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Generation of a Gal4-dependent gene recombination and illuminating mouse

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Abstract: Cell labeling technologies, including the Cre/loxP system, are powerful tools in developmental biology. Although the conventional Cre/loxP system has been extensively used to label the expression of specific genes, it is less frequently used for labeling protein-protein interactions owing to technical difficulties. In the present study, we generated a new Gal4-dependent transgenic reporter mouse line that expressed Cre recombinase and a near-infrared fluorescent protein, miRFP670. To examine whether this newly generated transgenic mouse line is applicable in labeling of protein-protein interaction, we used a previously reported transgenic mouse lines that express Notch1 receptor with its intracellular domain replaced with a yeast transcription factor, Gal4. Upon the binding of this artificial Notch1 receptor and endogenous Notch1 ligands, Gal4 would be cleaved from the cell membrane to induce expression of Cre recombinase and miRFP670. Indeed, we observed miRFP670 signal in the mouse embryos (embryonic day 14.5). In addition, we examined whether our Cre recombinase was functional by using another transgenic mouse line that express dsRed after Cre-mediated recombination. We observed dsRed signal in small intestine epithelial cells where Notch1 signal was suggested to be involved in the crypt stem cell maintenance, suggesting that our Cre recombinase was functional. As our newly generated mouse line required only the functioning of Gal4, it could be useful for labeling several types of molecular activities *in vivo*.
Key words: Cre/loxP system, developmental biology, miRFP670, non-invasive *in vivo* imaging, transgenic mouse

Introduction

Cell lineage tracing is an important field of developmental biology. For decades, significant effort has been made to genetically label gene expression using Cre recombinase and fluorescent reporters [1] as these methods are particularly powerful for labeling the expression of transcription factors. In contrast, the application of Cre recombinase-mediated labeling to protein-protein interactions has been difficult. For example, the application of Cre recombinase-mediated labeling to activated receptor proteins may be controversial, as Cre recombinase functions irrespective of ligand binding when it was expressed under the regulation of gene promoters. To explore the physiological function of receptor proteins, new strategies are necessary to label cells receiving sig-

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nals from ligand-bound receptors.

The Delta-Notch signaling pathway is a well-conserved ligand-receptor signaling pathway [2] and involved in many developmental processes, including those of the retina, thymus, lung, liver, pancreas, spleen, small intestine, and kidney [3]. Particularly, this signaling pathway is essential for stem cell maintenance in the small intestine [4]. In mammals, this signaling pathway is composed of five delta ligands and four Notch receptors. Upon binding of these ligands and receptors on opposing cell membranes, the intracellular domain of the Notch receptor (NICD) is cleaved and translocates to the nucleus, where it functions as a transcription factor [5].

This characteristic of receptor cleavage enables the specific labeling of ligand-receptor binding by replacing NICD with the yeast transcription factor Gal4 and herpes simplex virus protein VP16 [6]. In this study, we generated a new transgenic mouse line that expressed Cre recombinase and a near-infrared fluorescent protein, miRFP670 (UAS-Cre-T2A-miRFP670, UC2i), and examined whether this transgenic mouse line is applicable in labeling protein-protein interaction by using Notch1-Gal4VP16 expressing transgenic mouse line (N1-Gal4VP16) [6]. This strategy would enable labeling the past and ongoing Notch1 activity at a cellular level, using another previously established transgenic mouse line (R26GRR) that expressed EGFP and tandem dsRed before and after Cre recombinase-mediated recombination under regulation of the chicken actin gene (CAG) promotor inserted in the *ROSA26* locus [7]. In addition, as we used one of the brightest near-infrared fluorescent proteins (monomeric iRFP670; miRFP670) [8], we also hypothesized that ongoing Notch1 signal (miRFP670 signal) could be observed at the whole-body scale. In the current knowledge, there is no transgenic mouse line that enables Gal4-dependent Cre-mediated recombination or non-invasive *in vivo* imaging. We believe that this newly generated mouse line (UC2i) would be useful in many experimental contexts, including conditional gene recombination and cell labeling experiments. Finally, this new transgenic mouse line will be deposited in the National BioResource Project through RIKEN (RBRC11716 Crl:CD1(ICR)-Tg(UAS-cre/T2A/miRFP670)216Staka).

