portion of these overexpression (OE) vectors retains the *P<sub>TRE3G</sub>* promoter, only now followed by a multiple cloning site (MCS) linked to the *Gfp* reporter by a sequence encoding the self-cleaving peptide P2A. The constitutive expression of the transactivator *rtTA3* and the *mCherry* reporter are still driven by either the *PGK* or the *UBC* promoter (**Figure S4A**).

To test the efficacy of these inducible OE vectors we sought to clone a relatively large gene that also allows efficient detection in cells. Thus, we cloned firefly luciferase (*Luc*) into both as testing vectors and having constructs only with the *Gfp* sequence (without the MCS and P2A) as control. Similarly to what we previously described, these lentiviral vectors were first validated in K562 cells. Sorted mCherry<sup>+</sup> cells maintained in culture for 18 days in the presence of dox remained close to 100% (Figs. 4A and 4D). The vectors demonstrated excellent inducibility with virtually 100% of GFP<sup>+</sup> cells (Figs. 4B and 4E), and no GFP leakiness in the absence of dox (data not shown). Functional analysis was performed using a luminescence assay for luciferase quantification. Luciferase activity reached more than  $2 \times 10^6$  relative light units (RLUs) in lysates derived from dox-treated K562 cells, whereas lysates from untreated cells showed background levels of luminescence (Figs. 4C and 4F). This clearly underscored the inducibility capacity of both lentiviral vectors upon addition of dox, with averages around 8800-fold induction in cells transduced with *Luc-Gfp-PGK* and 4300-fold in cells transduced with *Luc-Gfp-UBC*. Subsequently, we validated this OE system in OCI-AML3 cells. Cells transduced with *Gfp-PGK/Luc-Gfp-PGK* or with *Gfp-*

*UBC/Luc-Gfp-UBC* maintained mCherry expression at ~ 100% after 18 days of culture (**Figures S4B, 4G and 4J**). Cells transduced with the *Luc-Gfp-PGK* vector had lower inducibility (~ 80% of GFP<sup>+</sup> cells) compared to cells transduced with the *Luc-Gfp-UBC* vector that resulted in ~ 90% of GFP<sup>+</sup> cells (Figs. 4H and 4K). Importantly, no GFP leakiness was observed in the absence of dox in all conditions. Consistent with the lower GFP levels observed in OCI-AML3 compared to K562 cells, luciferase activity in OCI-AML3 cells (Figs. 4I and L) reached more than 10<sup>6</sup> RLUs. Although lower compared to K562, induction of luciferase expression was 880-fold and 960-fold in cells transduced with *Luc-Gfp-PGK* and *Luc-Gfp-UBC*, respectively. Altogether, these results indicate that both vectors integrated stably in the genome of transduced cells and demonstrated strong inducibility and negligible leakiness with concomitant potent transgene expression induction.

## Efficient *in vivo* gene expression by inducible lentivirus vectors

As a proof of principle, we sought to test whether this newly developed all-in-one inducible lentiviral system could also be efficient in overexpressing a gene of interest *in vivo*. We used OCI-AML3 cells transduced with *Gfp-Luc-UBC* lentivirus and transplanted mCherry<sup>+</sup> cells into NSG mice. Taking advantage of the luciferase system we tested the efficiency of switching on and off luciferase expression by determining its activity using *in vivo* bioluminescence imaging (BLI; Fig. 5A). Background activity was determined (day 0; 9 days post-transplant) before animal treatment with vehicle (-dox; control) or dox for 3 days (Figs. 5A and B). Animals treated with dox demonstrated a ~ 37-fold luciferase gene induction compared to vehicle-treated mice (Fig. 5B). Subsequent withdrawal of dox in these animals significantly reduced the bioluminescence signal, and luciferase gene induction compared to control animals dropped to ~ 6-fold difference (Fig. 5B). These results showed that *Luc* expression could be switched off within 3 days of dox removal. We then re-treated the respective animal cohorts either with vehicle or dox for an additional 3 times. A ~ 214-fold induction of bioluminescence signal was observed at day10, which likely correlates with leukemic cell expansion *in vivo*. Despite modest levels of engraftment (~ 22%) in the bone marrow by day 10 (Fig. 5C, high bioluminescence signals were observed (Figs. 5A and 5B), indicating that this inducible lentivirus vector was also very efficient in overexpressing luciferase *in vivo* upon dox treatment. Importantly, the bioluminescence signals correlated well with the percentage of engraftment and GFP expression levels (Fig. 5D).

