Chapter 2: Design of Targeting Construct for Densin Deletion, Confirmation of Densin Knockout, and Initial Characterization of the Knockout Phenotype

Introduction

Derangements in synaptic transmission and plasticity are part of the pathology of numerous neurological and mental health diseases including epilepsy, schizophrenia, depression, and Alzheimer's disease. In excitatory synapses of the CNS, the postsynaptic reception, integration, and transduction of signals is mediated by the supermolecular complex of the postsynaptic density. Understanding the role that particular PSD proteins play in normal and pathological states will greatly enhance our knowledge of the underlying molecular mechanisms which contribute to overall mental health and well being.

A major step in the study of a protein's function in any biological system is the generation of a mutant phenotype that completely lacks expression of the protein. Numerous core proteins of the PSD have been studied in this manner, including PSD-95 [1], αCaMKII [2, 3], the GluR2 subunit of the AMPA receptor [4], SynGAP [5], δ-catenin [6], and Shank [7]. Knockouts have also been generated for all the subunits of the NMDA receptor, including the NR1 subunit [8, 9], NR2A subunit [10], NR2B subunit [11], NR2C subunit [12] and NR2D subunit [13]. Finally, transgenic animals have been generated with deletions in the cytoplasmic tails of the NR2A, NR2B, and

NR2C subunits of the NMDA receptor [14]. These mutant and transgenic animals have provided an immensely detailed understanding of their roles in synaptic transmission and plasticity. However, a more holistic understanding of how these core PSD proteins are functionally and structurally integrated into the supramolecular complex of the PSD still remains elusive.

As previously discussed, Densin is a core protein of the PSD and has been shown to complex with Maguin-1/ PSD-95, α-Actinin/ CaMKII, δ-catenin/ N-cadherin, Shank, β-catenin, and Nephrin. Given the facts that 1) PSD-95 and Shank are major scaffolding molecules for signaling complexes within the PSD, 2) CaMKII activity is of critical importance for integrating Ca^{2+} signaling in the PSD, 3) β-catenin, δ-catenin, N-cadherin, and nephrin are key players in mediating cell adhesion, spine morphology and dendritic arborization, and 4) α-Actinin plays a key role in actin cytoskeletal dynamics, Densin seems to sit at a major hub for cross-linking and integrating major signaling complexes within the PSD.

Here I describe the generation and initial characterization of a Densin knockout mutation in the mouse.

Material and Methods

2.1 Intron-Exon boundary structure and gene-targeting construct

Intron-Exon boundaries of Densin were determined by identifying all known cDNA and splice variants of Densin in the NCBI and Celera databases and mapping them onto the NCBI and Celera mouse genome databases.

A CITB mouse BAC library (Research Genetics) encoding genomic sequence of strain 129S1Sv (recently renamed 129S3Sv/ImJ) was screened with a cDNA probe encoding Densin Exon 3. CITB Mouse BAC Clone 456C10 hybridized with the probe for Exon 3. The BAC DNA insertion junctions were sequenced and aligned against the known genomic sequence of Densin. The presence of Exon 3 in the BAC clone was determined by PCR. A restriction map of the BAC clone was developed and a 9.9kb region, which included Exon 3 and its surrounding introns, was cloned into the pKO Scrambler 907 vector (Stratagene). The long arm of the Densin targeting construct contained a 7.8kb fragment including intron 2, Exon 3, and part of intron 3; the short arm contained a 2.1kb fragment of intron 3 (Fig 2.1). The first loxP site was inserted 4.8kb upstream of Exon 3. A hygromycin selection cassette, flanked on both sides by loxP sites (no. 2 and 3), was cloned into the short arm of the targeting sequence 1.1kb downstream from Exon 3 (Fig 2.1).