Materials and Methods

Vector construction

The UAS-Cre-2A-miRFP670 plasmid was created from the pUAS-Cre (Addgene plasmid #50797) and pSpCas9(BB)-2A-miRFP670 (Addgene plasmid #91854) plasmids. pUAS-Cre was a gift from Connie Cepko (Addgene plasmid #50797; http://n2t.net/addgene:50797; RRID: Addgene_50797) [9], and pSpCas9 (BB)-2A-miRFP670 was a gift from Ralf Kuehn (Addgene plasmid #91854; http://n2t.net/addgene:91854 ; RRID: Addgene_91854). pUAS-Cre was digested with FseI (New England Biolabs, Ipswich, MA, USA) and HpaI (Takara, San Jose, CA, USA) in CutSmart buffer (New England Biolabs), followed by bacterial alkaline phosphatase (Takara) treatment and Klenow fragment (Takara) treatment. The T2A-miRFP670 sequence was amplified from pSpCas9(BB)-2A-miRFP670 by PCR (PrimeSTAR GXL DNA Polymerase from Takara, forward primer: 5'-AGAATTCGGCAGTGGAGAGGG-3', 2A_miRFP670_Fw1 and reverse primer: 5'-AAAAGGTACCTCCCCAGCATGCCTGCTATT-3', 2A_miRFP670_Rv1), followed by T4 polynucleotide kinase (New England Biolabs) reaction. The PCR product was inserted into the digested pUAS-Cre vector using Ligation High (Toyobo, Osaka, Japan), and this product was used for the transformation of XL-10 Gold Ultracompetent Cells (Agilent, Santa Clara, CA, USA). Successful insertion was confirmed by PCR (forward primer: 5'-ATACCGGAGATCATGCAAGC-3', UAS_Cre_iRFP_Fw2; reverse primer: 5'-TCACCGCATGTTAGCAGACT-3', UAS_Cre_iRFP_Rv2). After direct sequencing, we removed redundant sequences to adjust the codon frame and introduced the -Gly-Ser-Gly- motif to the N-terminus of the T2A sequence to increase the cleavage efficiency [10] to avoid topological inhibition of Cre activity by conjugated miRFP670 protein, by using the KOD -Plus- Mutagenesis Kit (Toyobo) (forward primer: 5'-GGCAGTGGAGAGGGCAGAGGA-3', UC2i_KODmut_Fw1 and reverse primer: 5'-GGGCCCCAGATCTTCTTCAGAAATAAG-3', UC2i_KODmut_Rv1). This PCR product was digested with DpnI (Toyobo) to remove the PCR template plasmid and then self-ligated with Ligation High (Toyobo). The resultant plasmid was digested with KpnI (Toyobo) and NotI (Toyobo), and a 2.8kb DNA construct, which was gel-extracted using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), was injected into the fertilized eggs. The expected sequence of the UAS-Cre-T2A-miRFP670 plasmid is available in Supplementary Figs. 1 and 2.

In vitro experiments

To examine the Gal4-dependency of Cre and miRFP670 expression prior to injection into fertilized eggs, we performed an *in vitro* experiment using a Gal4 expression vector. The pCL1 plasmid harboring the Gal4-coding sequence was digested with HindIII (Takara).

The pcDNA3 plasmid was digested with HindIII (Takara), followed by treatment with bacterial alkaline phosphatase (Takara). The Gal4-coding sequence was inserted into the HindIII-digested pcDNA3 plasmid using LigationHigh (Toyobo). To visualize successful transfection, IRES-EGFP DNA sequence was excised from pIRES2-EGFP plasmid (Clontech, San Jose, CA, USA) by digestion with EcoRI (Takara) and NotI (Toyobo) and inserted to this pcDNA3_Gal4 plasmid and original pcDNA3 plasmid (negative control). The resulting plasmids (pcDNA3_Gal4_EGFP or pcDNA3_EGFP) were amplified using XL-10 Gold Ultracompetent Cell (Agilent).

293T cells were cultured in DMEM (Gibco, Waltham, MA, USA), supplemented with 10% FBS (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 2mM L-glutamine (Gibco), at 37°C and 5% CO_2. The UAS-Cre-T2A-miRFP670 plasmid (2 μg) was transfected into 293T cells using FuGENE (Promega) with either pcDNA3_Gal4_EGFP plasmid (2 μg) or pcDNA3_EGFP (2 μg), and the cells were photographed using BioRevo BZ-X810 (Keyence, Osaka, Japan) using a GFP or Cy5 filter. For statistical analysis, the numbers of the fluorescent protein positive cells were manually counted, followed by chi-square test (degree of freedom equals five).