## Discussion

RNAi-based systems for gene knockdown have revolutionised gene function studies and genetic screens in mammalian systems. Unlike conventional gene deletion, RNAi-based loss of function requires strong and stable shRNA expression throughout time. While early versions of RNAi systems performed poorly, with low knockdown efficiencies and substantial non-specific effects due to inefficient shRNA processing [2, 3], a better understanding of the *miRNA* processing machinery and new rules for shRNA design have led to reliable RNAi technology in the last decade [23, 25, 26]. Yet, shRNA expression vectors

are constantly improving to overcome technical challenges and broaden their applications, particularly for conditional systems [13, 14, 27, 22].

In this work, we aimed to develop an all-in-one inducible *miR30shRNA* expression system with dual colour for reliable efficiency monitoring. These lentiviral vectors comprise updated elements that allow tight regulation and, at the same time strong *miR30shRNA* expression. Firstly, these new vectors feature an upgraded inducible promoter containing an optimized TRE and modified minimal promoter ( $P_{TRE3G}$ ) shown to be virtually silent in the absence of induction [19]. Indeed, the new Tet-regulated promoter contains mutations that reduce background expression by 5–20 fold compared to the previous version (TRE2 or Ptight) used in the pINDUCER and TRMPVIR systems respectively [13, 14] thus, our new vectors do stand out over the others. Secondly, by placing the *miR30shRNA* sequence inside an *EF1a* intron upstream of the reporter gene, the pri-*miR30shRNA* can be spliced out from the transcript and follow the *miR30* processing pathways, stabilising this event and the translation of the reporter as previously demonstrated [23]. Thus, the developed inducible system allows a more stable *miR30shRNA* expression associated with tight and efficient inducibility that can be reliably monitored through GFP expression. Moreover, we confirmed that the newly optimized *miR30-E* backbone [23] was able to significantly improve the potency of *miR30shRNAs* up to ~ 20%. Indeed, by integrating the *miR30-EshRNA* into the *EF1a* intron, we achieved high reporter gene expression levels, unlike previous reports [23]. Importantly, we demonstrated that this new system performed extremely well in silencing Notch1 receptor expression *in vivo*.

During the development of the gene silencing vectors, we observed that the construct with constitutive *rtTA3* expression under the *SFFV* promoter showed moderate performance, with a reduction of mCherry expression over time. This instability could be associated with the high levels of rtTA protein derived from the strong *SFFV* promoter (Carmelo, personal observations). The presence of the activating domain of the herpes virus simplex viral protein 16 (VP16) linked to the TetR repressor in the Tet-off or Tet-On systems can be toxic due to the sequestration of transcription factors required for cell growth (squenching) [28]. Therefore, it is paramount to consider the strength of the promoter for the rtTA expression. In line with this, the new Tet-On *miR30shRNA* constructs where the rtTA3 is constitutively expressed by weaker promoters (PGK and UBC) showed great long-term stability.

Taking the opportunity of the very good performance of the above vectors, we also modified them for gain-of-function studies. As previously mentioned, one of the major advantages of this dual colour inducible system, is the ability to select clones with optimal levels of fold-induction, *i.e.*, the highest signal-to-noise ratio upon expression induction. As demonstrated, they offer excellent association of GFP reporter levels with potent knockdowns and overexpression. In addition, the design of these vectors permits easy changes in the cassettes, thus enabling the cloning of a desired reporter. The dual colour system also allows monitoring of which cells have been targeted for knockdown or OE in addition to being transduced, a feature that is difficult to attain with the CRISPR/Cas9 system. Moreover, other available all-in-one inducible vectors have limited use in cells that are difficult to transduce since they are usually associated with low viral titres [22]. These newly developed vectors resulted in unconcentrated

viral titres of  $2-5 \times 10^5$  TU/ml. Importantly, this system allows temporal manipulation of gene expression and gene silencing (e.g. on and off), which is again difficult to achieve with the CRISPR/Cas9 system.

