Generation of FLAG-tagged *Arx* knock-in mouse model

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Abstract

Aristaless-related homeobox (ARX) is a paired-like homeodomain transcription factor playing important roles in brain development. Patients with mutations in *ARX* have a spectrum of neurodevelopmental disorders such as epilepsy, intellectual disability, and autism spectrum disorder, with or without structural abnormalities of the brain such as lissencephaly (smooth brain), microcephaly (small brain), and/or agenesis of corpus callosum. Mouse models have provided important clues on the pathophysiologic roles of ARX in these disorders. However, successfully isolating specific *in vivo* complex of ARX, with DNA and proteins, has remained as a challenge. To facilitate in *vivo* detection of ARX complexes, we generated a mouse line containing one epitope of FLAG-tag (1 x FLAG) targeted at the translational start site of the endogenous *Arx* gene using CRSPR/Cas9 strategy. Homozygous *Flag-Arx* mice are viable and fertile without gross abnormality, suggesting that the FLAG-tag does not perturb normal function of ARX. Using a FLAG antibody, we successfully detected ARX with immunofluorescent staining and pulled down ARX in embryonic brain tissues. This *Flag-Arx* mouse model will be a useful tool to isolate ARX complexes from mouse tissues for many applications.

Keywords

ARX; *Flag-Arx* knock-in mouse; CRSPR/cas9; brain development; detection of FLAG tagged-endogenous protein

1. INTRODUCTION

The aristaless-related homeobox (ARX) protein plays important roles in brain development and mutations are associated with a spectrum of neurodevelopmental disorders with structural and/or functional deficits (Friocourt, 2010; Kato et al., 2004; Matsuo et al., 2001; Shoubridge et al., 2010). The structural defects include lissencephaly (smooth brain), microcephaly, and agenesis of the corpus callosum, whereas the functional defects include

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intellectual disability, early infantile epileptic encephalopathies and other seizure types, dyskinesias (involuntary movements), and autism spectrum disorder.

ARX is expressed both in the dorsal and ventral forebrain but with distinct patterns during embryonic development (Colombo et al., 2004; Miura et al., 1997). In the dorsal forebrain (i.e., pallium), ARX is expressed in the proliferating cells of the ventricular zone (VZ), however its expression is not detected once cells exit the cell cycle and begin migrating out of the VZ (Colombo et al., 2004; Miura et al., 1997). In contrast, in the ventral forebrain (i.e., subpallium) ARX expression is weak in the VZ and stronger in the subventricular, intermediate, and mantle zones (Colombo et al., 2004; Miura et al., 1997). Interestingly, the expression of ARX in cells derived from the subpallium, unlike cells emanating from the pallial VZ, remains on even after the cells have migrated and differentiated into interneurons. Given that the cortical projection neurons are derived from the dorsal forebrain while the cortical interneurons originate from the ventral forebrain (Butt et al., 2007; Lodato and Arlotta, 2015; Ohtaka-Maruyama and Okado, 2015; Wonders and Anderson, 2006), this differential expression of ARX in the dorsal vs. ventral forebrain suggests distinct roles of ARX in these two populations of neurons, thus contributing differently to the spectrum of phenotypes (McKenzie et al., 2007; Olivetti and Noebels, 2012; Ruggieri et al., 2010; Shoubridge et al., 2010; Strømme et al., 2002). In fact, dorsal specific depletion of ARX function in mice leads to distinct phenotypes from those associated with ventral loss (Colasante et al., 2015; Marsh et al., 2009; Nasrallah et al., 2012; Simonet et al., 2015). Furthermore, we have shown that specifically ablating ARX expression in the dorsal progenitor cells, exhibit microcephaly but no seizures (Colasante et al., 2015), whereas loss of ARX in the ventral forebrain results in severe epilepsy without microcephaly (Marsh et al., 2009).

These findings prompt us to investigate the molecular basis of ARX function in distinct populations of cells. However, identification of transcriptional targets of ARX in mice via chromatin immunoprecipitation sequencing (ChIP-seq) has been challenging in part due to the performance of ARX antibodies, although ChIP-seq in Neuro2A cells transfected with *Arx*, or ChiP-qPCR and Chip-on-chip in mouse embryonic brains have been reported previously (Friocourt, 2011; Fulp et al., 2008; Quillé et al., 2011). In order to overcome this issue, we have generated a knock-in mouse model expressing FLAG-tagged ARX which facilitates pull-down of ARX protein *in vivo* via FLAG antibody. We verified that FLAG-tagged ARX reliably recapitulated most of the normal ARX expression in the embryonic mouse brains and the FLAG antibody successfully pulled down FLAG-ARX. Thus, this new mouse model will be a useful tool for the future studies involving isolation of ARX complex with DNA or with other transcription-associated factors.