2.2 Generation of mouse embryonic stem cells for injection into blastocysts

The linearized targeting construct (25μg) was electroporated into $1x10^7$ cells/cuvette of mouse ES (CJ7) cells. Transfected ES cells were grown in the presence of hygromycin (200μg/ml) for 7-8 days to select for homologous recombinants. Two recombinant clones with both 5^1 and 3^1 construct integration were identified and confirmed by PCR. One clone (2G8) exhibited a normal karyotype and was expanded for the generation of Densin transgenic ES cells. Ninety-eight 129B6 blastocysts injected with ES cells from the 2G8 clone were implanted into seven pseudo-pregnant mothers.

Chimeric pups exhibiting a >90% agouti coat color were used for subsequent breeding. Preparation of ES cells for electroporation, injection, blastocyst implantation and breeding of chimeras were performed under the direction of Dr. Shirley Pease in the Transgenic Mouse Core Facility at Caltech (Pasadena, CA).

2.3 Knockout animal breeding strategy

Eight adult male chimeras were freely mated to C57BL6 *EIIaCre*$^{+/+}$ expressing female mice (Jackson Laboratory, ME) developed in the laboratory of H. Westphal (NIH, Bethesda. MD; Lakso et al., 1996). F1 generation offspring were screened for mosaic *Cre*-recombination patterns by PCR. F1 animals exhibiting genomic mosaicism were subsequently mated to wild-type C57BL6 mice (Fig 2.2). Segregation of the *EIIaCre* transgene and *Cre*-recombined alleles was monitored by PCR in the F2 generation. F2 generation *EIIaCre*$^{-/-}$, Densin$^{+/-}$ (total excision loxP1/3 recombination pattern) males were liberally mated to wild-type C57BL6 females to generate a large F3 population of Densin$^{+/-}$ animals for subsequent production of Densin$^{-/-}$ null mutants for experimentation.

2.4 Genotypic verification of knockout

Genomic DNA was isolated from mouse ear punch or tail samples and used for PCR. For Densin knockout genotyping a set of three primers was used: one recognizing a sequence 5^{1} of the first loxP site (LoxPrayUp; 5^{1}-GAGATGCTCTCAAGATAGACATG-3^{1}), one recognizing a sequence 3^{1} of the first loxP site (LoxPrayLow; 5^{1}-CTCCAATTCTGAAGCCAGTAG-3'), and one recognizing a sequence 3^{1} to the third loxP site (PostHygro2; 5^{1}-ACAGAACTGGCTTCTGTCCAC-

3[1]); the LoxPrayUp-LoxPrayLow and LoxPrayUp-PostHygro2 banding patterns recognize wild-type or knockout genotypes, respectively. The PCR protocol used for genotyping the Densin knockout line was as follows: 10 cycles of 95ºC/ 30sec denaturation, 58ºC/ 30sec annealing, 72ºC extension followed by 20 cycles of 95ºC/ 30sec denaturation, 56ºC/ 30sec annealing, 72ºC extension, followed by a final extension at 72ºC for 5 min. Correct banding patterns of a single 187bp fragment for *wt*, 187/ 257bp PCR fragments for heterozygous animals, and a single 257bp PCR fragment from knockout animals were observed (Fig 2.3A).

2.5 Forebrain homogenization and Immunoblot verification of knockout

Forebrains were isolated from five 6-8 week old Densin wt-*ko* pairs. Forebrains were individually homogenized (Potter-Elvehjem homogenizer) in 2.4ml buffer (4mM HEPES-NaOH pH 7.4, 0.32M sucrose, Roche Complete EDTA-free protease inhibitors, adjusted to pH 7.4 with 1N NaOH) at 900 rpm in Teflon-glass homogenizers. Homogenate was centrifuged for 10 min/ 1000 g in a Sorval SM24 rotor. Supernatant was flash frozen in liquid nitrogen and stored in 400 μl aliquots at -80ºC.

Aliquots of individual forebrain homogenates were assayed upon use for total protein concentration by the bicinchoninic acid method (Pierce, Rockford, IL) with bovine serum albumin (Thermo Fisher, Rockford, IL) as a standard, and analyzed on a Versa-Max microplate reader (Molecular Devices, Sunnyvale, CA). Equal amounts of forebrain protein (10 μg or 50 μg), adjusted for total volume, were dissolved in SDS-PAGE sample buffer, boiled at 90ºC for 5 min, and centrifuged for 2 min in an Eppendorf microcentrifuge.

