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CRISPR-mediated Labeling of Cells in Chick Embryos Based on Selectively Expressed Genes

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[Abstract] The abilities to mark and manipulate specific cell types are essential for an increasing number of functional, structural, molecular, and developmental analyses in model organisms. In a few species, this can be accomplished by germline transgenesis; in other species, other methods are needed to selectively label somatic cells based on the genes that they express. Here, we describe a method for CRISPR-based somatic integration of reporters or Cre recombinase into specific genes in the chick genome, followed by visualization of cells in the retina and midbrain. Loci are chosen based on an RNA-seq-based cell atlas. Reporters can be soluble to visualize the morphology of individual cells or appended to the encoded protein to assess subcellular localization. We call the method eCHIKIN for **e**lectroporation- and **C**RISPR-mediated **H**omology-instructed **K**nock-**IN**.

Keywords: Chick embryo, Cre, CRISPR, GFP, Homologous recombination, Electroporation

[Background] Many functional, structural, molecular, and developmental analyses in model organisms now rely on the abilities to mark and manipulate specific cell types. In a limited number of species – *Mus musculus* (mice), *Danio rerio* (zebrafish), *C. elegans* (worms), and *Drosophila melanogaster* (flies) – this can be accomplished by germline transgenesis. For example, transgenic animals can be generated in which regulatory elements from a gene selectively expressed by a cell type of interest drive the expression of a reporter.

Alternatively, the transgene can encode an effector such as Cre recombinase, which enables selective expression of a second transgene provided either in the germline or to somatic cells via a viral or other vector (Luo *et al*., 2018).

For other model organisms in which germline transgenesis is currently infeasible or limited, few methods are available for the selective labeling of somatic cells. One option is to incorporate cell type-specific promoters into viral vectors, but to date, this approach has generally resulted in labeling groups of related cell types (discussed in Domenger and Grimm, 2019; Juttner *et al*., 2019). Genome editing using CRISPR (clustered regularly interspaced short palindromic repeats) (Jinek *et al*., 2012; Cong *et al*., 2013; reviewed in Pickar-Oliver and Gersbach, 2019; Nishizono *et al*., 2020) provides an alternative approach. The CRISPR-associated endonuclease Cas9 is delivered to specific sites in the genome by a single guide RNA (sgRNAs) to generate double-strand breaks. The breaks can be repaired by non-homologous end-joining (NHEJ) or homology-directed repair (HDR) (Sander and Joung, 2014; Yeh *et al*., 2019). If cDNAs encoding reporters are flanked by the sequences flanking the cut site, HDR can be harnessed to insert them into the genome at defined sites. Because homologous recombination

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activity is largely confined to mitotically active cells, methods involving HDR are generally applied to dividing cells (Heidenreich and Zhang, 2016).

The HDR method has primarily been used to generate germline “knock-ins” (Hsu *et al*., 2014; Nishizono *et al*., 2020) but can also be used to label somatic cells. By transducing dividing cells in the mouse brain with CRISPR-Cas9-based HDR tools using *in utero* electroporation, Mikuni *et al*. (2016) developed a method called SLENDR (single-cell labeling of endogenous proteins by CRISPR-Cas9-mediated homology-directed repair). This method introduces a plasmid encoding an sgRNA and Cas9, along with a single-stranded DNA (ssDNA) encoding a protein tag embedded between sequences that flank the sgRNA recognition site; this results in the HDR-mediated introduction of the tag at the desired genomic location. Related methods have been reported by Uemura *et al*. (2016), Nishiyama *et al*. (2017), and Matsuda and Oinuma (2019); Suzuki *et al*. (2016) devised a method in which homology-independent target integration by NHEJ allows tagging in post-mitotic cells.

Here, we modified these methods for use in chick (*Gallus gallus*) embryos. Chicks have been used for embryological studies for over a century and remain a well-used model (Stern, 2005). However, while germline transgenesis has been demonstrated in chicks, it remains a difficult and rarely used method (Lee *et al*., 2017). Moreover, even though the method is likely to improve, few laboratories are equipped with the facilities for the avian husbandry that would be needed to generate or maintain transgenic lines.

Our own studies on the development of the chick retinotectal system have used viral and electroporation methods for gene transfer into chick embryos (*e.g.*, Gray *et al*., 1988; Galileo *et al*., 1992; Leber *et al*., 1996; Yamagata *et al*., 2002; Yamagata and Sanes, 2008a). However, none of these enabled reliable labeling of specific cell types because neither cell type-specific promoters nor inventories of selectively expressed genes were available. Recently, we addressed the second of these problems by generating a cell atlas of the chick retina using high-throughput single-cell RNA-seq, which provided a set of genes selectively expressed by each of its ≥136 cell types (Yamagata *et al*., 2021). We therefore attempted to use the CRISPR-based methods described above to insert reporters or tags into some of these genes.

The efficiency of those methods was, unfortunately, unworkably low, leading us to optimize the method in ways detailed here. Innovations include those described next. (A) Whereas previous methods for editing somatic cells introduced Cas9 via a cDNA, sufficient Cas9 protein production may occur too late for efficient editing. For this reason, CRISPR-dependent methods for germline HDR have introduced Cas9 mRNA (Yang *et al*., 2013; Takahashi *et al*., 2015), avoiding the need for transcription or translation of the Cas9 protein (Aida *et al*., 2015; Chen *et al*., 2016; Troder *et al*., 2018; Gurumurthy *et al*., 2019). We adopted this approach using the Cas9 protein to generate a Cas9-ribonucleoprotein complex. (B) In *Streptococcus pyogenes,* Cas9-based CRISPR uses an RNA that targets a genomic locus (crRNA; 35-36 nt in length) and a sequence that recruits Cas9 (tracrRNA; 67 nt). For most gene editing applications, these are fused to form an sgRNA of 99-100 nt. We used separate crRNA and tracrRNA because they are cheaper to purchase and likely to act more efficiently. (C) Because the brain vesicles of chick embryos are far larger than zygotes, far more ssDNA is required. To produce

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sufficient quantities, we used asymmetric PCR (Marimuthu *et al*., 2012). (D) We added reagents to enhance HDR – carrier DNA and a DNA ligase IV inhibitor (Hu *et al*., 2018). We call the method eCHIKIN (**e**lectroporation- and **C**RISPR-mediated **H**omology-**i**nstructed **K**nock-**IN**).

Figure 1 summarizes the key steps of eCHIKIN. The method begins with the design and preparation of CRISPR HDR reagents (Steps 1-3), which must be finished prior to *in ovo* electroporation. On the day of electroporation, a reagent cocktail including the CRISPR-Cas9 ribonucleoprotein (RNP) complex is prepared (Steps 4-6) and used for injection and electroporation (Steps 7 and 8). At later stages, tissues are dissected, fixed, and analyzed histologically (Step 9).

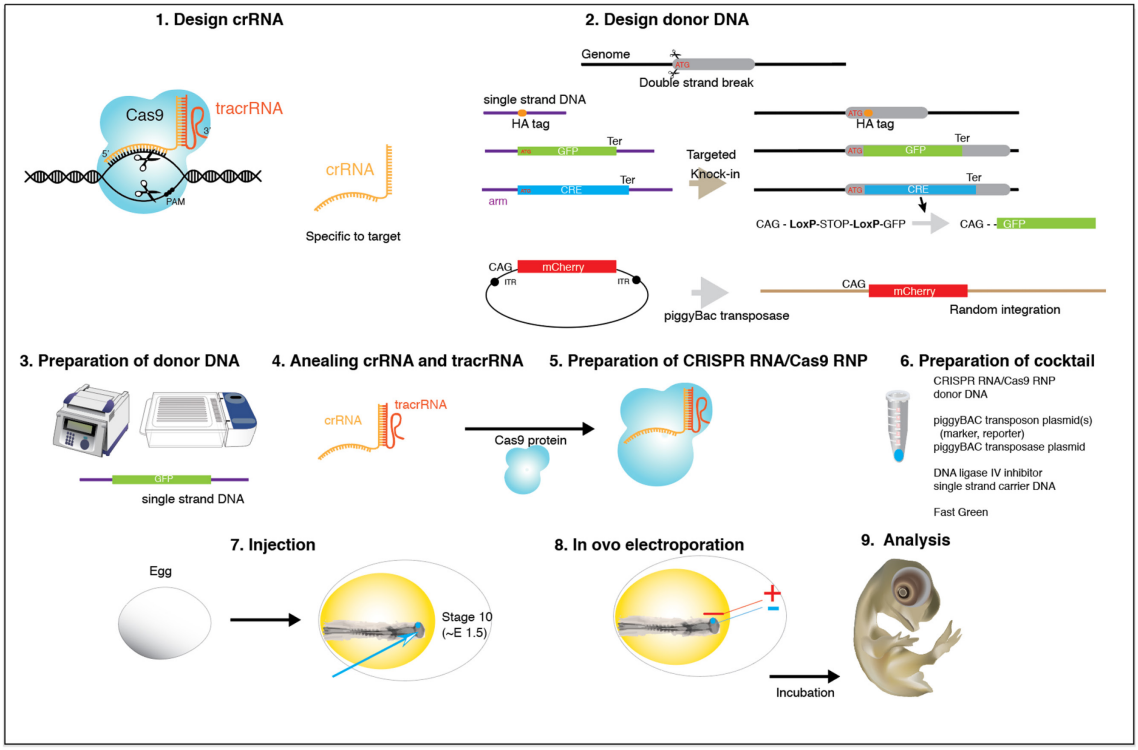

Figure 1. Overview of the eCHIKIN workflow. The main steps are the design and preparation of RNA and DNA reagents (Steps 1-3), preparation of the CRISPR-Cas9 cocktail (Steps 4-6), *in ovo* injection and electroporation (Steps 7 and 8), and immunohistochemical analysis (Step 9). Some panels are from https://togotv.dbcls.jp/en/.