2. RESULTS AND DISCUSSION

Flag-Arx knock-in mouse line was generated by introducing one epitope of the FLAG (GAC TAC AAG GAC GAC GAT GAC AAG) sequence into the first exon of *Arx*, immediately after the initiation codon, using the CRISPR/Cas9 system (Fig. 1A). N-or C-terminal tagging of the FLAG is often used to facilitate the endogenous detection of proteins, whose antibodies are not available or with poor performance (Ferrando et al., 2014). However,

usually more than one copy of the FLAG epitope is tethered to the protein to improve detection of often low endogenous expression of the target proteins. However, the addition of multiple FLAG sequences increases the risk of disrupting the structure and function of the tagged protein. Therefore, to minimize potentially disrupting ARX function, a single copy of FLAG sequence was introduced into the *Arx* locus.

The genotype of each mouse was confirmed by PCR amplification followed by NcoI restriction enzyme digestion, which generates digested fragments only in *Flag-Arx* allele (Fig. 1B). We have also verified the N-terminal insertion of the *Flag* sequence into *Arx* locus by Sanger sequencing (Fig. 1C). Homozygous *Flag-Arx* knock-in mice are viable and reproduce normally. Expected phenotypes with reduced or loss of ARX include microcephaly, agenesis of corpus callosum, and epilepsy, but homozygous *Flag-Arx* knock-in mice did not exhibit these phenotypes in multiple litters produced over five generations bred to C57BL/6, suggesting that FLAG-tagged ARX functions normally.

Next, we examined the expression of FLAG-ARX by immunofluorescent labeling using both anti-FLAG and anti-ARX antibodies. The detection of the single FLAG tag, as predicted, required antigen retrieval and fluorescent amplification methods (Fig. 2 and see Materials and Methods). We successfully detected FLAG-ARX with anti-FLAG antibody in E14.5 embryonic brains. Furthermore, the specificity of the FLAG antibody labeling was confirmed by demonstrating co-localization with anti-ARX labeling. Detection of the FLAG tagged ARX was observed in all expected brain regions including the subventricular zone (SVZ) and outer SVZ of the ganglionic eminence (ventral telencephalon), as well as in the marginal zone (MZ) and cortical plate of the dorsal telencephalon (Figure 2). It is known that ARX expression is much lower in the cells of the cortical ventricular zone (VZ) when compared to GE cells. This was the one populations of cells we could not detect with FLAG antibody labeling, while a weak staining with anti-ARX antibody was detected (Figure 2) as previously reported (Colombo et al., 2004; Miura et al., 1997).

At E16.5 more interneurons have migrated from the GE to the cortex, and, as noted above, these cells continue to express ARX. Anti-FLAG and anti-ARX immunofluorescent labeling identified the migrating cells in the marginal zone and those that had reached the cortical plate (Figure 3).

To determine if the FLAG tagged ARX could be detected in biochemical assays, Western blot analysis of embryonic brain lysates were performed. At E12.5 few cells have migrated from the GE to the dorsal forebrain (Faux et al., 2012). Thus, lysates from isolated dorsal or ventral E12.5 forebrain were interrogated with anti-ARX (Figure 4A) and anti-FLAG (Figure 4B) antibodies. Both antibodies detected comparable levels of protein in both the dorsal and ventral forebrain, with slightly lower levels dorsally as expected (Figure 4A, B). Knowing that the vast majority of ARX positive cells in the dorsal telencephalon at this embryonic stage are derived from and located in the VZ, this result supports the conclusion that FLAG antibody can detect FLAG-ARX present in the cortical VZ by Western blot analysis, where we were not able to detect FLAG-ARX by immunofluorescence (data not shown).

Finally, we performed immunoprecipitation of ARX using embryonic brain lysates from the *Flag-Arx* knock-in mouse or wild type control (E14.5) (Figure 4C, D). The anti-FLAG antibody specifically pulled down ARX in the brain lysates prepared from *Flag-Arx* KI, but not from WT mouse (Figure 4D). Immunoprecipitation of ARX with an anti-ARX antibody was also performed as a control (Figure 4C).