Generation of UAS-Cre-T2A-miRFP670 (UC2i) mouse line

Pregnant mare serum gonadotropin (5 units) and human chorionic gonadotropin (5 units) were intraperitoneally injected into female ICR mice (Charles River Laboratories Japan, Yokohama, Japan) at 48 h intervals, and mated with male ICR mice (Charles River Laboratories Japan). We collected zygotes from the oviducts of mated females, and the UAS-Cre-T2A-miRFP670 transgene (liner, 5 ng/μl) were microinjected to the zygotes. Subsequently, the surviving injected zygotes were transferred into the oviducts of pseudopregnant females, and newborns were obtained.

Wild-type ICR mice were purchased from Charles River Laboratories Japan and used for the propagation of mouse lines. F0 female mice were mated with wild-type male mice and F1 male mice were mated with female wild-type mice to exclude the possibility of integration of UAS-Cre-T2A-miRFP670 gene elements onto the sex chromosomes. Mice after the F2 generation were used for subsequent experiments. Integration and germ line transmission of UAS-Cre-T2A-miRFP670 were examined by PCR using tail DNA from F0 and F1 mice, respectively (forward primer: 5'-ATACCGGAGATCATGCAAGC-3', UAS_Cre_iRFP_Fw2 and reverse primer: 5'-TCACCGCATGTTAGCAGACT-3', UAS_Cre_iRFP_Rv2). A summary of our genotyping strategy is provided in Supplementary Data 3.

Animal experiments

N1-Gal4VP16; UC2i embryos (embryonic day 14.5, E14.5) were collected and immediately photographed using an *in vivo* imaging system (IVIS; Perkin Elmer, Waltham, MA, USA) with an excitation wavelength of 640 nm and an emission wavelength of 680 nm, and then subjected to PCR to examine their genotypes. For immunohistochemistry, sagittal formalin-fixed paraffin-embedded sections of E14.5 embryos were incubated with rabbit monoclonal anti-Cre recombinase antibody (AB_2798694, Cell Signaling Technology, Danver, MA, USA), followed by HRP-mediated DAB detection and hematoxylin counterstaining.

Adult N1-Gal4VP16; UC2i; R26GRR mice were euthanized by cervical dislocation and perfused with 4% paraformaldehyde (PFA). For fluorescent imaging, tissues were embedded in OCT compound and counterstained with Hoechst33342 (Molecular Probes, Eugene, OR, USA). Images were acquired using a BioRevo BZ-X810 (Keyence).

Animal welfare

Animal experiments were carried out in accordance with the Regulation for Animal Experiments in our university and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology. Approval was obtained from the Institutional Animal Care and Use Committee and the DNA Experiment Committee of the University of Tsukuba (Approval Numbers for Animal Experiments: 20-325 and 21-184) (Approval Number for DNA Experiments: 200044).

Results and Discussion

Reporter system and DNA construction

Figure 1A provides a summary of the reporter system. This system consists of three transgenic elements: N1-Gal4VP16 [7], UC2i (present study), and R26GRR [8]. Since the Gal4-VP16 transcription factor is cleaved upon *trans*-interaction of endogenous Delta ligands and Notch1-Gal4VP16 protein, this *trans*-interaction would yield both Cre recombinase and miRFP670 protein. To label the cells that had experienced the *trans*-interaction, we used the R26GRR mouse line, where the fluorescent expression is converted from green (EGFP) to red

