Biochemical and Biophysical Research Communications 441 (2013) 777–781

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Retina-specific gene excision by targeted expression of Cre recombinase

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ARTICLE INFO

Article history:
Received 21 October 2013
Available online 5 November 2013

Keywords:
Cre recombinase
Rho
Transgenic mice
Retina

ABSTRACT

The use of Cre recombinase for conditional targeting permits the controlled removal or activation of genes in specific tissues and at specific times of development. The Rho–Cre mice provide an improved tool for studying gene ablation in rod photoreceptor cells. To establish a robust expression of Rho–Cre transgenic mice that would be useful for the study of various protein functions in photoreceptor cells, a total 11,987 kb fragment (pNCHS4 Rho–NLS–cre) containing human rhodopsin promoter was cloned. The Rho–Cre plasmid was digested with EcoR1 and I Ceu-1, and the 9.316 kb fragment containing the hRho promoter and Cre recombinase gel was purified. To generate transgenic mice, the purified DNA fragment was injected into fertilized oocytes according to standard protocols. ROSA26R reported the steady expression of Rho–Cre especially in photoreceptor cells, allowing further excising proteins in rod photoreceptors across the retina. This Rho–Cre transgenic line should thus prove useful as a general deletor line for genetic analysis of diverse aspects of retinopathy.

1. Introduction

Gene-targeting strategies have been used to study varies genes that are important in ocular function and retina disease [1–3]. However, some genes that function in the eye are also critical for normal development and homeostasis of the animal and are therefore not feasible to simple knockout techniques [4,5]. The Cre/LoxP recombination system has become a powerful tool for allowing gene manipulation in vivo [6], especially with the increasing availability of cell and tissue specific Cre transgenic mouse lines [7].

Photoreceptors are the primary transducers of visual stimulation [8,9]. The light responses of rod cells in the vertebrate retina are extremely precise because of the extraordinary regulation of enzymes and protein regulators [10]. The signaling characteristics of the retina pigments modulate the unique physiological functions of rods [11]. Many indispensable proteins have been certified to perform a role in the cells, while their physiological function remained unclear. By genetic manipulation of rod photoreceptors, the typical function of a featured protein can be directed, using rod-specific promoters. To further detect the unknown characteristic of a proper protein, we investigate a new panel of synthetic promoters by combining pNCHS4–CBPA as a vehicle with rhodopsin as the promotor, which contains the Cre series. Thus a more sturdy and differential expression of Cre is established. We use a classical Cre–loxP mutagenesis to test the function of conditional knockout gene specifically in rod cells [12,13].

2. Materials and methods

2.1. Construction of transgenic Rho–Cre mice

For the construction of transgenic mice, Rho promoter was ordered from Addgene No. 13779 (Cambridge, USA). The purified plasmids pRho–Cre was digested with the restriction enzymes Sal I/EcoR I (Takara Biotechnology (Dalian) Co., Ltd.) at 37 °C to isolate the 2301 bp human Rho promoter fragment [14]. A Cre gene was cloned into the Sal I/Not I (Takara Biotechnology (Dalian) Co., Ltd.) sites of pNCHS4–CBPA (Jiangsu Animal Experimental center of Medical and Pharmaceutical Research, Nanjing, China) synchronously and named pNCHS4 Rho–NLS–cre. The Cre gene was excised as an 1110 bp EcoR I/Not I (Takara Biotechnology (Dalian) Co., Ltd.) site of purified plasmids pCAG–Cre–NLS (Jiangsu Animal Experimental center of Medical and Pharmaceutical Research, Nanjing, China). The pNCHS4 Rho–NLS–cre also contains a pair of cHS4 Prime. This total 11.987 kb Cre expression vector excised by EcoR1 and I CEU-1 (New England Biolabs, U.S.A.), respectively was further purified and microinjected into the

Abbreviations: PCR, polymerase chain reaction; SPF, specific-pathogen-free; PFA, paraformaldehyde; PBS, phosphate buffer saline; OCT, opti-mum cutting temperature compound; EGTA, ethyleneglycol bis(2-aminoethylether)tetraacetic acid.