In summary, we have generated a *Flag-Arx* KI mouse line and successfully detected FLAG-ARX with immunostaining as well as with Western blot using FLAG antibody. Furthermore, FLAG antibody successfully pulled down ARX *in vivo* from the brain lysates isolated from *Flag-Arx* KI mouse, with higher efficiency than the anti-ARX antibody. We anticipate this mouse line will be a useful tool for other analyses requiring ARX complex isolation (e.g., ChIP-seq) as the function of this gene is further elucidated in understanding a range of human neurodevelopmental conditions.

3. MATERIALS AND METHODS

3.1 Generation of *Flag-Arx* knock-in mouse

All animal experiments were performed in accordance with the relevant guidelines and regulations approved by the Brigham and Women's Hospital Institutional Animal Care and Use Committee. *Flag-Arx* knock-in mouse line was generated using CRISPR/Cas9 gene editing system. *Cas9* mRNA was purchased from TriLink BioTechnologies (San Diego, CA), and all the oligonucleotides were synthesized at IDT integrated DNA Technologies (Coralville, IA). Targeting sequence for *Cas9* within *Arx* genome locus was selected using an online tool (https://zlab.bio/guide-design-resources). The single guide RNA (sgRNA) was transcribed *in vitro* according to the manufacturer's instruction (HiScribe™ T7 High Yield RNA Synthesis Kit, NEB). The donor template DNA bearing FLAG tag sequence was prepared by annealing complementary Ultramer oligonucleotides. *Cas9* mRNA/sgRNA/ donor template were injected into C57BL/6 mouse eggs at the DF/HCC transgenic mouse core. In-frame insertion of FLAG tag at the N-terminus of ARX was confirmed by sequencing (Macrogen, USA). The sgRNA (5'-AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTT TAACTTGCTATTTCTAGCTCTAAAACGGTACTGATTGCTCATGGCTC-3') was transcribed using the following oligos:

Template oligo for *in vitro* transcription of sgRNA, 5'-AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTT TAACTTGCTATTTCTAGCTCTAAAACGGTACTGATTGCTCATGGCTCCCTATAGTGA GTCGTATTA-3',

T7 primer, 5'- TAATACGACTCACTATAGGG-3'.

Ultramer for donor template:

Top strand, 5'-CCCCAACACACACCCTTTTCATCCAACCCTCGAGGAGAGAGGAGCAGAGGTCCGC TCTCTGAGCCCAAGGAAAAAGCACCAGCCATGGACTACAAGGACGACGATGACA

AGAGCAATCAGTACCAGGAAGAGGGCTGCTCTGAGAGGCCGGAGTGCAAGAGTA
AATCTCCAACTTTGCTCTCCTCCTACTGCATC-3',

Bottom strand, 5'-
GATGCAGTAGGAGGAGAGCAAAGTTGGAGATTTACTCTTGCACTCCGGCCTCTCA
GAGCAGCCCTCTTCCTGGTACTGATTGCTCTTGTCATCGTCGTCCTTGTAGTCCAT
GGCTGGTGCTTTTTCCTTGGGCTCAGAGAGCGGACCTCTGCTCCTCTCTCCTCGA
GGGTTGGATGAAAAGGGTGTGTGTTGGGG-3'

For genotyping, mouse genomic DNA was isolated from tail biopsies by quick
NaOH extraction (Truett et al., 2000), and DNA fragment (Flag-Arx,462 bp; wild
type, 438 bp) and was amplified by PCR using the following oligos: Flag-Arx-
F, GGGACGGAAAGGAACAAAGATC, Flag-Arx-R, GGGACTTGTCAAGTTGGAGAC.
PCR-amplified DNA was digested with NcoI restriction enzyme which generates 142 bp and
320 bp DNA fragments only from the Flag-tag inserted allele.

A total of five founder mice were obtained as a result of Cas9 mRNA/sgRNA/donor
template injection into C57BL/6 mouse eggs. Among these, only one had the correct Flag
sequence targeted to the *Arx* locus, immediately after the initiation codon, and this line was
used for all analyses in this study after backcrossing to C57BL/6 for five generations. Two of
the other lines lost initiation codon, consequently producing 5' deletion (40 amino acid) in
Arx, and the other two were wild type. The characterized mouse line will be available to the
research community upon acceptance of the manuscript.