In summary, the developed all-in-one inducible system allowed potent knockdowns of over 90% of large transcripts with the *miR30-EshRNA* both *in vitro* and *in vivo* and up to  $1 \times 10^4$ -fold induced expression with the overexpression system with stable genome integration over time and constant levels of inducibility and negligible leakiness.

## Declarations

## Author Contribution

J.G.C. conceptualized, designed, executed, and interpreted experiments and data and wrote the paper; K.J.S. performed the *in vivo* experiments; F.P.L. helped design and perform experiments; R.W.E.C. provided the animal facility for the experiments; E.K.S. helped design experiments and wrote the paper; F.A.-A. conceptualized, supervised research, helped design, interpreted experiments and data and wrote the paper. All authors helped with the editing and approved the manuscript.

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## Figures

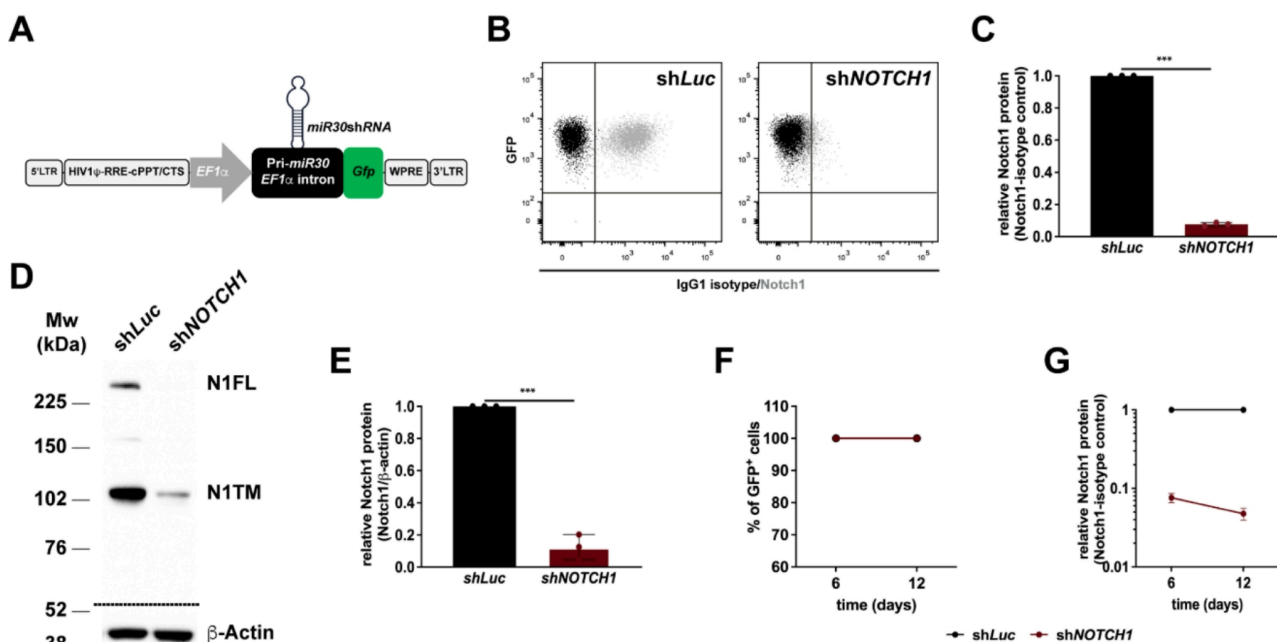


Figure 1

**Functional assessment of the lentiviral vector pLV.EF1a-miR30shRNA-Gfp.** (A) Schematic representation of the developed lentiviral vector for constitutive expression of *miR30*-based shRNAs. (B) Representative FCM dotplots of K562 cells after transduction with lentiviruses carrying shRNA against non-target *Luc* (sh*Luc*) as control or *NOTCH1* (sh*NOTCH1*). Cells were stained either with an anti-Notch1 (grey) or an isotype control (black) antibody. (C) Relative Notch1 receptor levels quantified by FCM as in (B) (n=3). (D) Representative immunoblot with K562 whole cell lysates harvested after transduction with the indicated lentiviral vectors. The membrane was cut (dotted lines) and probed separately with an anti-Notch1 or an anti-b-Actin antibody respectively. (E) Relative Notch1 receptor levels quantified as in (D). Notch1 transmembrane (N1TM) expression was used to quantify after normalising with the expression of b-Actin (n=3). (F) Percentage of transduced (GFP<sup>+</sup>) cells with the indicated lentiviruses throughout the denoted times in culture (n=3). (G) Relative Notch1 levels quantified by FCM on sh*Luc* or sh*Notch1* transduced cells (GFP<sup>+</sup>) throughout the denoted times in culture (n=3). Error bars shown represent S.D. \*\*\*p≤0.0003.