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http://dx.doi.org/10.1016/j.bbrc.2013.10.139

pronuclei of fertilized eggs of C57-B6/SJL mice (Shanghai Laboratory Animal Center, CAS) at Jiangsu Animal Experimental center of Medical and Pharmaceutical Research according to standard protocols, to generate transgenic mice. The resulting transgenic mouse line was named Rho–Cre. To evaluate the cell subtypes which demonstrated Cre recombinase activity in vivo, the F1 homozygote Rho–Cre line was crossed with mice B6.129S4-Gt(ROSA)26$^{Sortm1Sor/J}$ from Jackson laboratory (No. 003474) carrying the Cre-activatable lacZ reporter allele (ROSA26R) [15].

2.2. Animal treatment and generation and PCR of genomic DNA

The use of animals in this study was approved by the Institutional Animal Care and Jiangsu Animal Experimental Center of Medical and Pharmaceutical Research. Mice were housed and bred in the Experimental Animal Facilities at Nanjing Medical University under SPF conditions, and the animal experiments were conducted following the guidelines of Nanjing Medical University and have been approved by Ethics Committee of Nanjing Medical University. The animals were allowed free access to food and water at all times and were maintained on a 12 h light/dark cycle at controlled temperature (20–25 °C) and humidity (50 ± 5%). Founders were screened by PCR to identify the 9 founders carrying the Rho–Cre gene. DNA was prepared from mice tails [16] and PCR reactions were performed using the PCR kit (Promega, U.S.A.) in 20 μL volumes with tail DNA 1 μL, H_2O 12.5 μL, 10× buffer 2 μL, 25 mM $MgCl_2$ 2 μL, 10 mM dNTP 0.4 μL, forward and reverse primers 1 μL each, and rTaq polymerase 0.1 μL (Promega, U.S.A.). PCR diagnostic for the Cre transgene was performed with primers Cre-F (5′-TTGCCTGCATTACCGGTCGATGC-3′) and Cre-r1 (5′-TTGCACGTTCACCGGCATCAACG-3′) according to an established procedure [17], which amplified a 364 bp Cre fragment. PCR condition was as following: 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. PCR detection of the Cre-activatable lacZ reporter gene in ROSA26R mice were performed with primers Fzd9lacZ-F(5′-TAACCGTCAAGAGCATCATCCTC-3′) and Fzd9lacZ-R(5′-CCAGGTAGCGAAAGCCATTTTTTG-3′) to detect a 522 bp product in the lacZ structure gene, using the following condition: 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, according to an established procedure [18]. PCR products were separated in a 1% or 1.5% agarose gel and visualized by staining with ethidium bromide.

2.3. RT-PCR determination of Cre mRNA expression

Total RNA (2 mg) was extracted from various mouse organs using a Promega kit (Promega, U.S.A.). Subsequently, cDNAs were generated from 1 μg of total RNA, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, U.S.A.), and the cDNA was subjected to a 35-cycle PCR amplification. RT-PCR diagnostic for Cre was performed with primers Cre-F (5′-TTGCCTGCATTACCGGTCGATGC-3′) and Cre-r1 (5′-TTGCACGTTCACCGGCATCAACG-3′) as stated above, which amplified a 364 bp Cre fragment. PCR condition was as following: 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Amplified DNA products were separated on a 1.5% agarose gel and visualized by staining the gel with ethidium bromide.