3.2 Immunofluorescence staining and imaging

Embryonic brains were fixed with 4% paraformaldehyde overnight and processed for
cryosection as described previously (Lim et al., 2019). Coronal sections (14 μm) were used
for immunostaining. Briefly, sections were subjected to antigen retrieval (boiled for 10–15
min in antigen unmasking solution, citrate-based, Vector Lab) (Lim et al., 2019; Shi et al.,
2020), blocked with R.T.U. animal free-blocker (Vetor Lab) for 30 min, and incubated with
primary antibody (FLAG-M2, 1:1000, Sigma-Aldrich; anti-ARX, 1: 100, sheep polyclonal,
R&D Systems) overnight at 4°C. Next, the sections used for FLAG-M2 primary antibody
were incubated with EnVision Flex+ mouse linker (raised in rabbit) (DAKO, neat) for
30min, goat anti-rabbit IgG secondary antibody conjugated with polymer-HRP, neat)
for 30min, and finally Tyramide-594 (ThermoFisher) with H_2O_2 in amplification buffer
provided by the manufacturer, for 5 min. The sections used for ARX primary antibody were
incubated with biotinylated secondary antibody (goat anti-sheep IgG, ThermoFisher, 1:200)
for 1hr and then with streptavidin-Alexa-488 (1:1000, ThermoFisher) for 30 min. DAPI was
used for nuclei staining. Images were acquired on Zeiss Observer Z1 inverted microscope
equipped with a Hamamatsu ORCA-Flash4.0 camera using Zeiss Zen Pro software.

3.3 Immunoprecipitation and Western blot analysis

For E12.5 brains, the dorsal and ventral embryonic telencephalons were dissected out
separately and used for Western blot analysis as described previously (anti-ARX antibody,
1:1000, sheep polyclonal, R&D; anti-FLAG antibody, FLAG-M2, mouse monoclonal,
1:1000, Sigma Aldrich) (Shi et al., 2020). For ARX immunoprecipitation, either the dorsal

forebrains (Figure 4C) or the whole embryonic brains (Figure 4D) were isolated from C57BL/6 mouse (Figure 4C) or *Flag-Arx* KI (Flag-Arx/y) and WT (Arx/y) male mouse (Figure 4D) at E14.5. The brains were lysed in RIPA buffer (25 mM Tris-Cl, pH 7.4, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholic acid, 0.1% SDS,1 mM DTT, 1 mM EDTA, protease inhibitors; Roche Biochem) and sonicated on ice (Branson digital sonifier 250, 5 cycles of sonication at 2 sec "On" and 2 sec "Off" at 10% output). The lysates were spun at $13,000 \times$ g for 10 min at 4°C and the cleared lysates were incubated with an anti-ARX antibody (rabbit polyclonal, generated by ARX polypeptide, in Golden lab) for 1 hr at 44°C then protein G-conjugated beads (Invitrogen), or anti-FLAG M2 magnetic beads (Sigma Aldrich) for 1 hr at 4°C. The beads were washed with RIPA buffer five times and bound proteins were eluted using 2x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer by boiling for 5 min. For Western blotting, 2% input and 30% eluate were run on 4–15% SDS-PAGE gel (Bio-Rad, Mini-PROTEAN® Precast Gels) and blotted on PVDF membrane (Bio-Rad,Trans-Blot® Turbo™ Mini PVDF Transfer Packs) using Trans-Blot® Turbo™ Transfer System (Bio-Rad). The blot was probed with anti-ARX antibody (rabbit polyclonal, against 158–181 amino acid, 1:1000, a gift from Dr. Kitamura)(Kitamura et al., 2002) or rabbit anti-FLAG antibody (1:1,000; Cell signaling Technology) and developed using ECL kit (SuperSignal Chemiluminescent Substrate, Thermo Scientific). The image was scanned using ChemiDoc MP imaging system (Bio-Rad).