Materials and Reagents

1. 1.5-ml microcentrifuge tubes (DNase, RNase-free low adhesion microcentrifuge tubes) (USA Scientific, catalog number: 1415-2600)
2. Fertilized chicken eggs: Specific-pathogen-free chicken (SPF) eggs from Charles River Laboratories (Wilmington, MA, USA)
   Upon receipt, eggs should be stored at 16°C and used within 1 week. Embryonic development

resumes when the eggs are placed into a humidified incubator at 38°C. Embryos can be staged according to the system of Hamburger and Hamilton (1951).

Note: The temperature of the eggs is sometimes poorly controlled during shipping, which decreases their viability, and does so differentially during summer and winter.

3. Alt-R® CRISPR-Cas9 crRNA, 2 nmol, designed as detailed below (Integrated DNA Technologies (IDT))
4. Alt-R® CRISPR-Cas9 tracrRNA, 20 nmol (IDT, catalog number: 1072533)
5. Alt-R® S.p. Cas9 Nuclease V3, 100 µg (IDT, catalog number: 1081058)
6. Alt-R® Cas9 Electroporation Enhancer, 2 nmol (IDT, catalog number: 1075915)
7. Alt-R® HDR Enhancer, 100 µl (IDT, catalog number: 1081072)
8. 10× Reaction Buffer (with Cas9 nuclease from GenScript, catalog number: Z03386) (200 mM HEPES, 1 M NaCl, 50 mM $MgCl_2$, 1 mM EDTA, pH 6.5 at 25°C)
9. Nuclease-free water (tubes with Alt-R® CRISPR-Cas9 RNAs from IDT)
10. Hanks' Balanced Salt Solution (HBSS, without calcium, magnesium, or phenol red, ThermoFisher Scientific, catalog number: 14175095)
11. DNA oligo 100 nmol scale, designed as discussed in the protocol (IDT)
12. Q5 DNA polymerase (NEB, catalog number: M0491)
13. Deoxynucleotide (dNTP) Solution Mix (NEB, catalog number: N0447S)
14. Exonuclease I (*E. coli*) (NEB, catalog number: M0293S)
15. EconoTaq PLUS GREEN 2× Master Mix (Lucigen, catalog number:30033-1)
16. QIAquick Gel Extraction Kit (Qiagen, catalog number: 28704)
17. 2-Propanol BioReagent, for molecular biology, ≥99.5% (Sigma-Aldrich, catalog number: 19516)
18. Isoamyl alcohol, ≥98%, FG (Sigma-Aldrich, catalog number: W205702)
19. Ethanol Anhydrous 200 Proof (Koptec, catalog number: V1016)
20. pXL-BacII-CAG-Venus (plasmid containing piggyBac inverted terminal repeat (ITR) sequences flanking CAG and Venus) (Yamagata and Sanes, 2012)
21. pXL-BacII-CAG-mCherry (plasmid containing piggyBac ITRs flanking CAG and mCherry)
22. pXL-BacII-CAG-loxP-STOP-loxP-Venus (plasmid containing piggyBac ITRs flanking CAG, a loxP-STOP-loxP cassette and Venus)
23. pCAG-mPBorf (plasmid containing piggyBac transposase, codon-optimized)
24. tPT2A-Venus-pCR21 (plasmid containing tPT2A-Venus sequence) (This plasmid will be available from Addgene, plasmid 170521)
25. pCAG-Cre:GFP (Addgene, 13776)
26. Rabbit anti-GFP (Millipore, catalog number: AB3080P)
27. P-RAN-GFP1 Supernatant (Yamagata and Sanes, 2018a; Addgene plasmid, 106408)
28. TSA Plus Fluorescein Evaluation kit (PerkinElmer, NEL741E001KIT)

    Standard lab reagents for molecular biology (*e.g.*, 70% ethanol, agarose for gel electrophoresis)
29. Agarose (Agarose RA (Amresco); VWR Life Science, catalog number: 97064-258)

30. Buffer for agarose electrophoresis: 10× Tris-Acetate-EDTA (TAE) (Mediatech, catalog number: SC45001-074)
31. Kanamycin sulfate (Sigma-Aldrich, catalog number: K1377) (see Recipe 1 below for HBSS-kanamycin)
32. Fast Green FCF, Dye content ≥85% (Sigma-Aldrich, catalog number: F7252) (see Recipe 2 below for stock solution)
33. Chloroform, contains 100-200 ppm amylenes as a stabilizer, ≥99.5% (Sigma-Aldrich, catalog number: C2432) (see Recipe 3 below for Chloroform-isoamyl alcohol (25:1))
34. Phenol, BioUltra, for molecular biology, ≥99.5% (GC) (Sigma-Aldrich, catalog number: 77608) (see Recipe 4 below for Phenol-Chloroform (1:1))
35. Plasmids (#20, #21, #22) are available from Addgene (Addgene plasmids 170518, 170519, 170520), but current restrictions prevent Addgene from distributing plasmids encoding piggyBac transposase (https://www.addgene.org/terms/1118/). Phenol-chloroform treatment is required for all the plasmids for injection (see Recipe 5 below).

Equipment

1. Egg incubator
   Many incubators are available commercially; we use a 1550 HATCHER (GQF MFG, Savannah, GA). The incubator should be kept humidified AND set to 38°C. Cartons in which eggs are delivered can be used as holders for incubation and electroporation.
2. ECM 830 square wave electroporation system (BTX; Holliston, MA)
3. Tungsten wire (0.1-mm diameter)
4. Glass capillaries, 3-inch length, 1-mm diameter (World Precision Instruments, 1B100F-3)
5. Genetrodes, 3-mm L-Shape (GOLD TIP) 45-0116
   Accessories for holding and connecting electrodes to the electroporator are available from several vendors, including BTX and NEPAGENE.
6. Scotch transparent tape 2592 (25.4-mm width)
   Warning: Chemical adhesives in some tapes from other vendors are toxic to embryos. Suspect tape if the survival rate is low.
7. 18 ½ G needles
8. 10-ml syringes
9. Sharp-edged curved surgical scissors (~4 inches)
10. #5 Forceps
11. Mastercycler Pro Thermal Cycler (Eppendorf, 950040015)
12. Strips of eight tubes, 0.2-ml (Corining PCR-0208-A and PCR-02CP-A)
13. Dissection stereoscope (for electroporation)
    Carl Zeiss Stemi 2000 (444036-9000) equipped with 10×/23 eye pieces (magnification range: 6.5-50×). The viewing magnification should be adjusted as desired

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14. Fluorescence stereozoom microscope (Olympus, SZX12 equipped with the cubes for observing GFP/fluororescein and mCherry/rhodamine fluorescence
15. Spectrophotometer (ThermoFisher, model: NanoDrop 2000) to measure OD_{260}

Procedure

A. Preparation of critical reagents
   1. CRISPR-RNA (crRNA) (IDT)
      Guidelines for designing crRNA are described in Note 1.
   2. ssDNA donor
      Guidelines for designing the ssDNA donor are described in Note 2.
      a. Generate a double-stranded DNA template bearing homology arms.
         Create a double-stranded DNA template (for an example, see Figures 2A and 2B) using two 90-bases ssDNA primers (for an example, see Figure 2C) and a GFP template (for example, pXL-BacII-CAG-Venus). We use a high-fidelity PCR enzyme (Q5 DNA polymerase), perform a 100-µl reaction in a thermocycler, run agarose gels, and purify the appropriately sized major PCR product (Left arm + GFP + right arm: ~850 bp; Figure 2C) using the QIAquick Gel Extraction Kit.