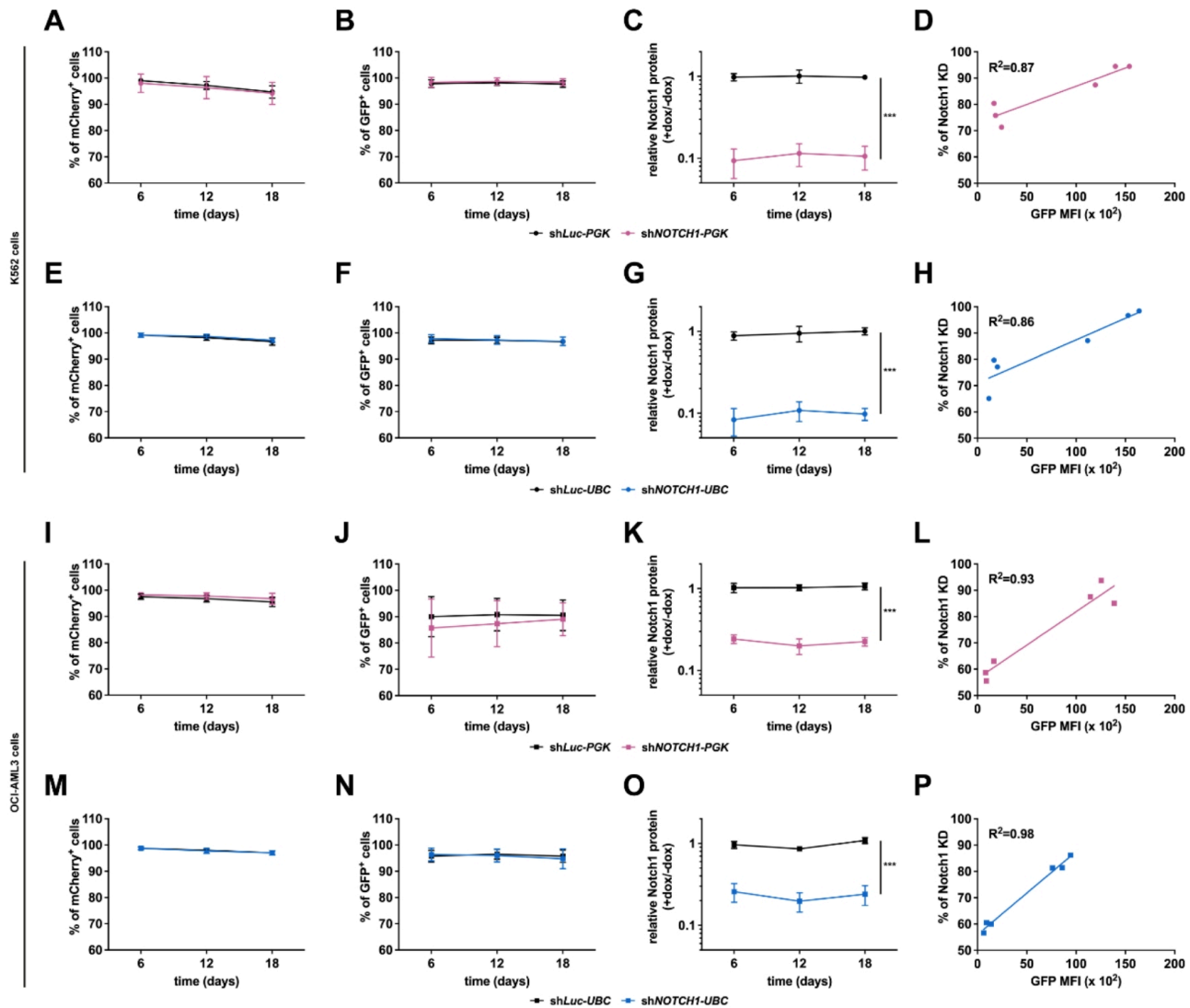


**Figure 2**

### Stability and inducibility evaluation of the *miR30*-based shRNA-PGK and shRNA-UBC lentiviral vectors.

K562 (A-H) and OCI-AML3 (I-P) cells were transduced with the denoted lentiviruses carrying shRNA against non-target *Luc* (shLuc) as control or targeting *NOTCH1* (shNOTCH1). (A, E, I and M) Percentage of transduced (mCherry<sup>+</sup>) cells throughout the indicated times in culture (n=3). (B, F, J and N) Percentage of GFP<sup>+</sup> cells throughout the indicated times in culture in the presence of doxycycline (dox; n=3). (C, G, K and O) Relative Notch1 levels quantified by FCM on transduced cells (mCherry<sup>+</sup>) in the presence of dox relative to the Notch1 expression levels in the absence of dox (n=3). (D, H, L and P) Linear regression analysis between the mean fluorescent intensity (MFI) of GFP expression with the percentage of Notch1 knockdown derived from day 6 post-transduction. This was calculated based on the 25% of the highest and 25% of the lowest GFP expression from each transduced population. Error bars shown represent S.D. \*\*\*p≤0.0003.