Acknowledgments

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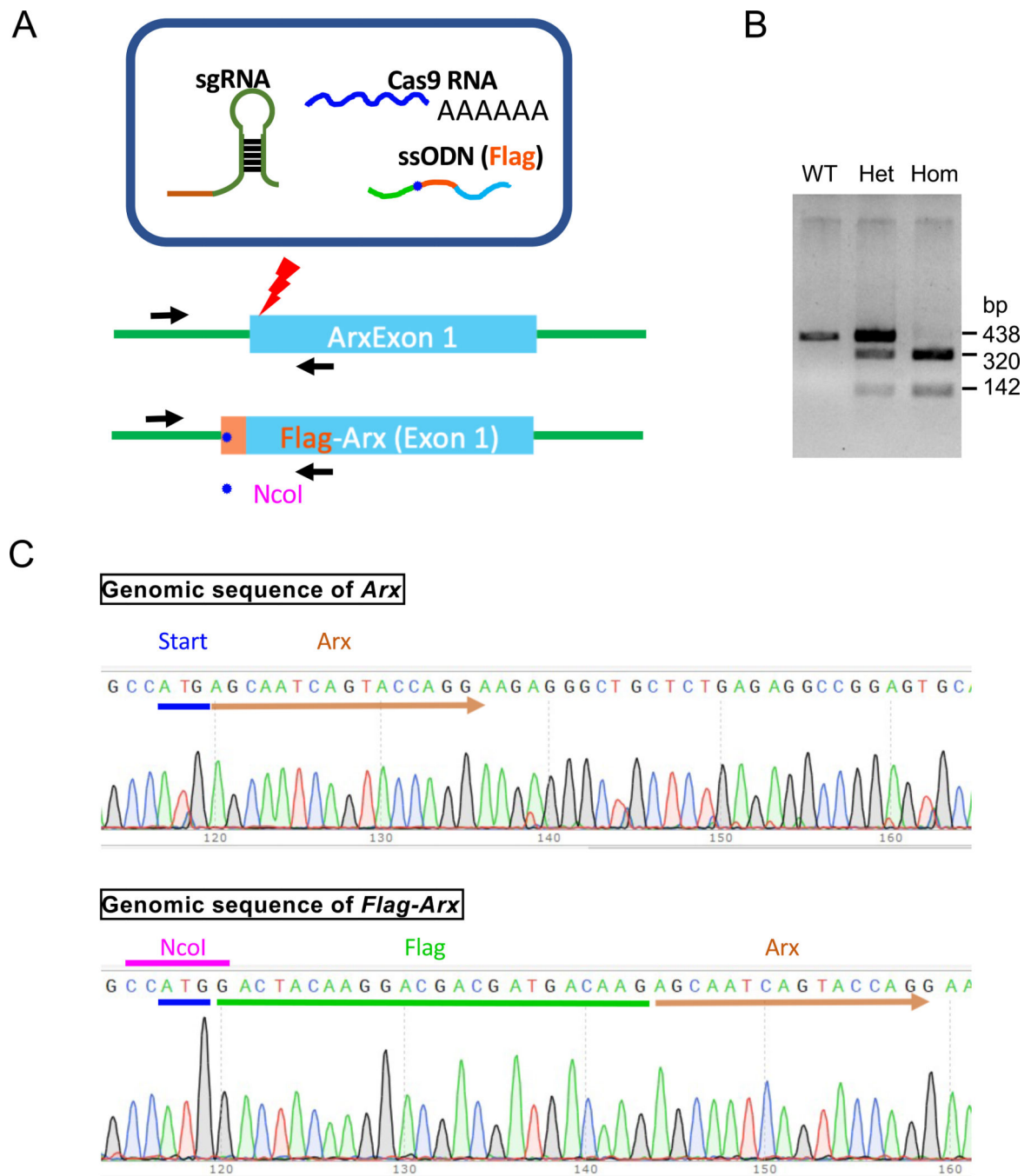
Figure 1.
Generation of *Flag-Arx* knock-in (KI) mouse with CRISPR/Cas9 system. **A.** CRISPR/Ca9-mediated genome editing strategy to generate *Flag-Arx* KI mouse. sgRNA, single guidance RNA; ssODN, single strand oligonucleotide donor. NcoI, restriction enzyme site recognized by NcoI. **B.** Genotyping results of wild type (WT), *Flag-Arx* KI het (Het), and *Flag-Arx* KI homo (Hom). bp: base pair. C. Sequencing results of genomic DNA from wild type (*Arx*) and *Flag-Arx* KI (*Flag-Arx*) mice.
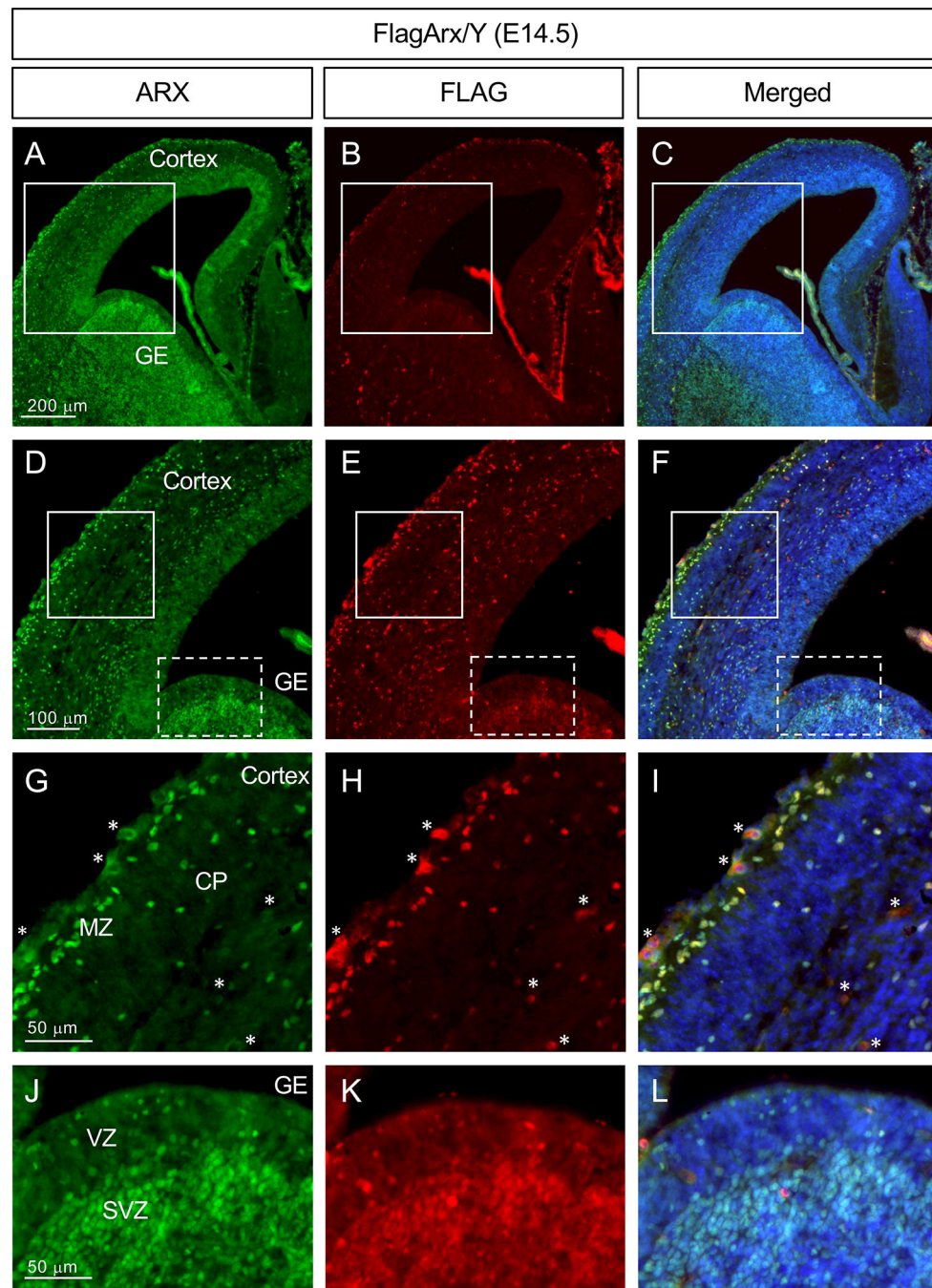
Figure 2.
FLAG-tagged ARX expression detected by anti-FLAG immunofluorescent staining.
Representative images of the embryonic cortex and ganglionic eminence (E14.5, coronal
section) of the *Flag-Arx* KI mouse stained with anti-ARX (A, D, G, J; green)- or anti-FLAG
(B, E, H, K; red) antibody (C, F, I, L: merged images). DAPI (blue) was used for nuclear
staining. D,E,F: magnified images of the boxed area in A, B, C; G,H,I: magnified images
of the solid boxed areas in D, E, F; J, K, L: magnified images of the dotted boxed areas
in D, E, F. Abbreviations: CP, cortical plate; GE, ganglionic eminence; MZ, marginal zone;