Samples were fractionated by SDS-PAGE on 7.5 or 9% acrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell) in transfer buffer (25mM Tris, 200mM glycine, 20% methanol). Membranes were blocked with 5% milk in TBS buffer (20mMTris, 150mM NaCl) for 1 hour at room temperature followed by overnight incubation with primary antibody in TBS + 0.1% Tween 20 blocking buffer at 4ºC. Primary antibodies were used at the following dilutions: Densin CT245 (1:2500), M2 (1:2500), and LRR (1:1000). All Densin antibodies are described by Apperson, et al., 1996. Membranes were washed three times with TBS + 0.1% Tween 20 blocking buffer at room temperature. Bound antibodies were detected with IRdye700- or IRdye800- (1:10,000) secondary antibodies (Rockland, Gilbertsville, PA). Membranes were then washed three times with TBS + 0.1% Tween 20, followed by two washes with TBS. Blots were visualized with the Odyssey Infrared Imaging System (Fig 2.3B; Li-Cor Bioscience, Lincoln, NE).

2.6 RNA seq confirmation of exon 3 deletion and expression

Ten adult males (five wt:ko sibling pairs), 11 weeks of age were killed by cervical dislocation. Forebrains were dissected from two wt:ko sibling pairs (four animals total) and hippocampal tissues was dissected from three wt:ko sibling pairs (six animals total). The forebrain and hippocampal tissue were not harvested on the same day, but all subsequent processing was the same.

Once the brain tissue was harvested, it was immediately flash frozen in liquid nitrogen. Brain tissue was weighed and placed in mirVana lysis buffer (Cat # AM1560, Ambion, Foster City, CA). The tissue was minced and then sequentially sheared by

passage through 20g and 25g needles. The samples were allowed to sit at room temperature for 5 minutes at which time, each was supplemented with 75µl MicroRNA homogenate additive (mirVana kit). Samples were shaken by hand for 15 seconds and subsequently incubated on ice for 10 minutes. Total RNA was extracted using phenol/ chloroform, isolated using mirVana Filter Cartridges as per the manufacturers recommendations, eluted with mirVana elution buffer, and supplemented with ScriptGuard (Epicentre Biotechnologies, Madison, WI). Sample concentrations were measured using the Nanodrop quantification system (Thermo Scientific, Waltham, MA).

Residual genomic DNA was enzymatically removed using Baseline Zero DNase (Epicentre Biotechnologies). RNA was re-extracted using phenol/ chloroform and precipitated O/N with ethanol. The RNA was dried, re-suspended in 50µl dH_2O supplemented with ScriptGuard, and its concentration measured using Nanodrop. RNA was subsequently stored at -80ºC.

Oligo(dT) selection from total RNA, cDNA preparation, sequencing and read mapping was performed as previously described [15]. Analysis of the expression level of the gene loci was performed with ERANGE software as previously described [15]. Unique RPKM (reads per kilobase of exon model per million mapped reads) counts were used for subsequent statistical analysis.

Results

2.7 Genomic organization of the Densin gene

The Densin gene was located on mouse chromosome 3 at position 161,551,730 to 161,910,689, with a length of 358,959 bp. The gene contains 27 exons. Two start

codons were identified within exon 3. The first start codon is formed by the last base pair of exon 2 and the first two base pairs of exon 3. The second start codon is +16 base pairs from the first. No other methionine start codons were identified. Exons 2, 7, 9, 17, 22, 23, and 24 are predicted to be alternatively spliced. A predicted homology site for a matrix attachment region/ scaffold attachment region (MAR/SAR) was identified at -2000bp from the start of the Densin gene. Such regions are known to flank transcriptionally active chromosome regions and may contain a concentration of transcription factor binding sites [16]. Contained within exon 22 is a cDNA sequence reported from a macaque brain cDNA library (gi:9967402).