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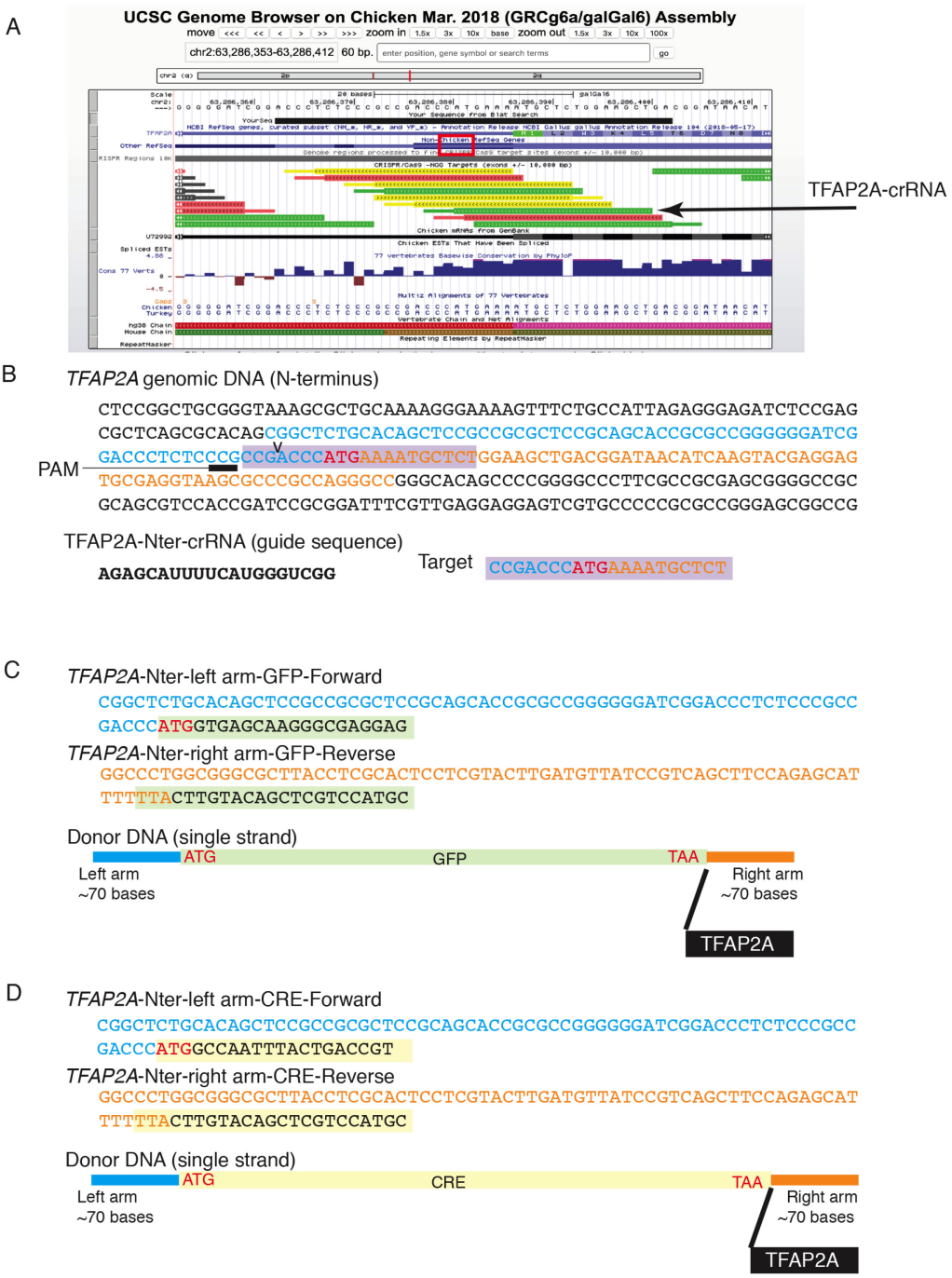

Figure 2. Designing crRNA and the ssDNA donor: *TFAP2A* is given as an example. (A) The *TFAP2A* gene in the UCSC Genome Browser. (B) Genomic sequence of *TFAP2A* in the targeted region and *TFAP2A*-crRNA as shown in A. (C) 90 nucleotide primer sequences that anneal to the 5' (ATG) and 3' ends of GFP and the structure of the single-strand donor DNA used for inserting GFP into the *TFAP2A* locus. (D) Primers and single-strand donor DNA for inserting CRE into the *TFAP2A* locus. See Table 1 for sequences.

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This PCR uses standard methods and equipment. A typical reaction is for 25 cycles (2 min, 95°C; 25 cycles of 94°C, 30 s/60°C, 30 s/72°C, 1 min + 1 s extension at 72°C/cycle; 72°C, 7 min; 4°C). If a clean single PCR band is not obtained, try using buffers optimized for GC-rich templates and/or adding DMSO.

Table 1. Primer sequences (see Figure 2 and Figure 8)

TFAP2A-Nter-left arm-GFP-Forward	CGGCTCTGCACAGCTCCGCCGCGCTCCGCAGCACCGCGCCGGGGGGATCGGACCCTCTCCCGCCGACCCATGGTGAGCAAGGGCGAGGAG
TFAP2A-Nter-right arm-GFP-Reverse	GGCCCTGGCGGGCGCTTACCTCGCACTCCTCGTACTTGATGTTATCCGTCAGCTTCCAGAGCATTTTTTACTTGTACAGCTCGTCCATGC
TFAP2A-Nter-left arm-CRE-Forward	CGGCTCTGCACAGCTCCGCCGCGCTCCGCAGCACCGCGCCGGGGGGATCGGACCCTCTCCCGCCGACCCATGGCCAATTTACTGACCGT
TFAP2A-Nter-right arm-CRE-Reverse	GGCCCTGGCGGGCGCTTACCTCGCACTCCTCGTACTTGATGTTATCCGTCAGCTTCCAGAGCATTTTTTACTTGTACAGCTCGTCCATGC
TFAP2A-Cter-left arm-GFP-Forward	AGCAACAACCCCAACAGCCACACAGACAACAGCACCAAAAGCAGCGACAAAGAGGAGAAGCACCGAAAGATGGTGAGCAAGGGCGAGGAG
TFAP2A-Cter-right arm-GFP-Reverse	GGGAGGAGGGAGATGAATGGACTGATGGGCTGCGAGGGGGGGAAGGGAGAGGAGTGGGGGGGAATCCTCACTTGTACAGCTCGTCCATGC
TFAP2A-Cter-left arm-tPT2A-GFP-F	AGCAACAACCCCAACAGCCACACAGACAACAGCACCAAAAGCAGCGACAAAGAGGAGAAGCACCGAAAGGGCAGCGGCGCCACAAACTTC
TFAP2A-Cter-right arm-GFP-Reverse	GGGAGGAGGGAGATGAATGGACTGATGGGCTGCGAGGGGGGGAAGGGAGAGGAGTGGGGGGGAATCCTCACTTGTACAGCTCGTCCATGC

b. Optimize PCR for ssDNA generation

Before carrying out a large-scale preparation of ssDNA by asymmetric PCR, it is helpful to optimize the reaction on a small scale. We use a robust and low-price PCR enzyme mixture for this purpose. First, using the double-stranded DNA template prepared above, we set up two reactions containing different molar ratios of primers: Reaction #1, 1 forward:100 reverse; Reaction #2, 100 forward:1 reverse.

Reaction #1 and Reaction #2 (2 tubes)
50 µl EconoTaq PLUS GREEN 2× Master
47 µl dH_2O
1 µl ~0.01 µg/µl template from Step A2a
1 µl 0.01 mM primer: (Reaction #1) Forward or (Reaction #2) Reverse

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1 µl 1 mM primer: (Reaction #1) Reverse or (Reaction #2) Forward
**Note that typical PCR uses 0.1 mM primer stocks. Split into two PCR wells (50 µl each).*
94°C, 2 min
40 cycles of 94°C, 30 s/60°C, 30 s/72°C, 1 min + 1 s extension at 72°C/cycle
72°C, 7 min
4°C

Run 1%(w/v) agarose gels (with ethidium bromide). Note that ssDNA is less effectively stained with ethidium bromide than is double-stranded DNA.

A typical result is shown in Figure 3A. In this case (*TFAP2A*), Reaction #1 gave one intense band (~850 bp) and several faint bands, including one that co-migrated with a ~500 bp double-strand DNA marker. Reaction #2 gave two clean bands: the slow-migrating band (asterisk) corresponds to double-stranded DNA (~850 bp) based on its predicted size. However, electrophoretic migration of ssDNA is often anomalous in regular non-denaturation electrophoresis buffers, making it difficult to be sure which band corresponds to ssDNA species. It is therefore useful to incubate a portion of the PCR product (2 µl) with *E. coli* exonuclease I, which specifically digests linear ssDNA. As shown in Figure 3B, some bands from the digest, including part of the diffuse band at ~500 bp, were resistant to *E. coli* exonuclease I digestion, suggesting that some double-strand DNAs were present at ~500 bp. By contrast, in Reaction #2, the corresponding band was less diffuse and was completely digested by *E. coli* exonuclease I, indicating that it contained only ssDNA (arrow). Therefore, we used the conditions of Reaction 2 for large-scale preparation.