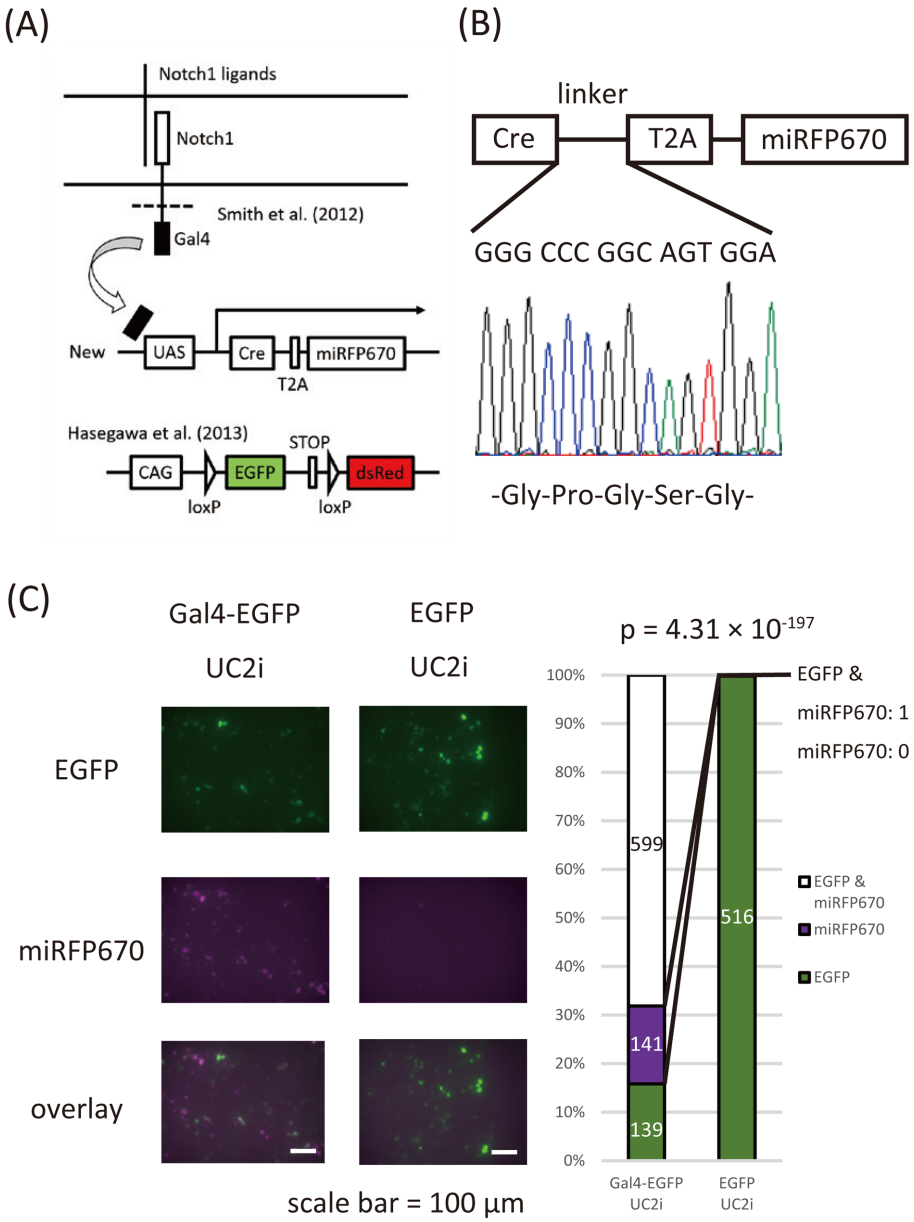

Fig. 1. Generation of UAS-Cre-T2A-miRFP670 (UC2i) mouse line. (A) Schematic representation of the reporter system. Trans-interaction of Delta ligands and Notch1-Gal4VP16 fusion protein induces translocation of Gal4VP16 to the nucleus and expression of Cre recombinase, which converts the signal from EGFP to dsRed, also triggering expression of miRFP670. Therefore, dsRed and miRFP670 signals represent past and ongoing Notch1 activity, respectively. (B) The result of direct DNA sequencing of the linker elements of UAS-Cre-T2A-miRFP670 plasmid. Note that a -Gly-Ser-Gly- coding sequence is present at the N terminus of T2A sequence. (C) *In vitro* examination for Gal4 dependency of the expression of miRFP670. 293T cells were transfected with the indicated expression vectors and photographed with the GFP or Cy5 filter. The cumulative bar graph showed the result of statistics of the transfected cells.

(dsRed) by Cre recombinase action.