2.4. Localization and functional analysis of inducible Cre expression

To evaluate the cell subtypes which demonstrated Cre recombinase activity in vivo, the homozygote Rho–Cre line was crossed with mice carrying the Rosa26 reporter allele [15]. The β-galactosidase (βgal) activity resulting from Cre-mediated recombination of the ROSA26R location was monitored by X-gal staining (biodee, Beijing, China) [19]. Rho–Cre mice were killed by cardiac perfusion with 4% PFA (wuhan Boster Bio-engineering, Wuhan, China.) in phosphate-buffered saline (PBS). The eyes apart from the body were penetrated in 4% PFA in PBS overnight at 4 °C and the anterior segment were removed, 30% sucrose overnight for dehydration. Eye cups sank to the bottom, frozen in OCT freezing medium were sliced at 7 μm. Then the specimens were treated with raising buffer three times for 10 min, including 5 mM EGTA, 0.01% Deoxycholate, 0.02% NP40, and 2 mM $MgCl_2$ in PBS. The processed samples were staned overnight at 37 °C in 1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$ in PBS. Stained samples were rinsed in PBS and postfixed at 4 °C in 4% PFA in PBS buffer for over 2 h, then attentively washed by PBS and preserved in glycerine. Retinas were examined and photographed with microscope (BX41; Olympus, Tokyo, Japan) equipped with color digital cameras.

3. Results

3.1. Generation of inducible rod-specific Cre transgenic mice

Gene recombination technology has been successfully employed to analysis gene function in different differential tissues and cells [20,21]. We generated rod-specific Cre transgenic mice to further explore the specific effect of gene knockout in rod photoreceptors. The pNCHS4 Rho–NLS–cre were prosperously constructed (Fig. 1A) and reappraised by EcoR1 (reveal the 4524/7463 bp segments, Fig. 1B) or I CEU-1 (reveal the 9316/2671 bp segments, Fig. 1C) digestion. According to standard protocols, this Cre expression vector was microinjected into the pronuclei of fertilized eggs of C57-B6/SJL mice to generate a single chromosome, which co-integrated of both transgenes. The generated mice behaved normally. PCR analysis using primers Cre-F/Cre-r1 (amplifying a 364 bp Cre fragment) obtained the nine funders of Rho–Cre line, which were candidates that implied to cross with ROSA26R mice. The upshot F1 Rho–Cre mice carried Cre-activatable lacZ reporter gene in funder ROSA26R mice were performed with primers Fzd9lacZ-F and Fzd9lacZ-R, amplifying a 522 bp LacZ fragment (Fig. 2). No retina disability found. In the sequel, mice can be legal for further characterize.

To confirm that Cre expression was specially restricted to retina, RNA was prepared from various organs, reverse transcription-mediated PCR for detection of Cre showed retina-specific expression (Fig. 3). No other tissues reflect the expression, along with normal controlled mice and negative control (pure water).

3.2. Cell subtypes exhibited Cre recombinase activity

To further test the function and specificity of Rho–Cre line in vivo, we crossed founders with ROSA26R mice. ROSA26R progeny showed strong, diffuse lacZ expression while in most other strains of mice, rod cells, along with all other retina tissues and organizations including the live, heart, lung, kidney, uterus, were negative. X-gal staining of retina tissues from Cre-mediated recombination of the ROSA26R progeny was observed with rod photoreceptors (Fig. 4). The cell types with the β-gal activity showed positive signals corresponded to those in which the Rho promoter is active, indicating that β-gal expression was dependent on the presence of the Cre recombination [22].

4. Discussion

It is thorough known that gene is important for vital activity raging from survival, apoptosis and cell death. Gene function has been studied in various physiologic and pathological patterns [23]. The development of conditional knockout strategies has enabled the study of objective genes. The use of Cre and other recombinases for conditional targeting permits the controlled removal or