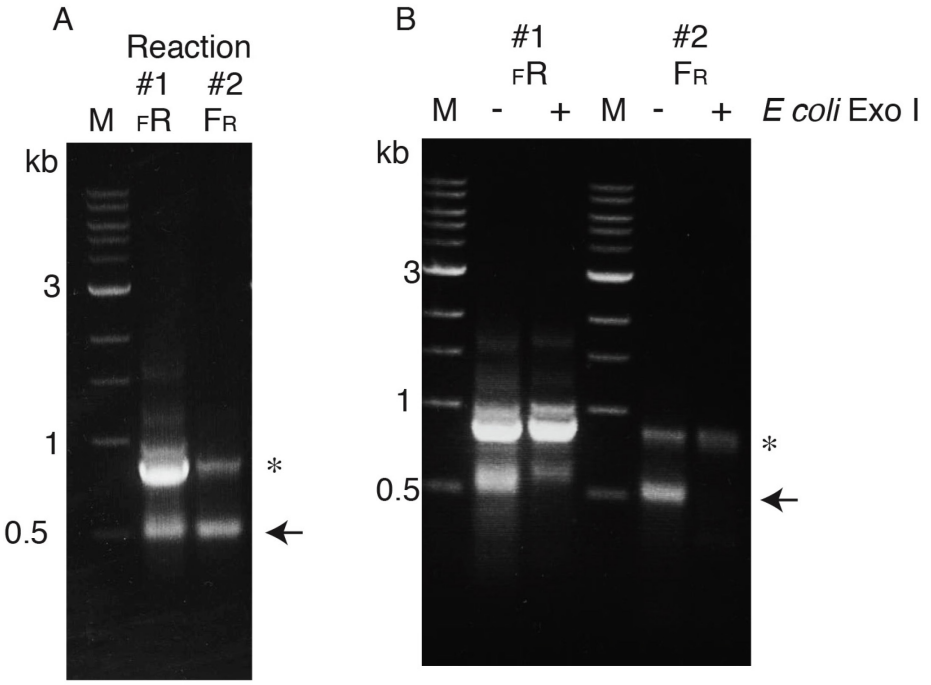

Figure 3. Assessing the quality of ssDNA. (A) Products generated by asymmetric PCR (molar ratio of primers (forward/reverse): 1/100 in Reaction #1; 100/1 in Reaction #2). (B) PCR products were digested with *E. coli* Exonuclease I (*E. coli* Exo I), which specifically digests single-strand DNA (arrow). An additional band (asterisk) corresponds to double-strand DNA. See text for details.

c. Large-scale preparation of the ssDNA donor

Scale-up the reaction optimized in b and then purify the ssDNA donor.

500 µl EconoTaq PLUS GREEN 2× Master
470 µl dH_2O
10 µl ~0.01 µg/µl template from Step A2a
10 µl 0.01 mM Reverse primer
10 µl 1 mM Forward primer
Split into 20 PCR wells (50 µl each)
94°C, 2 min
40 cycles of 94°C, 30 s/60°C, 30 s/72°C, 1 min +1 s extension
72°C, 7 min
4°C

Run a large agarose gel and purify ssDNA using a QIAquick Gel Extraction Kit.

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It is important to use thin wells so that the bands are clearly separated. Typically, we use an 11-cm wide × 13-cm long × ~7-mm thick gel. After solubilizing the agarose in the QG solution (QIAquick Gel Extraction Kit), add 20% volume 2-propanol, and load onto 4 mini spin-columns. Elute each tube with 100 µl EB (QIAquick Gel Extraction Kit) and 50 µl EB, successively. Add 1:10 volume 5 M NaCl, extract twice with phenol-chloroform, then twice with chloroform, and finally ethanol-precipitate by adding 2.5 volumes -20°C ethanol. Rinse the pellets three times with 70% ethanol at -20°C. Spin down, completely remove the residual ethanol, dry, and resuspend in RNase-free water (total 20 µl).

Estimate the DNA concentration using a conventional or NanoDrop™ (Thermo Fisher) spectrophotometer. Note that OD_{260} of ssDNA is lower due to hyperchromicity (40 µg/ml for OD_{260} as opposed to 50 µg/ml for double-stranded DNA). Adjust the concentration to 1 µg/µl, freeze, and store at -20°C until use.

B. Equipment setup

Figure 4 shows a stereomicroscope setup for injection and electroporation (Figure 4A and 4B).

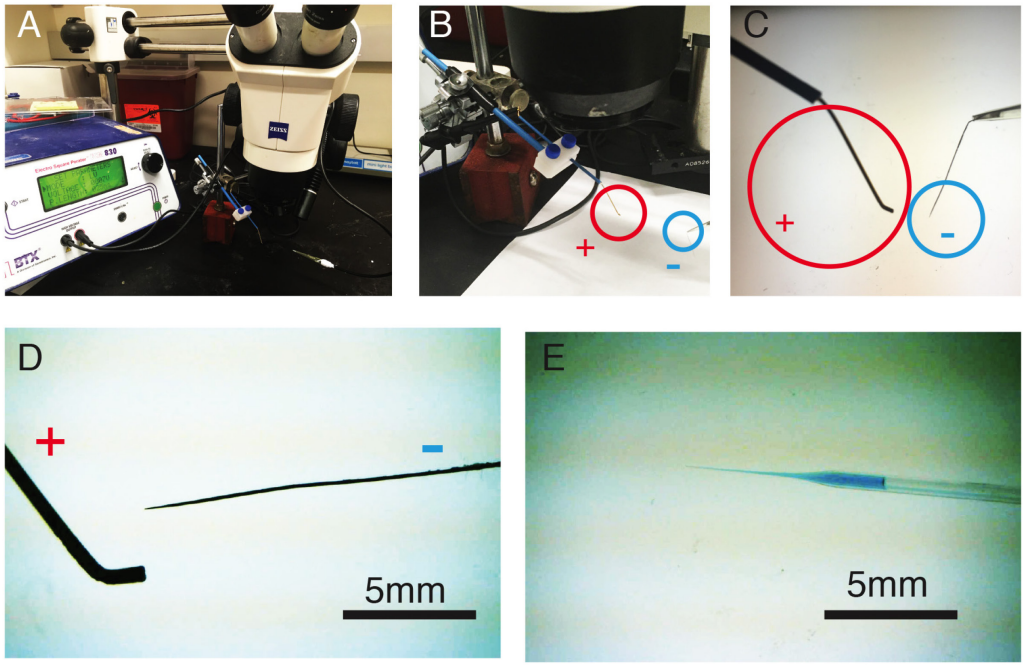

Figure 4. Equipment and instruments required for eCHIKIN. A. Setup of the electroporater, dissection microscope, and electrode holder. B-D. Cathode (+) and tungsten anode (-). E. Glass capillary loaded with eCHIKIN cocktail plus Fast Green.

1. Tungsten needle

   Cut a 0.1-mm tungsten wire to an appropriate length (~2 cm) and sharpen the tip. We sharpen it electrically (Brady, 1965) (see Figure 4C and 4D).

2. Glass capillaries for injection

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Make glass capillaries for injection. We use home-made micropipettes that are finely drawn from glass capillaries (World Precision Instruments, 1B100F-3 (3-inch, 1-mm) using a puller. Break off the last few mm of the tip to allow facile injection while maintaining sharpness (Figure 4E).

C. Incubation of eggs

At appropriate times prior to injection and electroporation (see below), place the eggs in the incubator. Eggs need to be incubated on their side (horizontally; Figure 5A). Mark the top of each egg since the embryo will float on top of the yolk. Incubate more eggs than you plan to inject because some are likely to be infertile or have embryos poorly placed for injection.