SVZ, subventricular zone; VZ, ventricular zone. Asterisks (*) in G, H and I are examples of non-specific blood vessel staining.
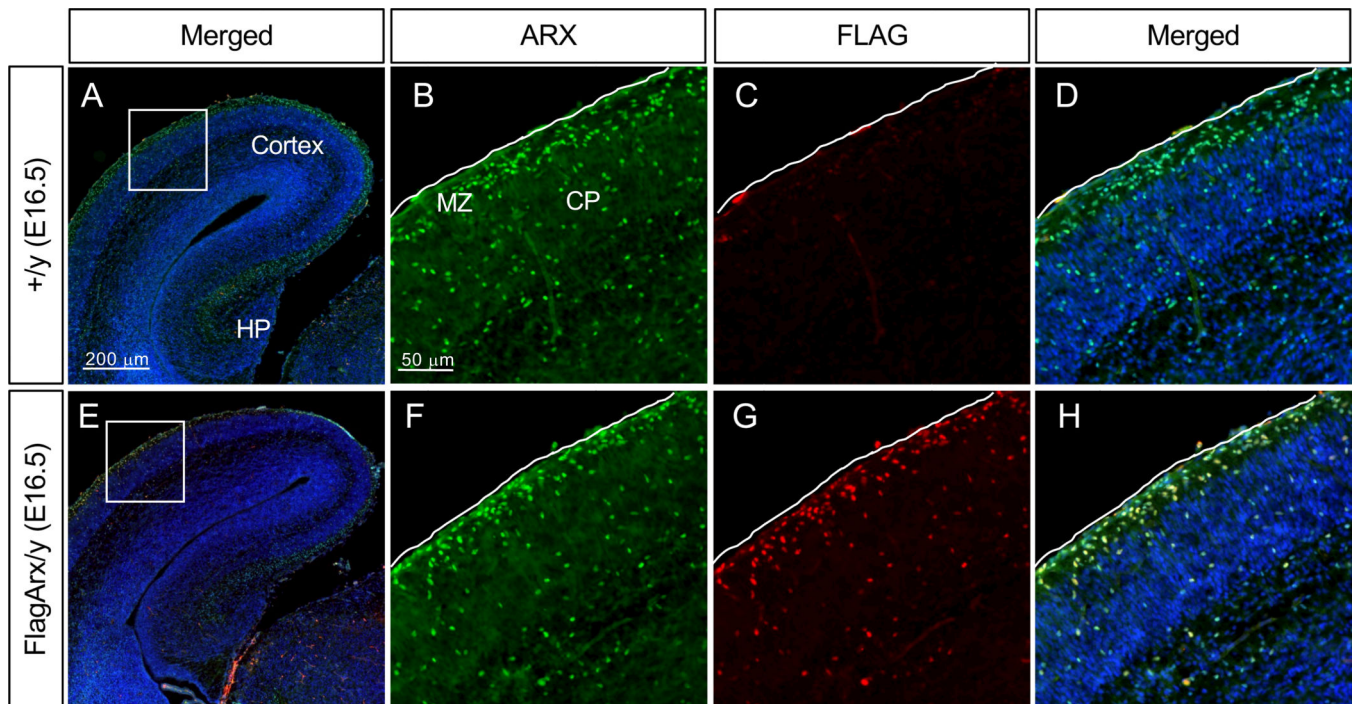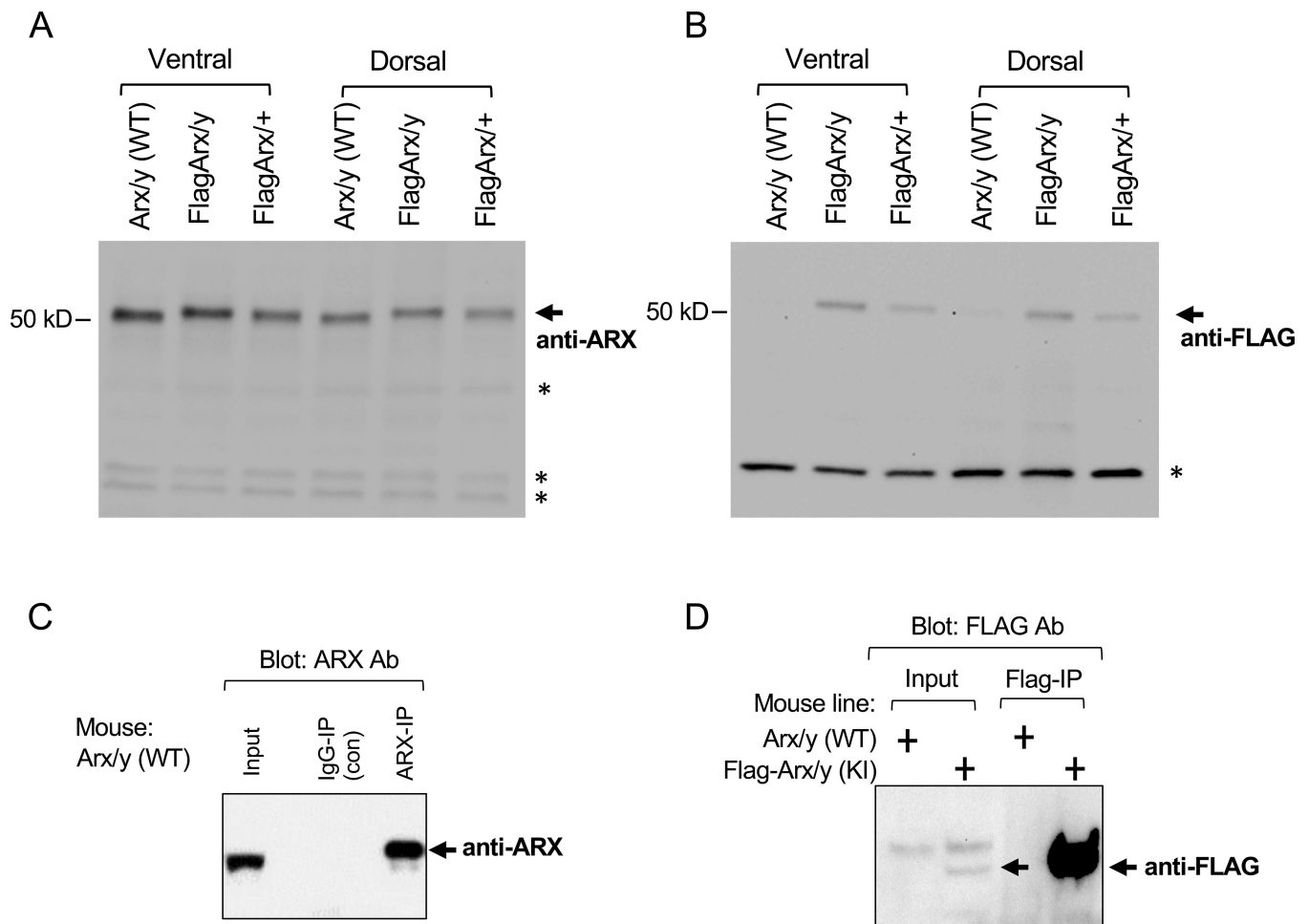
Figure 3.
FLAG-tagged ARX recapitulates normal ARX expression. **A-D**, Representative images of the wild type (+/y) embryonic cortex stained with ARX (B) or FLAG (C) antibody (E16.5). B, C, D are magnified images of the boxed area in A (merged). **E-H**, Representative images of the Flag-Arx KI (FlagArx/y) embryonic cortex stained with ARX (F) or FLAG (G) antibody (E16.5). F, G, H are magnified images of the boxed area in E (merged). DAPI (blue): nuclear staining. CP, cortical plate; HP, hippocampus; MZ, marginal zone. Pial surface is outline with white line in each image.