Because we used the T2A sequence for the bicistronic expression of Cre recombinase and miRFP670, we examined its linker sequence by direct DNA sequencing of the UAS-Cre-T2A-miRFP670 plasmid (Fig. 1B). We confirmed that the miRFP670 sequence was in-frame following Cre recombinase and that a -Gly-Ser-Gly- coding sequence was inserted at the N-terminus of the T2A sequence. Before injection into fertilized eggs, we examined miRFP670 fluorescence *in vitro* (Fig. 1C). We observed miRFP670 fluorescence with the Gal4 expression vector (P=4.31 × 10^{-197}), suggesting that expression of miRFP670 is dependent on the presence of Gal4. We considered that we observed miRFP670 single positive

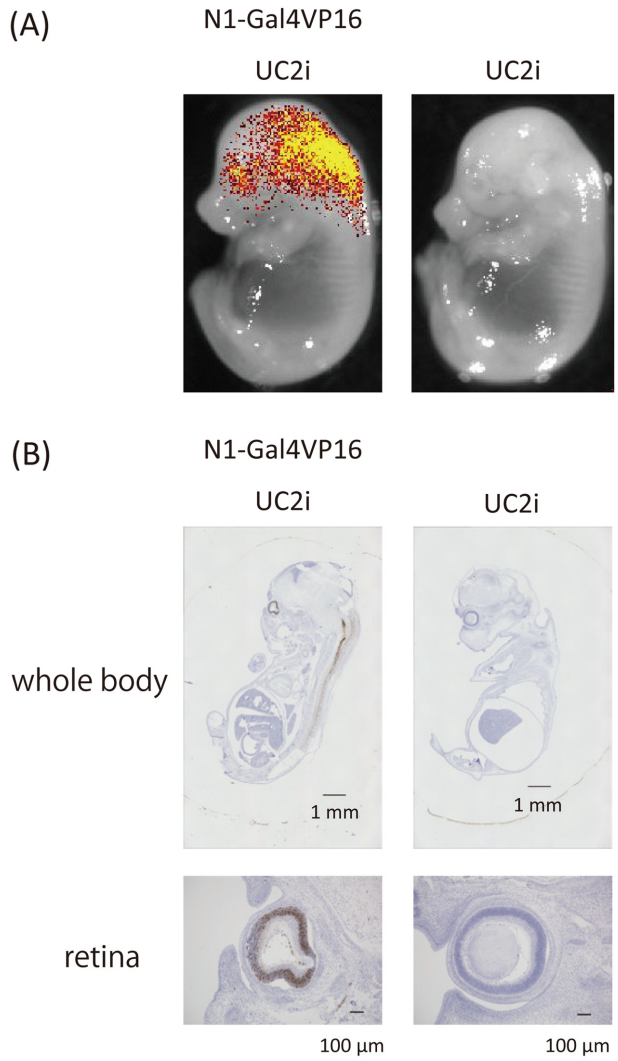

Fig. 2. Whole body imaging of miRFP670 fluorescence imaging signal. (A) The eye and a part of the head region of N1-Gal4VP16; UC2i embryos (E14.5) showed strong miRFP670 signals in the eye, whereas that of UC2i embryos showed no signals. (B) Immunohistochemistry for Cre recombinase. Note that the neural cells of the retina of N1-Gal4VP16; UC2i embryos were positive for Cre recombinase.