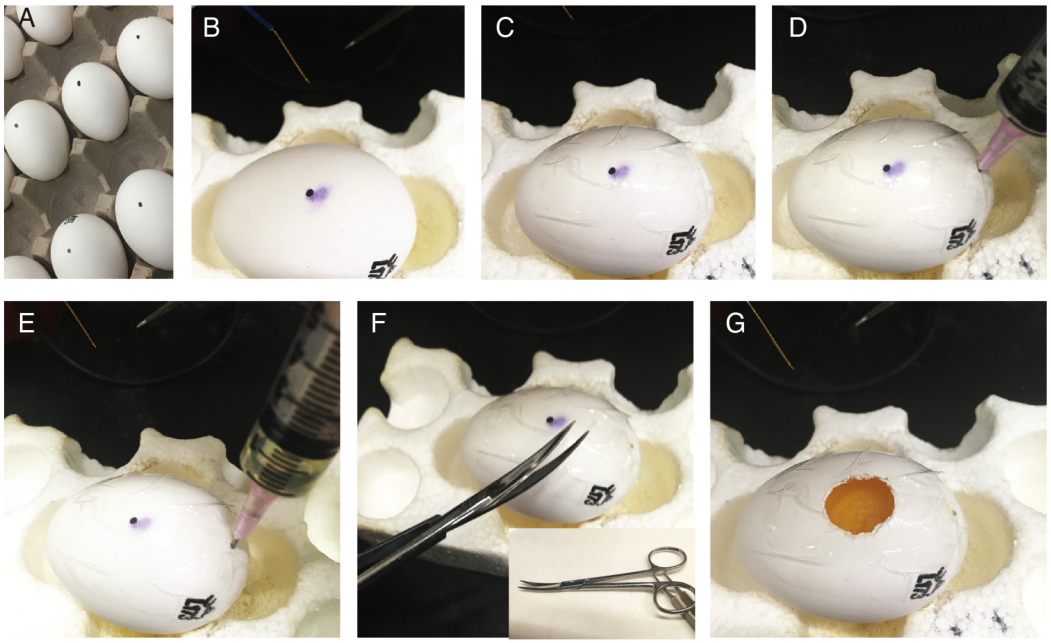

Figure 5. Preparation of chick embryos for injection and electroporation. A. Eggs incubated on their side. B-E. Placing plastic tape on the top of an egg and removing egg albumin from the round side of the egg. F, G. Using surgical scissors with curved blades, a small window (~5-mm diameter) is made on top of the egg by cutting through the tape and shell together.

D. Injection and *in ovo* electroporaton

1. Preparation of injection cocktail

On the day of *in ovo* electroporation, prepare the cocktail for injection. All plasticware and reagents must be RNase-free.

a. crRNA:transcrRNA/Cas9 RNP (Solution "R")

To reconstitute the guide RNA duplex, the target-specific crRNA (35-36 nt) and tracrRNA (67 nt) are combined, heat-denatured, and annealed by slowly chilling to room temperature. In a 1.5-ml tube, add these reagents in this order:

2.5 µl dH_2O
0.5 µl 10× Reaction Buffer
1 µl crRNA (2 nmol/20 µl dH_2O; concentration, 100 µM)
1 µl tracrRNA (2 nmol/20 µl dH_2O; concentration, 100 µM)

Spin briefly and incubate for 5 min at 95°C in a heat block.
Leave at room temperature so that cooling is slow, maximizing annealing.
Leave for 5 min, briefly spin, and incubate for 30 min at room temperature.

Add 0.8 µl 10 mg/ml Cas9 protein (*Streptococcus pyogenes* Cas9 Nuclease V3), mix, spin briefly, and incubate for 60 min at room temperature. This solution is Solution "R."

b. Denaturation of the ssDNA donor
The ssDNA donor should be prepared in advance and stored at -20°C until use.
Thaw the frozen aliquot, heat at 95°C for 5 min, and rapidly cool on ice. This denaturation can be carried out simultaneously with the annealing of crRNA:tracrRNA/Cas9 RNP described in the previous step. However, in contrast to slow annealing of the crRNA:tracrRNA, this tube needs to be cooled quickly by placing on ice immediately. Spin the tube briefly before use.

c. Dilution buffer with HDR enhancer (Solution "D")
In an RNase-free 1.5-ml tube, add these reagents in this order:

21.5 µl dH_2O
2.5 µl 10× Reaction Buffer
1 µl Alt-R® HDR Enhancer (available as a solution suspended in DMSO). Mix well and spin briefly.

d. piggyBac plasmid mixture (Solution "P")
For GFP reporter eCHIKIN, mix in a 1.5-ml tube:
45 µl 1 µg/µl pXL-BacII CAG-mCherry
5 µl 1 µg/µl pCAG-mPB

For Cre reporter eCHIKIN, mix in a 1.5-ml tube:
22.5 µl 1 µg/µl pXL-BacII CAG-mCherry
22.5 µl 1 µg/µl pXL-BacII CAG-loxP-STOP-loxP-Venus
5 µl 1 µg/µl pCAG-mPB
Note that these plasmid DNAs must be RNase-free (see Recipe 5 and Note 3).

e. Final injection cocktail

To a tube of Solution "R" (total 5.8 µl), add these reagents in this order (see Note 4):
11 µl Solution "D"
3 µl 1 µg/µl ssDNA donor (denatured at 95°C in advance)
3 µl Alt-R® Cas9 Electroporation Enhancer (2 nmol/50 µl; concentration, 40 µM)
3 µl Solution "P"
2 µl 0.1%(w/v) Fast Green

Mix well and place this solution on ice. Use within 5-6 h.

2. Preparation of eggs and embryos
   a. Embryos reach stage 9-10 in ~40 h at 38°C. Thus, if eggs are placed in the incubator at 8 pm (Day 0), they should be ready to electroporate at noon on Day 2. However, embryos should be staged according to Hamburger and Hamilton (1951) because the developmental rate is sensitive to small changes in temperature and also varies among eggs. To slow development when planning to inject many embryos, eggs can be removed from the incubator and maintained at room temperature for up to 5-6 h without compromising viability.
   b. Spray eggs with 70% ethanol and allow to dry. Always keep the previously marked top side of the egg facing upward (Figure 5B).
   c. Apply a piece of transparent tape to the top of each egg (Figure 5C).
   d. Insert an 18 ½ G needle on a 10-ml syringe into the rounded side of each egg (Figure 5D); this side contains the air sac.
   e. Remove 1-2 ml egg albumin depending on the size of the eggs (Figure 5E). Some researchers omit this step to obtain better survival.
   f. Make a small window (~5-mm diameter) on top of the egg by cutting through the tape and shell using surgical scissors with curved blades (Figure 5F and 5G).
3. Injection and *in ovo* electroporation
   a. If necessary, gently rock the egg so that the embryo sits on top of the yolk. Make the window larger if needed (Figure 6A). In some cases, the translucent membrane beneath the shell needs to be carefully removed using #5 forceps.

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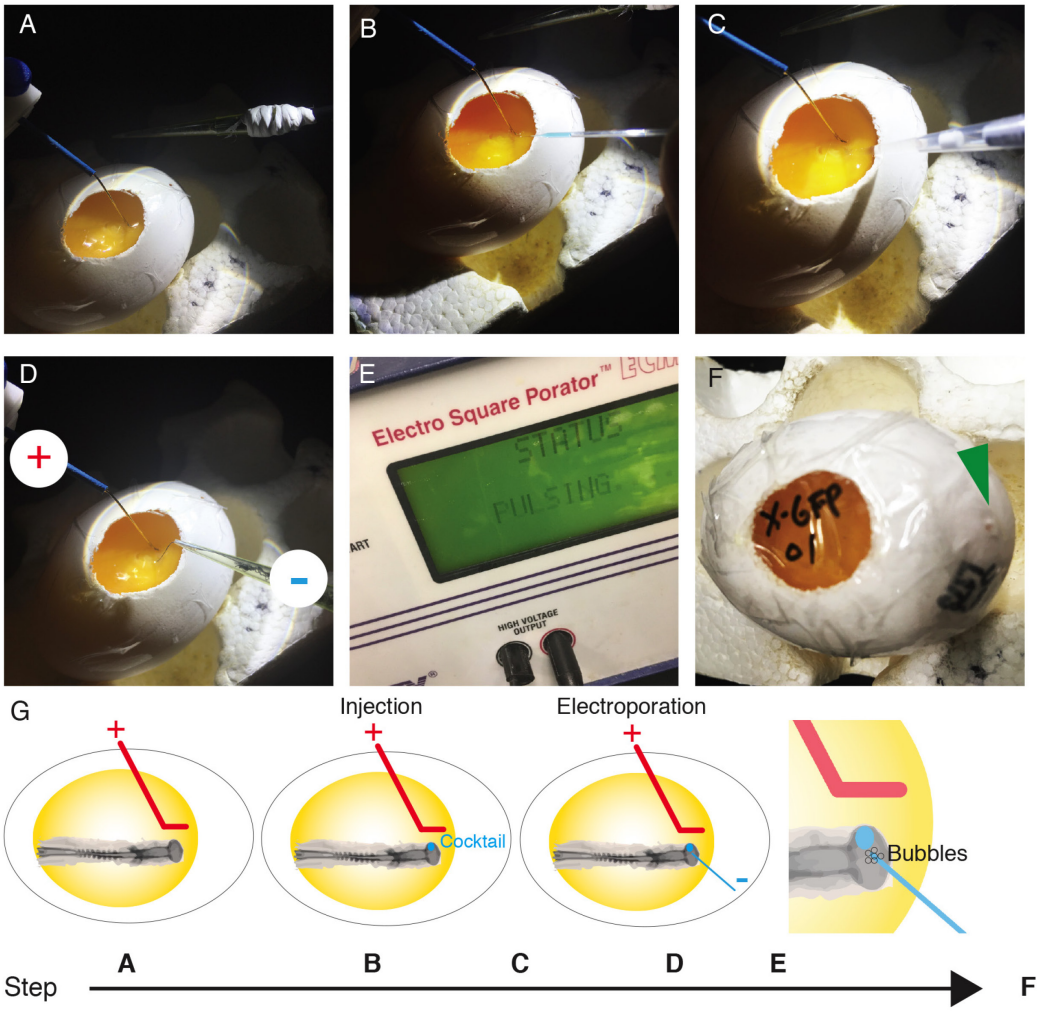