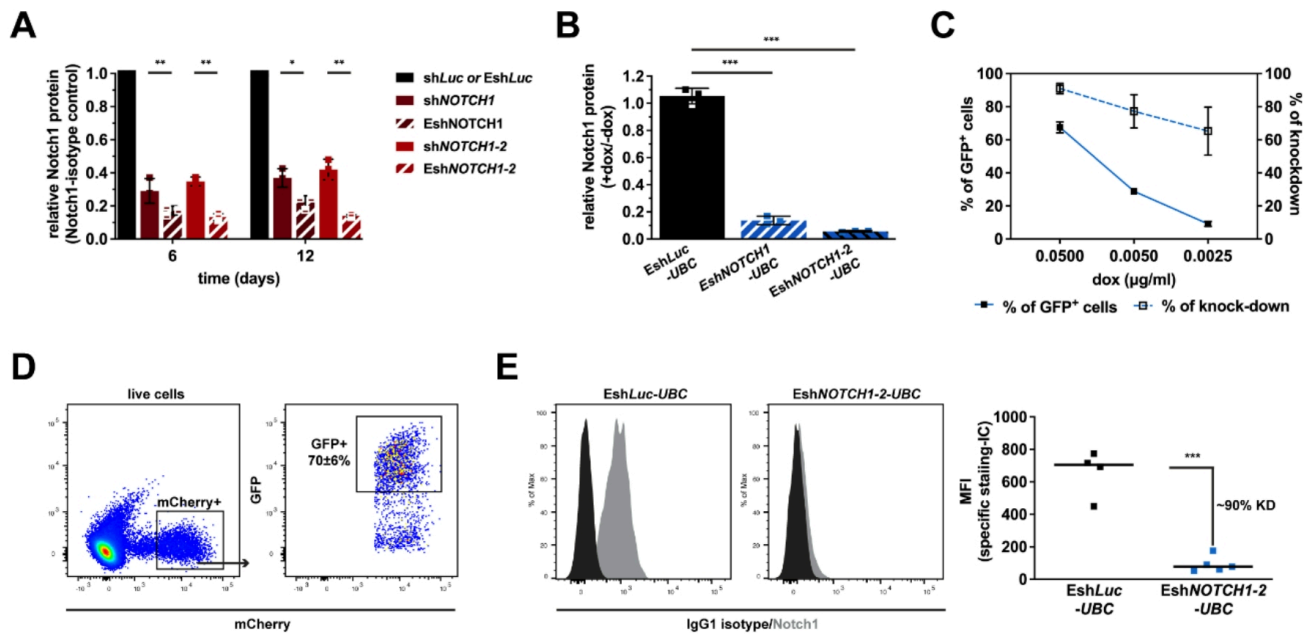


**Figure 3**

***In vitro* and *in vivo* knockdown efficiencies of the different lentiviral vectors carrying *miR30*-based or *miR30-E*-based shRNAs in OCI-AML3 cells.** (A) Relative Notch1 receptor levels quantified by FCM on cells transduced with different lentiviruses (shRNA/EshRNA; n=3). (B) Relative Notch1 receptor levels quantified by FCM on cells transduced with different lentiviruses (EshRNA-UBC) expressing the indicated inducible *miR30-E*-based shRNAs (n=3). (C) Percentage of GFP<sup>+</sup> cells and Notch1 knockdown induced with different dox concentrations in cells transduced with the EshNOTCH1-2-UBC vector. (D) Representative FCM dot plots depicting bone marrow cells engrafted with OCI-AML3/EshLuc-UBC (mCherry+) cells and the percentage of induction (GFP<sup>+</sup>) cells after 4 days of dox treatment. ± shown is the S.D for all mice in both conditions (n=9 mice). (E) Representative FCM histograms of OCI-AML3 cells transduced with the indicated lentiviral vectors showing extracellular Notch1 receptor expression on the cells recovered from transplanted NSG mice. Bone marrow cells were stained either with an anti-Notch1 (grey) or an isotype control (black) antibody and the analysis was performed on mCherry<sup>+</sup> (engrafted) and GFP<sup>+</sup> (induced) cells. The graph represents the data from all mice. Knockdown efficiency was calculated using the mean of the MFI from each group. Error bars shown represent S.D. \*p≤0.03, \*\*p≤0.003 and \*\*\*p≤0.0003.