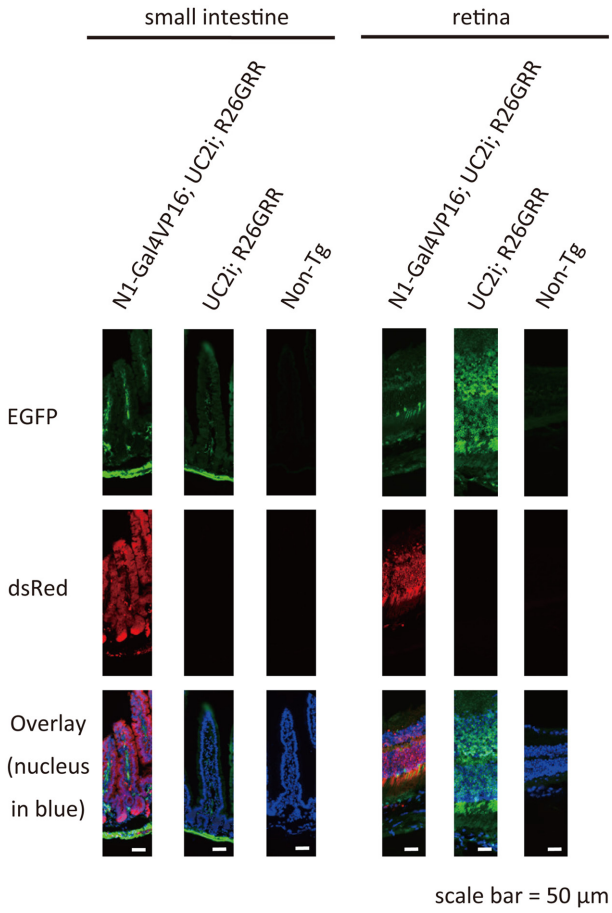

Fig. 3. Green to Red conversion after Gal4-dependent Cre recombination. Fluorescence imaging of adult intestinal epithelial cells (left panels) and retina cells (right panels). Because the cryptic stem cells of the small intestine receive Notch signaling, the intestinal epithelial cells of N1-Gal4VP16; UC2i; R26GRR mice expressed dsRed, which was absent in the UC2i; R26GRR mice and non-transgenic mice (negative controls). In addition, the retina cells of N1-Gal4VP16; UC2i; R26GRR mice expressed dsRed, which was absent in the UC2i; R26GRR mice and non-transgenic mice (negative controls).

cells (141 cells out of 879 cells; 16.0%) because EGFP was under degradation when miRFP670 signal emerged owing to Gal4 action.

In vivo examination of the miRFP670 signal

Next, we examined the miRFP670 signal *in vivo*. We collected E14.5 mouse embryos and observed strong miRFP670 signals in the eye and a part of the head region of N1-Gal4VP16; UC2i embryos, which was lacking in those of UC2i embryos (n=3) (Fig. 2A). To examine this Gal4 dependency at a histological level, we conducted immunohistochemistry for Cre recombinase. The eye and a part of the central nervous system of N1-Gal4VP16; UC2i embryo had a strong DAB signal while the other parts of the body had relatively weak DAB signals (n=3) (Fig. 2B). With a higher magnification, strong DAB signal in the retina cells of N1-Gal4VP16; UC2i embryos was detected while that of UC2i embryos (negative controls) was not detected. This result supports the hypothesis that miRFP670 expression is dependent on the presence of Gal4 *in vivo*.

Examination of Cre recombinase activity *in vivo*

Finally, we examined Cre recombinase activity in our UC2i mouse line using the N1-Gal4VP16 and R26GRR mouse lines. Because Notch signaling was reported to be involved in the maintenance of stem cells in the crypts of the small intestine [4], we expected a dsRed signal in the epithelial cells of the small intestine. A dsRed signal was observed in the differentiated intestinal epithelial

cells of N1-Gal4VP16; UC2i; R26GRR mice (n=3) (Fig. 3 left panels). Importantly, this dsRed signal was absent in the UC2i; R26GRR mouse and non-transgenic mouse (negative controls), supporting the hypothesis of Gal4 dependency of UC2i function *in vivo*. Thus, we concluded that Cre recombinase in the UC2i mouse line is functional and dependent on the presence of Gal4. Unfortunately, the expected miRFP670 fluorescent signal, in addiction to DAB signal in immunohistochemistry for Cre recombinase, in the crypt cells was hard to detect (unpublished observation).

To examine whether our transgenic system could also visualize the cells with past Notch1 signals, we collected eyes from adult mice and showed that the retina cells of N1-Gal4VP16; UC2i; R26GRR were positive for dsRed (n=3) (Fig. 3 right panels), suggesting that this transgenics system could visualize both ongoing and past Notch1 signals at a cellular level.

In summary, we established a new transgenic mouse line (UC2i) that enabled Cre recombination and non-invasive *in vivo* imaging using the miRFP670 reporter. Because of the capability of non-invasive *in vivo* imaging of the ongoing Notch1 signaling by using miRFP670, we consider this transgenic mouse line could be useful in time-lapse experiments. In addition, this reporter mouse line could potentially be combined with many Gal4-expressing mouse lines, although we have only validated its use to label past and ongoing Notch1 signaling. Finally, we will deposit the UC2i mouse line into the National BioResource Project through RIKEN (RBRC11716 Crl:CD1(ICR)-Tg(UAS-cre/T2A/miRFP670)216Staka).

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