Figure 6. Key steps of injection and *in ovo* electroporation. (A) Locate the embryo and expand the window. (B) Place the cathode as in G and inject the reagent cocktail (blue) into the embryo. (C) Wet the cathode and embryo with Hanks' + kanamycin. (D-E) Insert the anode as in G and electroporate. After successful electroporation, small bubbles appear at the anode. (F) Seal the egg with plastic tape. It is important to seal the small hole (green) that was made for removing albumin. (G) Summary.

b. Place the egg under the dissection microscope. For beginners, it may be challenging to see enough detail in the embryos for accurate injection. One aid is to inject a few µl black India ink beneath the embryo. Images in Hamburger and Hamilton (1951) are useful for orientation.
c. Place the L-shaped cathode parallel to the embryo (Figure 6A and 6G).
d. Load the CRISPR cocktail into a micropipette (Figure 4E) and inject ~0.1-0.2 µl using a mouth pipette or microinjector (*e.g.*, FemtoJet, Eppendorf). To transduce the retina, inject to one side of the optic vesicle of stage 9-10 embryos (Figure 6B and 6G). To transduce the optic tectum, injection should be targeted to the midbrain vesicle at stage 11-12.

e. Wet both the cathode and embryo with 20 µl HBSS + kanamycin using a pipette tip (Figure 6C).
f. Insert the fine tungsten anode of the electroporator into the head of the embryo (Figure 6D and 6G).
g. Deliver square wave pulses (Figure 6E and 6G). For optic vesicles, we use 6 pulses (7 V/25 ms) at 1-s intervals. A sign of successful electroporation is the appearance of numerous tiny bubbles at the anode.
h. Close the window with transparent tape (Figure 6F). It is important to also seal the small hole (green arrowhead in Figure 6F) that was made to remove the albumin.
i. Place the eggs back in the 38°C incubator.
This technique (Steps D2 and D3) relies heavily on the performer's skill and requires considerable practice. Experts can inject one embryo every few minutes or ~120 embryos in a 4-h session.

E. Histological analysis

Chick embryos hatch at E21; however, mortality increases greatly from E17. This is a common consequence of manipulations that introduce a window in the eggshell; it is not specific to eCHIKIN. Thus, it is best to retrieve tissue by E16, if possible. Both the retina and optic tectum are fairly mature by that age.

Although other methods can be used, the main analytical method is immunohistology. Embryos are retrieved, and the appropriate regions (retina or tectum in the cases described here) are dissected. The tissues are fixed with 4% (w/v) paraformaldehyde/PBS for 15 h, sunk in 15% (w/v) and 30% (w/v) sucrose/PBS successively, embedded in Tissue Freezing Medium (Triangle Biomedical Sciences), frozen at -80°C, and sectioned at 20 µm in a cryostat. Sections are then double-stained with anti-GFP and antibodies against cell class- or type-specific markers. Commercially available rabbit polyclonal anti-GFP, mouse monoclonal anti-GFP, and anti-GFP nanobody-reporter conjugates (RANbodies; Yamagata and Sanes, 2018a) can be used. However, commercially available chicken anti-GFP IgY antibodies display high background in chick tissues and should be avoided. Detailed immunohistochemical methods have been described in previous publications (Yamagata and Sanes, 2018b and 2019; Yamagata *et al*., 2021).

A problem that we encountered was finding cells marked by eCHIKIN, both because they were rare and because in some cases expression levels were low. To circumvent this limitation, we co-electroporated a second reporter (usually a red fluorescent protein) expressed from a strong, ubiquitous (CAG) promoter. To guarantee maintained expression through multiple cell divisions, we ensured stable genomic integration using a piggyBac transposon/transposase system. In most cases, fluorescence from the CAG-driven fluorescent protein was readily detectable in tissue using a dissection microscope equipped with fluorescence. Regions identified in this way could then be dissected, sectioned, and immunostained.

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A potential confounding factor is that HDR could lead to integration into, and thus inactivation of, both copies of a targeted allele, resulting in alterations in the target cell. We did not encounter this problem: labeled cells that we were able to visualize by alternative methods (*e.g.*, immunohistochemistry) were not detectably abnormal. Nonetheless, we cannot dismiss it and therefore tested two methods to circumvent it. In one, we maintain the function of endogenous protein by targeting the C-terminus to generate a fusion protein. For example, we appended GFP to the C-terminus of the TFAP2A coding region (Figure 7E, Figure 8A, and 8B). This method reveals the subcellular distribution of the tagged protein. In the case shown, the TFAP2A protein is a nuclear transcription factor, so the fusion protein is localized in the nucleus (Figure 7F). Subcellular localization can be either advantageous or disadvantageous depending on the purpose of the experiment. As a second approach, when cell filling is desired, we use the self-cleavable 2A peptide to generate the inserted proteins and, separately, GFP (Figure 7E). We use a tandem fusion of two 2A sequences (tPT2A) comprising P2A (2A from porcine teschovirus-1) and T2A (2A from thosea asigna virus) (Liu *et al*., 2017) to separate TFAP2A from GFP. In this case, GFP remained soluble and filled the cytoplasm (Figures 7G and 8C).

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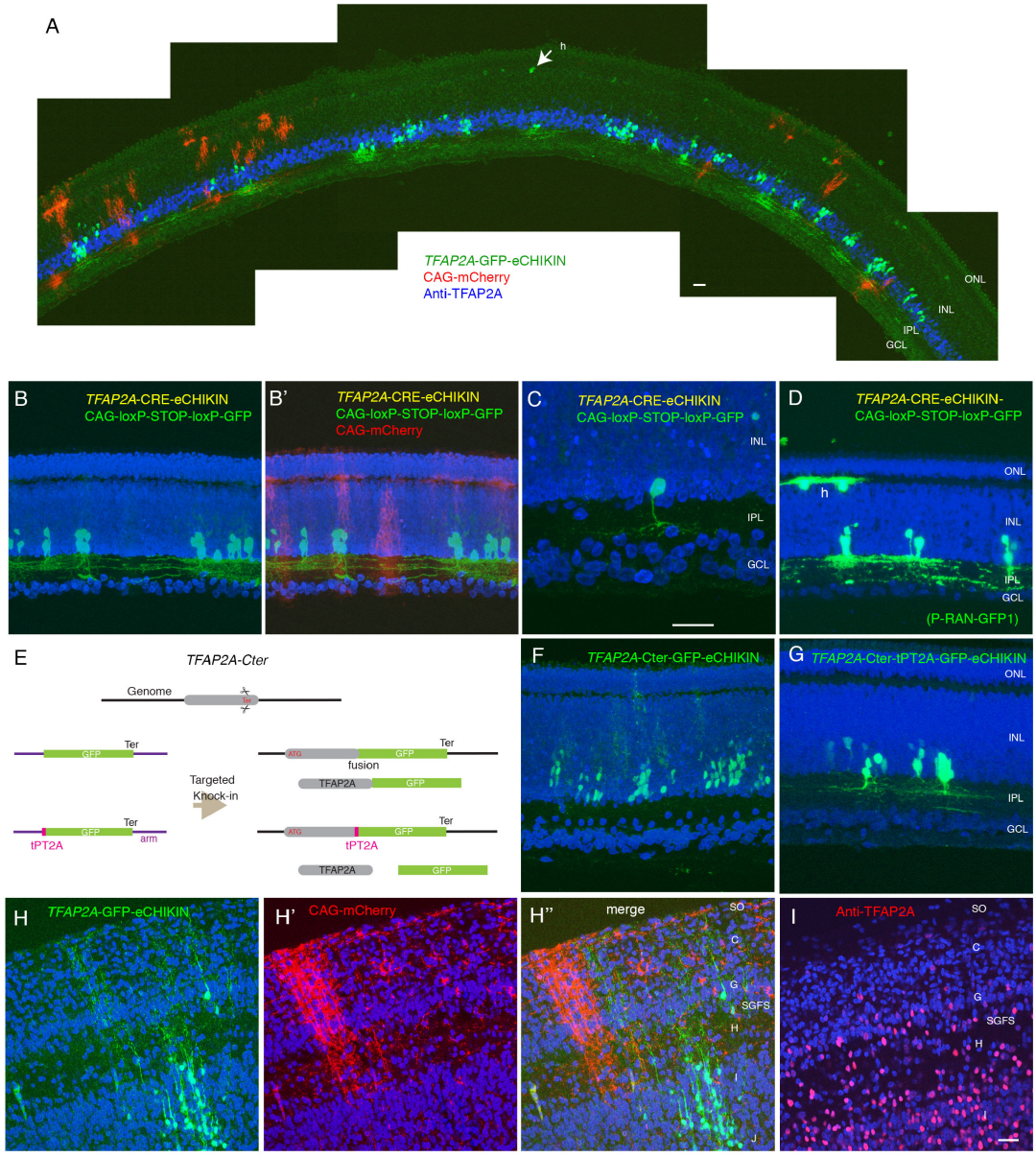