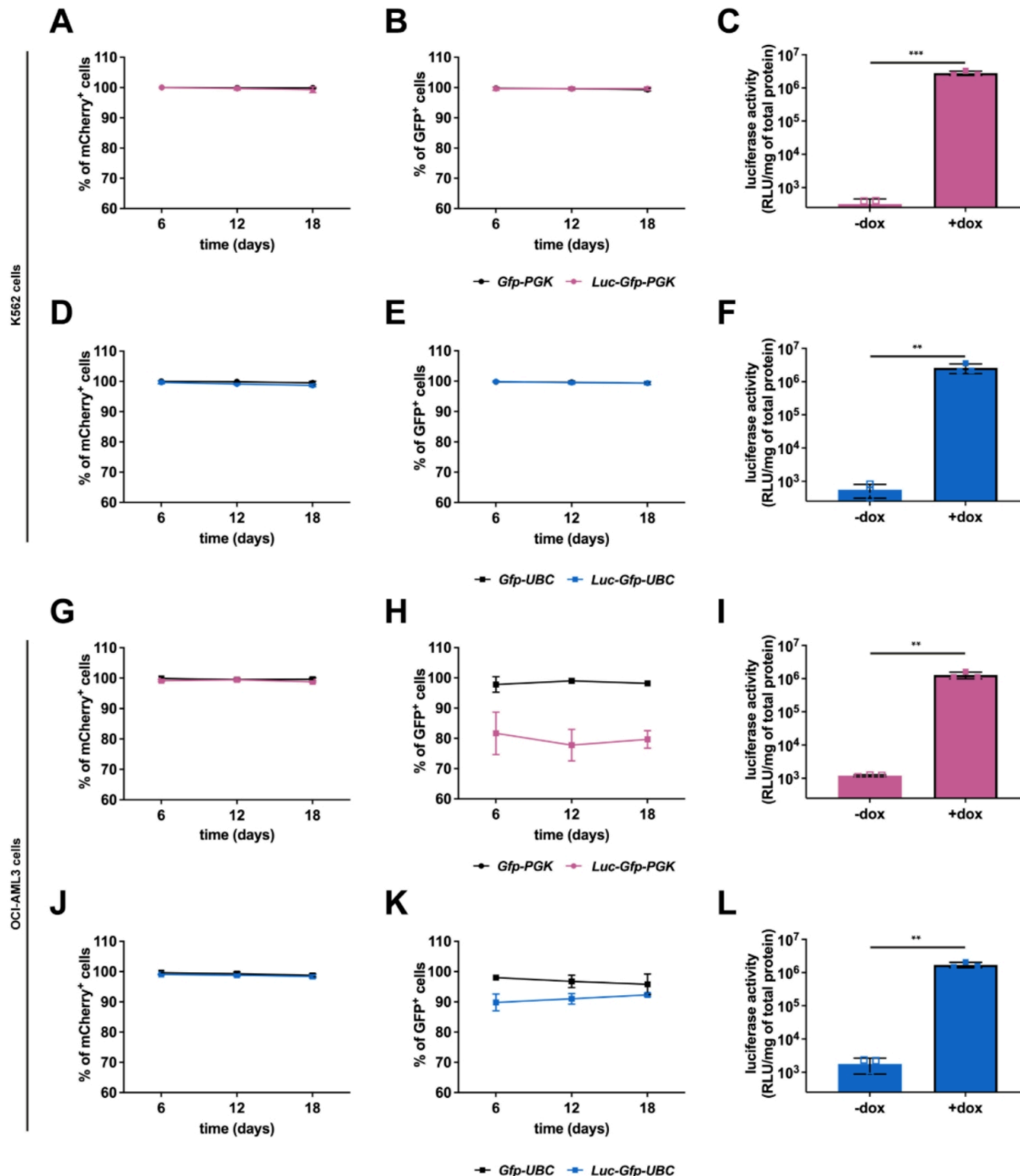


Figure 4

**Evaluation of the stability and inducibility of the pLV.*P<sub>TRE3G</sub>*-Gfp-/Luc-Gfp-PGK and pLV.*P<sub>TRE3G</sub>*-Gfp-/Luc-Gfp-UBC lentiviral vectors.** K562 (A-F) and OCI-AML3 (G-L) cells were transduced with the denoted lentiviruses carrying *Gfp* or *Luc-Gfp*. (A, D, G and J) Percentage of transduced (mCherry<sup>+</sup>) cells throughout the indicated times in culture (n=3-4). (B, D, H and K) Percentage of GFP<sup>+</sup> cells throughout the denoted times in culture in the presence of dox (n=3-4). (C, F, I and L) Quantification of luciferase

activity in cells transduced with the inducible *Luc-Gfp-PGK* (C and I) or *Luc-Gfp-UBC* (F and L) lentiviral vectors cultured in the absence (-dox) or presence (+dox) of doxycycline. Relative luciferase units (RLU) were normalised to total protein (n=3). Error bars shown represent S.D. \*\*p≤0.003 and \*\*\*p≤0.0003.