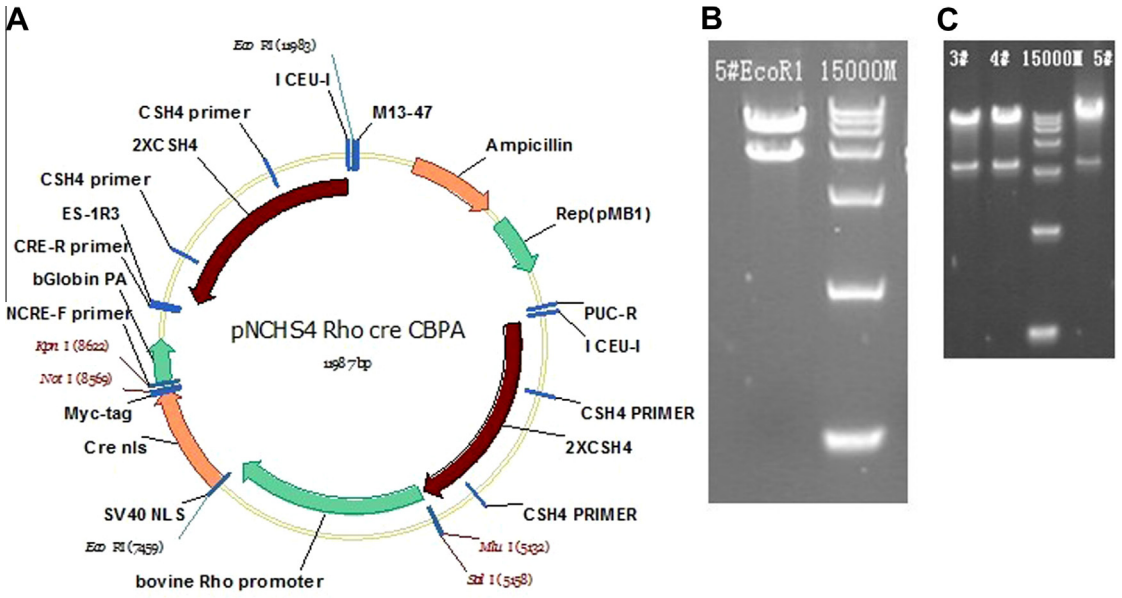

Fig. 1. The pNCHS4 Rho–NLS–cre CBPA. (A) Construction of pNCHS4 Rho–NLS–cre CBPA: pRho–Cre, pCAG–Cre–NLS and pNCHS4–CBPA were cut by proper restriction enzymes (Sal1/EcoR1/Not1), respectively and 3 required pieces were concatenated; (B), and (C) Total 11.987 kbp Cre expression vector excised by EcoR1 and I CEU-1, respectively for further purify. The 4524/7463 bp segments were digested by EcoR1 and the 9316/2671 bp segments were digested by I CEU-1.

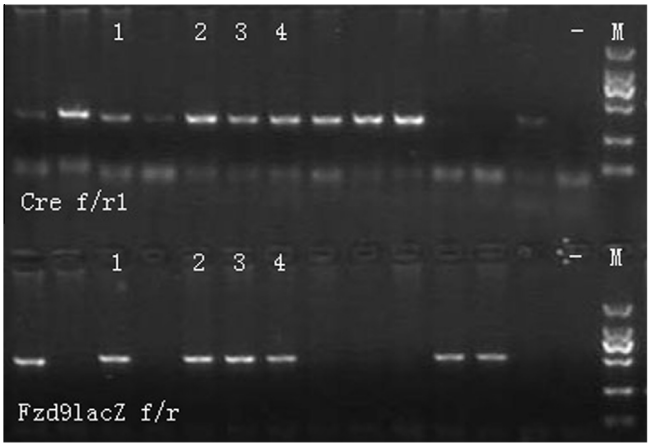

Fig. 2. F1 Rho–Cre mice tails carried Cre-activatable lacZ reporter gene. PCR analysis: primers Cre-F/Cre-r1 amplifying a 364 bp Cre fragment; Fzd9lacZ-F and Fzd9lacZ-R, amplifying a 522 bp LacZ fragment. 1–4: positive; –: negative; M: Marker(2000 bp).