Figure 7. eCHIKIN for TFAP2A. (A) Section of an E14 retina transduced with TFAP2A-eCHIKIN-GFP (green) and CAG-mCherry (red). Endogenous TFAP2A protein was stained with an anti-TFAP2A antibody (blue). TFAP2A marks most amacrine cells in the retina and is also weakly expressed by horizontal cells (arrow). (B, C) Sections of an E14 retina transduced with TFAP2A-eCHIKIN-CRE, CAG-loxP-STOP-loxP-GFP (green), and CAG-mCherry (red, shown only in B'). Nuclei were counterstained with NeuroTrace 635 (blue). (D) Similar to C but stained with a RANbody to GFP (P-RAN-GFP1). Note that this method (Yamagata and Sanes, 2018a) results in efficient labeling of horizontal cells (H). (E) Schematic of TFAP2A-Cter-eCHIKIN-GFP and TFAP2A-Cter-tPT2A-GFP-eCHIKIN. (F) Section of an E12 retina transduced with TFAP2A-Cter-eCHIKIN-GFP. Since GFP is fused to the C-terminus of the TFAP2A protein, GFP is localized to the nucleus. Weak staining in the upper INL is bleed-through from the mCherry

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channel. (G) Section of an E12 retina transduced with TFAP2A-Cter-tPT2A-GFP-eCHIKIN. GFP is rendered soluble in this method, resulting in the cytoplasm being filled with GFP. (H) Section of an E12 optic tectum transduced with TFAP2A-eCHIKIN-GFP (green) and CAG-mCherry (red). (I) Section of an E12 optic tectum stained with an anti-TFAP2A antibody; positive cells are in several laminae of the stratum griseum fibrosum superficiale (SGFS). Laminar distribution matches that of transduced cells in H. ONL, outer nuclear layer, containing photoreceptors. INL, inner nuclear layer, containing interneurons (horizontal, bipolar, and amacrine cells). IPL, inner plexiform layer, containing processes of retinal neurons. GCL, ganglion cell layer, containing retinal ganglion cells and some amacrine cells. Bar in I, 10 μm for B and F-I; Bars in A and C are 10 μm.

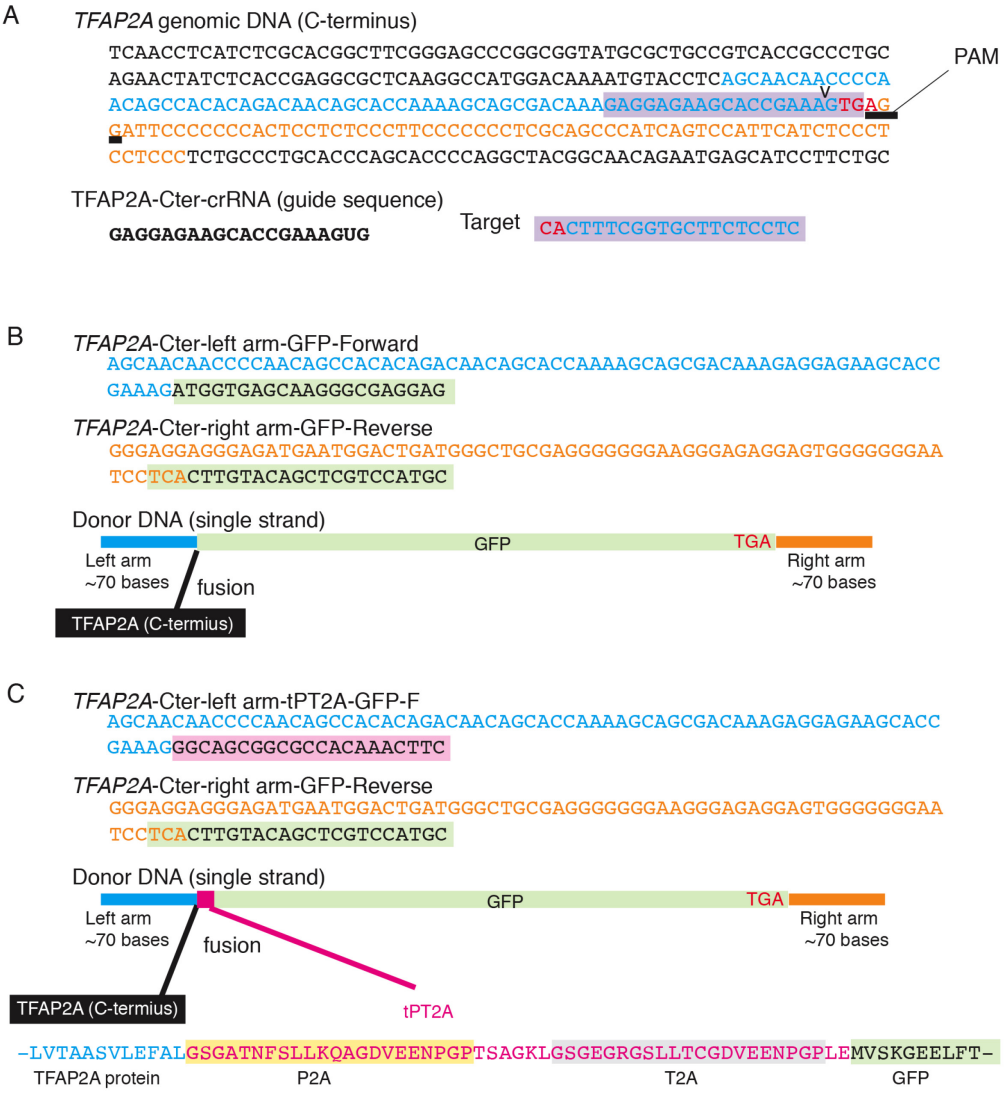

Figure 8. eCHIKIN for TFAP2A: Fusion protein and tPT2A. (A) Genomic sequence of *TFAP2A* flanking its C-terminus. TGA (red) is a termination codon. The target sequence

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(opposite strand) of TFAP2A-Cter-crRNA is shown below. (B) To generate a donor DNA template with 70 bases arms, two 90-nt primers were used. One primer (TFAP2A-Cter-left arm-GFP-Forward) links to the 5'end of the GFP sequence (ATG), the other primer (TFAP2A-Cter-right arm-GFP-Reverse) links to the 3' end of GFP. (C) To insert the tPT2A self-cleavable peptide sequence between TFAP2A and GFP, the donor DNA template was generated with two primers using a tPT2A-GFP plasmid as a template. See Table 1 for sequences.

EXAMPLES

Figure 7 shows examples of TFAP2A-expressing chick cells labeled using the eCHIKIN method. *TFAP2A* encodes a transcription factor expressed strongly by amacrine cells and weakly by horizontal cells in the inner nuclear layer of the retina. Figure 7A, from a retina transfection with a TFAP2A/GFP knock-in construct, shows that integration events were in some cases quite frequent. It is apparent that eCHIKN-labeled cells are all present in the inner nuclear layer, where amacrine cells are localized, whereas CAG-mCherry labeled cells are distributed among all retinal layers. The number of cells per layer is likely influenced by the number of divisions between the integration of the piggyBac transposon plasmid and later analysis; this varies among cell classes and types. In this image, there are more eCHIKIN-GFP-labeled cells than mCherry-labeled cells, but this is atypical. Figure 7B-D shows sections from retinas labeled by co-electroporation of TFAP2A-cre and a cre-dependent reporter. Cells in Figure 7B and 7C were labeled with anti-GFP, while cells in Figure 7D were labeled with an anti-GFP RANbody (P-RAN-GFP1; Yamagata and Sanes, 2018a). The RANbody provides more intense labeling than conventional indirect immunofluorescence (Yamagata and Sanes, 2018a). Figure 7F and 7G, described above, shows cells labeled with TFAP2A-Cter-eCHIKIN-GFP and TFAP2A-Cter-tPT2A-GFP-eCHIKIN, respectively.