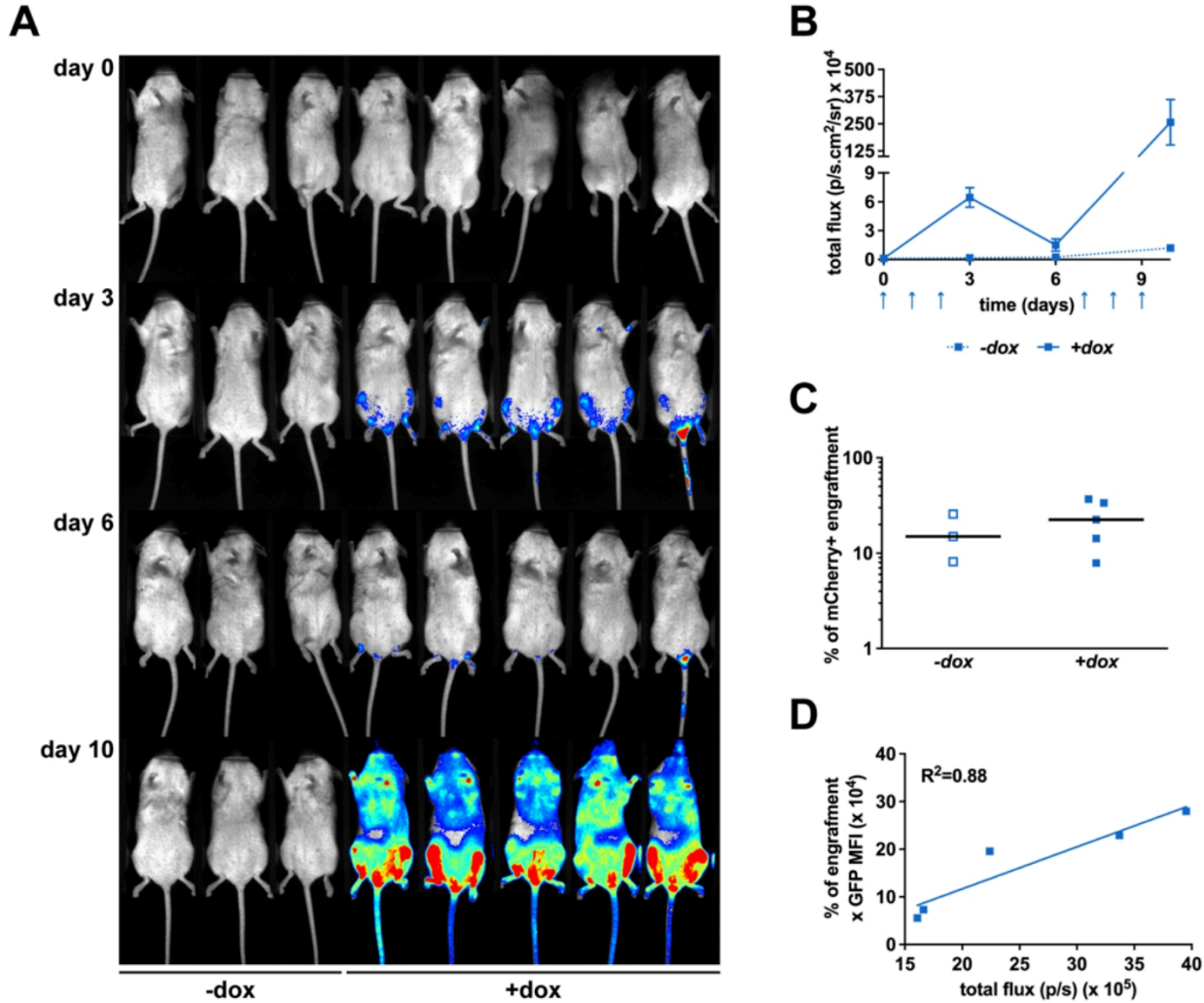


**Figure 5**

**Gene overexpression efficiency of the pLV.P<sub>TRE3G</sub>-Gfp-Luc-UBC lentiviral vector *in vivo*.** (A) BLI of NSG mice over the indicated times started 9 days post-transplant with OCI-AML3 cells transduced with the *Gfp-Luc-UBC* vector. (B) BLI signal quantification as seen in (A). Values were derived from the region of the right legs and arrows indicate the administration of the vehicle or dox (once in the indicated time points). (C) Percentage of engraftment (mCherry<sup>+</sup>) in the marrows of the mice (3 bones from the right legs) at day 10 post-dox administration (day 19 post-transplant). (D) Linear regression analysis between the MFI of GFP expression and the % of engraftment with the BLI quantification derived from the dox-treated mice. Each dot represents a mouse.

## Supplementary Files

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