Figure 4.
Biochemical analysis of *Flag-Arx* KI embryonic brain. ***A-B,*** Western blot analysis of the embryonic brain lysates (E12.5) probed with anti-ARX (A) or anti-FLAG (B) antibody. Dorsal and ventral telencephalon lysates from the WT male (*Arx*/y), *Flag-Arx* KI male (*FlagArx*/y), and *Flag-Arx* KI het female (FlagArx/+), were probed with sheep anti-ARX or mouse anti-FLAG antibody. Arrow indicates expected size of ARX or FLAG-ARX band. Asterisks indicate non-specific bands. ***C-D,*** Immunoprecipitation of ARX using wild type (C, D) and *Flag-Arx* KI embryonic brain lysates (D) (E14.5). For immunoprecipitation of ARX with anti-ARX antibody using WT brain lysates (IP with rabbit anti-ARX; Western with rabbit anti-ARX 1–221 from ref. Kitamura et al.), 2% of input and 30% of IP eluate were loaded (~3% of ARX was precipitated) (C). For immunoprecipitation of ARX with anti-FLAG antibody using WT and Flag-Arx KI brain lysates (IP with mouse anti-FLAG-M2; Western with rabbit anti-FLAG), 2% of input and 30% of IP eluate were loaded (~30% of ARX was precipitated) (D).