Application of eCHIKIN to 15 other genes, expressed by all retinal neuronal classes – photoreceptors, horizontal cells, bipolar cells, amacrine cells, and retinal ganglion cells – is illustrated in Yamagata *et al*. (2021). In our hands, eCHIKIN successfully labels ~90% of genes, although the efficiency of labeling is quite variable. In this fast-moving field, new methods can be added to the eCHIKIN protocol to enhance efficiency (Yeh *et al*., 2019; Broeders *et al*., 2020).

We also applied eCHIKIN to the optic tectum. Figure 7H shows labeling of cells with TFAP2A-GFP in the same laminar distribution as those labeled with anti-TFAP2A (Figure 7I). This result provides encouragement that eCHIKIN could be applied to multiple tissues in chick embryos and possibly to other birds. From our experience to date, we believe that the main key to the success of the technique is to achieve robust electroporation and choose appropriate CRISPR reagents; thus, the protocol may need to be modified for other tissues or species.

Notes

1. CRISPR-RNA

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This protocol uses the CRISPR/Cas9 system from *Streptococcus pyogenes* because it is the most commonly used. Moreover, target-specific RNAs and Cas9 protein are readily procured from commercial vendors.

Genome editing performance of a crRNA is highly dependent on its sequence and secondary structure, as well as the chromatin status of the targeted locus (Doench *et al*., 2014; Xu *et al*., 2015). Here, we illustrate the selection process using the design of the TFAP2A-GFP vector (Figure 2 and Figure 8) as an example. One constraint of the CRISPR/Cas9 system is that a protospacer adjacent motif (PAM) must be incorporated into the design of the CRISPR; for *Streptococcus pyogenes* Cas9, the PAM is the trinucleotide NGG. The targeted CRISPR/Cas9 endonuclease generates blunt ends three nucleotides upstream of NGG. The target sequence needs to be designed such that the intended insertion is close to this double-strand break, preferably within <10 bp. First, sequences are retrieved from the most recent genome assembly (currently *Gallus gallus* GRCg6a). It may be helpful to confirm the reported sequence because some genes are still incompletely annotated. Ensembl (https://ensembl.org) offers a function, "Export data," which allows easy retrieval of sequences flanking the searched sequence. The target sequence is then selected based on a panel of specificity and efficiency scores using publicly available tools (*e.g.*, CRISPR 10K Super-track in the UCSC genome server https://genome.ucsc.edu) (Figure 2A). Multiple recent methods are available; however, describing them is beyond the scope of this protocol [see Hanna and Doench (2020) for a comprehensive review]. Targets should not be in repetitive elements spread throughout the genome, but it is not necessary to rigorously rule out potential off-target crRNA cutting sites because HDR greatly enhances the specificity of integration at the targeted locus.

2. Design and preparation of the donor DNA for HDR

For HDR, single-stranded DNA (ssDNA) is widely used as a donor. To achieve HDR in high efficiency, ssDNA typically contains flanking sequences around 50-80 bases on each side homologous to the nuclease cleavage site (Chen *et al*., 2011; Yang *et al*., 2013; Richardson *et al*., 2016; Quadros *et al*., 2017). For eCHIKIN, we use 70 base homology arms because commercial synthesis of 90 bases DNA is available and affordable (see below). It is also possible to use double-strand circular plasmid DNA or double-strand linear DNA as HDR donors. However, they require much longer homology arms (200 bp-2 kbp or longer), which can be prepared by molecular cloning (*e.g.,* Baker *et al*., 2017). In addition, linearized double-strand DNAs often integrate randomly into the genome rather than specifically at CRISPR-mediated breaks, possibly leading to non-specific patterns of expression.

Short ssDNA can be obtained as single-strand oligonucleotides (*e.g.*, up to 200 nt; IDT's Ultramer) that can incorporate short epitope tags with some flanking sequences (*e.g.*, 27 nt for a HA tag) as well as both left and right arms (2 × 70 nt). Chemical synthesis can be used for longer ssDNA, sufficient to encode homology arms plus reporters (*e.g.*, GFP [~700 nt] or Cre [~1,000 nt]); however, these methods are costly due to the large amount of ssDNA needed. Instead, we generate a template using two 90-bp DNA oligonucleotides (70 bases of the left and

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right arms, and 20 bases complementary to the 5' and 3' ends of the reporter cDNA, respectively) (Figure 1, Figure 2D-F). To prepare a large amount of long ssDNA using this template, we use asymmetric PCR, in which one primer is added at a 100-fold greater concentration. The amplified ssDNA can be purified by agarose gel electrophoresis, treated with phenol-chloroform and then chloroform, ethanol-precipitated, and stored at -20°C.

Although other methods have been used to prepare long ssDNA (*e.g.*, a combination of T7 RNA polymerase/Reverse transcriptase/RNase H, or lambda exonuclease digestion of PCR products produced with one phosphorylated primer), our experience is that the asymmetric PCR protocol is more consistent, robust, and inexpensive.

It was demonstrated that an ssDNA donor complementary to the nontarget strand is slightly better for HDR than an ssDNA donor complementary to the target strand (Richardson *et al*., 2016). Thus, the sequence corresponding to the target would be the first choice for ssDNA, although the opposite ssDNA is also effective in our hands. We choose either ssDNA based on the ease of purification from agarose gels.

3. piggyBac reporters

   To monitor the success of electroporation, we add a piggyBac transposon plasmid equipped with CAG (CMV, beta-actin promoter/enhancer/globin leader)-driven mCherry together with a CAG-driven piggyBac transposase (Yamagata and Sanes, 2012). This transposon system is used to label the electroporated area stably by integration with the aid of the transposase. Randomly integrated mCherry is used to distinguish GFP, which is integrated specifically at the target locus. When Cre is used for HDR, we also use piggyBac to introduce the Cre-dependent GFP reporter (loxP-STOP-loxP-GFP).

4. HD enhancers

   Two additional reagents are added to enhance HD and electroporation. A DNA ligase IV inhibitor (Alt-R® HDR Enhancer, an SCR7-related proprietary product from IDT) inhibits NHEJ, thereby leading to HDR. SCR7 has been used in several studies to improve HDR (Maruyama *et al*., 2015; Hu *et al*., 2018); although, this approach has not yet become common practice (Yeh *et al*., 2019). For chick embryos, this reagent appears to be non-toxic. We also use the Alt-R® Cas9 Electroporation Enhancer from IDT; this is a mixture of purified carrier DNA fragments that improve the delivery of CRISPR/Cas9 RNP by electroporation. We found that inclusion of Alt-R® HDR Enhancer and Alt-R Cas9 Electroporation Enhancer in the injection cocktail improves the efficiency of eCHIKIN.

Recipes

1. HBSS with kanamycin

   Add 50 µg kanamycin sulfate per ml HBSS

   Keep at 4°C

2. 0.1% (w/v) Fast Green FCF

Dissolve 100 mg Fast Green FCF in 1 ml RNase-free water

Filter slowly through a 0.45-µm filter

Keep at 4°C

3. Chloroform-isoamyl alcohol (25:1)

   25 chloroform:1 isoamyl alcohol

   Keep at room temperature.

4. Phenol-chloroform (1:1)

   1 phenol:1 chloroform-isoamyl alcohol.

   Phenol needs to be saturated with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Keep at 4°C.

5. Plasmids

   All plasmids need to be RNase-free. After purification using kits (*e.g.*, Qiagen), plasmids should be treated as follows:

   a. Add 1:10 volume of 5 M NaCl and the same volume of phenol-chloroform (1:1) and vortex for 30 s.
   b. Spin for 5 min at the maximum speed of a mini centrifuge (*e.g.*, 16,100 × *g*) at room temperature. Recover the aqueous phase.
   c. Repeat the phenol-chloroform treatment.
   d. Add chloroform-isoamyl alcohol (25:1), vortex, spin, and recover the aqueous phase.
   e. Repeat the chloroform-isoamyl alcohol treatment.
   f. Add 2.5 volumes of ethanol to precipitate the plasmid DNA.
   g. Pellet and rinse three times with 70% ethanol (prechilled at -20°C).
   h. Dry and resuspend in RNase-free water to make a 1 µg/µl stock solution.

      Keep at 4°C for use within a month or dispense into aliquots and freeze at -20°C for long-term storage.

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Competing interests

The authors report no competing interests.

Ethics

Animals were used in accordance with NIH guidelines and protocols approved by the Institutional Animal Use and Care Committee at Harvard University.

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