activation of genes in specific tissues and at specific times of development [12,24,25]. In the present study, we used Cre–LoxP methods to construct gene deletion, which requires two different genetically engineered mouse strains: one containing a cell-type specific promoter with Cre recombinase, the other containing a floxed gene, which flanked by LoxP sequences that are Cre recognition sites [26–28]. The combination of these two mouse strains propagated a new mouse strain with gene deletion in a cell-specific tissue. Marszalek JR [29] used IRBP as a rod-specific promoter to create IRBP–Cre mice strain, although his study demonstrated a requirement for kinesin-2 in photoreceptor cell protein transport and viability, gene excision was incomplete and asynchronous across each retina, and its extent varied among different animals, thus limiting the usefulness of this approach. Jimeno D [30] used Cre–loxP mutagenesis to test for motor transport by kinesin-2 in photoreceptor cells. However, our objective of this study is to establish a robust expression of Rho–Cre transgenic mice. Transgenic mice that specifically express Cre in retina were obtained, and a cross with these mice induced rod cell-specific DNA recombination in mice, carrying a reporter for loxP-dependent DNA excision. The occurrence of Cre/loxP-mediated recombination was unique in retina tissues by means of X-gal staining. The variability between transgenic lines has been obviously reduced by flanking a tyrosinase minigene with tandem copies of the chicken β-globin 5HS4 insulator, the most characterized vertebrate element, which also reduced the number of transgenic founders required here [31]. Insulators are DNA sequences that can function as directional blocking elements either by interfering with promoter enhancer interactions when positioned in the intervening sequence or by reducing position effects imparted on transgenes when flanking the integrated transcription units[32–35]. By using site-specific recombinases, a recombination plasmid pNCHS4 Rho–NLS–cre were generated and specific Cre activity were observed. Transgenic mice that performed Cre in rod photoreceptors were obtained. The result shows a practicable process to create recombinase trans-genes expressing at high levels in rod photore-

Fig. 3. Reverse transcription-mediated PCR for detection of Cre mRNA: H, heart; Li, liver; S, spleen; Lu, lung; K, kidney; B, brain; T, testis; R, retina; C, choroid; Le, lens; M, Marker(2000 bp). Retina shows 364 bp segment, no other tissues reflect the expression. Control mice and water alone (–) were negative.

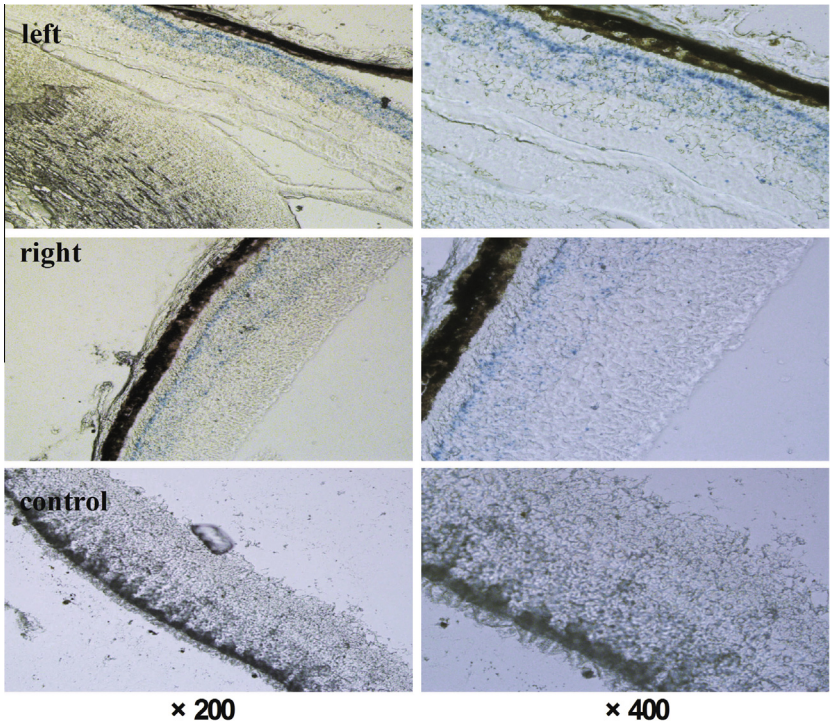

Fig. 4. Distribution of β-galactosidase in the retina of Rho–Cre mice. Retina sections from Rho–Cre mice and control mice were histochemically analyzed for the presence of β-galactosidase by staining with X-gal.

ceptors, which would be useful for the study of various protein functions specific works in photoreceptor cells.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (NFSC, Nos. 81170855, 81100413) and the National Basic Research Program of China (973 Program, No. 2011CB510200).

References

[1] H. Cao, R.S. Molday, J. Hu, Gene therapy: light is finally in the tunnel, Protein Cell 2 (2011) 973–989.
[2] J.J. Alexander, W.W. Hauswirth, Prospects for retinal cone-targeted gene therapy, Drug News Perspect. 21 (2008) 267–271.
[3] T.S. Rex, Rescue of sight by gene therapy – closer than it may appear, Ophthalmic Genet. 28 (2007) 127–133.
[4] A. Nagy, L. Mar, G. Watts, Creation and use of a cre recombinase transgenic database, Methods Mol. Biol. 530 (2009) 365–378.
[5] T.L. Saunders, Inducible transgenic mouse models, Methods Mol. Biol. 693 (2011) 103–115.
[6] K. Akagi, V. Sandig, M. Vooijs, M. Van der Valk, M. Giovannini, M. Strauss, A. Berns, Cre-mediated somatic site-specific recombination in mice, Nucleic Acids Res. 25 (1997) 1766–1773.
[7] E. Bockamp, R. Sprengel, L. Eshkind, T. Lehmann, J.M. Braun, F. Emmrich, J.G. Hengstler, Conditional transgenic mouse models: from the basics to genome-wide sets of knockouts and current studies of tissue regeneration, Regen. Med. 3 (2008) 217–235.
[8] D. Dell'Orco, A physiological role for the supramolecular organization of rhodopsin and transducin in rod photoreceptors, FEBS Lett. 587 (2013) 2060–2066.
[9] A. Wand, I. Gdor, J. Zhu, M. Sheves, S. Ruhman, Shedding new light on retinal protein photochemistry, Annu. Rev. Phys. Chem. 64 (2013) 437–458.
[10] J.I. Korenbrot, Speed, sensitivity, and stability of the light response in rod and cone photoreceptors: facts and models, Prog. Retin. Eye Res. 31 (2012) 442–466.
[11] V.J. Kefalov, Rod and cone visual pigments and phototransduction through pharmacological, genetic, and physiological approaches, J. Biol. Chem. 287 (2012) 1635–1641.
[12] A. Nagy, Cre recombinase: the universal reagent for genome tailoring, Genesis 26 (2000) 99–109.
[13] C.H. Kos, Cre/loxP system for generating tissue-specific knockout mouse models, Nutr. Rev. 62 (2004) 243–246.
[14] T. Matsuda, C.L. Cepko, Controlled expression of transgenes introduced by in vivo electroporation, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 1027–1032.
[15] P. Soriano, Generalized lacZ expression with the ROSA26 Cre reporter strain, Nat. Genet. 21 (1999) 70–71.
[16] J. Attal, M. Cajero-Juarez, L.M. Houdebine, A simple method of DNA extraction from whole tissues and blood using glass powder for detection of transgenic animals by PCR, Transgenic Res. 4 (1995) 149–150.
[17] Y. Le, B. Sauer, Conditional gene knockout using cre recombinase, Methods Mol. Biol. 136 (2000) 477–485.
[18] Y. Le, S. Gagneten, T. Larson, E. Santha, A. Dobi, D. v Agoston, B. Sauer, Far-upstream elements are dispensable for tissue-specific proenkephalin expression using a Cre-mediated knock-in strategy, J. Neurochem. 84 (2003) 689–697.
[19] O. Igoucheva, V. Alexeev, K. Yoon, Targeted gene correction by small single-stranded oligonucleotides in mammalian cells, Gene Ther. 8 (2001) 391–399.
[20] M. Hellstrom, A.R. Harvey, Retinal ganglion cell gene therapy and visual system repair, Curr. Gene Ther. 11 (2011) 116–131.
[21] A.R. Harvey, M. Hellstrom, J. Rodger, Gene therapy and transplantation in the retinofugal pathway, Prog. Brain Res. 175 (2009) 151–161.
[22] X. Wang, Cre transgenic mouse lines, Methods Mol. Biol. 561 (2009) 265–273.
[23] J. Hosch, B. Lorenz, K. Stieger, RPGR: role in the photoreceptor cilium, human retinal disease, and gene therapy, Ophthalmic Genet. 32 (2011) 1–11.
[24] R. Hoess, K. Abremski, N. Sternberg, The nature of the interaction of the P1 recombinase Cre with the recombining site loxP, Cold Spring Harbor Symp. Quant. Biol. 49 (1984) 761–768.
[25] H. Bouabe, K. Okkenhaug, Gene targeting in mice: a review, Methods Mol. Biol. 1064 (2013) 315–336.
[26] R. Oh-McGinnis, M.J. Jones, L. Lefebvre, Applications of the site-specific recombinase Cre to the study of genomic imprinting, Brief. Funct. Genomics 9 (2010) 281–293.
[27] P. Argos, A. Landy, K. Abremski, J.B. Egan, E. Haggard-Ljungquist, R.H. Hoess, M.L. Kahn, B. Kalionis, S.V. Narayana, L.S. Pierson 3rd, et al., The integrase family of site-specific recombinases: regional similarities and global diversity, EMBO J. 5 (1986) 433–440.
[28] J. Gao, X. Wu, J. Zuo, Targeting hearing genes in mice, Brain Res. Mol. Brain Res. 132 (2004) 192–207.
[29] J.R. Marszalek, X. Liu, E.A. Roberts, D. Chui, J.D. Marth, D.S. Williams, L.S. Goldstein, Genetic evidence for selective transport of opsin and arrestin by kinesin-II in mammalian photoreceptors, Cell 102 (2000) 175–187.
[30] D. Jimeno, L. Feiner, C. Lillo, K. Teofilo, L.S. Goldstein, E.A. Pierce, D.S. Williams, Analysis of kinesin-2 function in photoreceptor cells using synchronous Cre–loxP knockout of Kif3a with RHO–Cre, Invest. Ophthalmol. Vis. Sci. 47 (2006) 5039–5046.

[31] W. Potts, D. Tucker, H. Wood, C. Martin, Chicken beta-globin 5′HS4 insulators function to reduce variability in transgenic founder mice, Biochem. Biophys. Res. Commun. 273 (2000) 1015–1018.
[32] A.C. Bell, G. Felsenfeld, Stopped at the border: boundaries and insulators, Curr. Opin. Genet. Dev. 9 (1999) 191–198.
[33] J.H. Chung, A.C. Bell, G. Felsenfeld, Characterization of the chicken beta-globin insulator, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 575–580.
[34] T.I. Gerasimova, V.G. Corces, Boundary and insulator elements in chromosomes, Curr. Opin. Genet. Dev. 6 (1996) 185–192.
[35] S. Rivella, J.A. Callegari, C. May, C.W. Tan, M. Sadelain, The cHS4 insulator increases the probability of retroviral expression at random chromosomal integration sites, J. Virol. 74 (2000) 4679–4687.