2.8 Targeting construct and breeding

Following electroporation with the exon 3 targeting construct, two ES clones were identified to have undergone homologous recombination with one showing a healthy karyotype. The mutant ES clone was injected into 98 host blastocysts and subsequently implanted into seven pseudo-pregnant females. 40 pups (F0) were born of which 14 exhibited chimeric coat coloration. Three chimeras with greater than 90% agouti coat color were mated to C57B6 females expressing *cre*-recombinase under the EIIa promoter. 8 out of 19 pups that were littered (F1) exhibited germline transmission and genomic mosaicism as determined by PCR (data not shown). Three male mosaic pups were subsequently bred to C57B6 wild type females. Litters exhibited an assortment of monogenic genotypes. Pups that were heterozygous for the complete deletion of exon 3 and the hygromycin selection cassette were denoted knockout founder animals (Fig 2.2; F2 generation). Mice retaining the LoxP flanked exon 3 but showing deletion of the

hygromycin selection cassette were denoted conditional/ floxed founder animals (F2; Fig 2.2). Both sets of F2 heterozygous founder animals were liberally bred to wt C57B6 animals to generate a large F3 population that was subsequently interbred to produce full knockout animals for experimentation.

2.9 Verification of the homozygous ko mouse

The chromosomal deletion of exon 3 in knockout mice was verified by performing PCR on genomic DNA isolated from ear punches and tail clippings (Fig 2.3a). RNA Seq analysis demonstrated that the Densin transcript is stably expressed, but lacks exon 3 and the start codon necessary for protein translation (Fig 2.4a). Immunoblots with antibodies targeting the n-terminal LRR domain, the Mucin homology domain, and the c-terminal PDZ domain confirmed that no Densin protein was expressed (Fig 2.3b)

To examine if the homologous recombination of the targeting construct and deletion of exon 3 resulted in aberrant expression of genes flanking the Densin locus, we performed an RNA seq analysis on two wt:ko sibling pairs at 11 weeks of age. No significant changes in the transcript expression levels of the seven genes flanking the Densin locus were observed (Fig 2.4b). These data indicate that the homologous recombination of the targeting construct and deletion of exon 3 do not disrupt or alter the expression levels of those genes most proximal to the Densin locus. Consequently, the observed phenotypes in this animal will not be confounded by alterations in promoter/ enhancer regions resulting from the insertion of the targeting construct.

2.10 Homozygous Densin knockout mice show a runted phenotype

Crossing of Densin heterozygous mice results in progeny with a Mendelian distribution of 1.2 wt : 2.4 het : 1 ko (n=328). Thus, the Densin mutation does not result in embryonic lethality. Once born, knockout animals are viable and are able to compete for nutritional resources as demonstrated by the presence of an abdominal milk spot (image not shown). On P0, knockout animals are indistinguishable from their wildtype or heterozygous litter mates. However, by P4 knockout animals are significantly smaller. Of 74 knockout animals born, 51 showed runted phenotypes and 23 were of normal weight and size when compared to wt and heterozygous litter mates (data not shown). This data suggests that the runt phenotype is not fully penetrant.

At three weeks of age, knockout animals are still significantly smaller compared to their litter mates (weight= -44.95%, $p<0.01$; length= -15.48%, $p<0.01$). By the time of weaning (3-4 weeks of age) 20% of the knockouts had died (11 out of 51). By 11 weeks of age the size disparity between knockout and wild type animals significantly decreases, but was still statistically significant (weight= -11.86%, $p<0.05$; length= -4.92%, $p<0.05$) (Fig 2.5).

2.11 Densin knockout mice have seizures when injected with Nembutal

Twelve wt:ko sibling pairs were injected perinatally with 100μg/gram body weight of Nembutal. The animals were immediately placed into a small fishbowl ~40cm (l) x ~15cm (w) ~21cm (h), and their behavior recorded with a high speed video camera.

Animals were injected without prior knowledge of their genotype. The onset of seizures typically occurred within 1 min 15 seconds to 1 min 45 seconds. This delay is probably associated with the length of time it takes for the Nembutal to reach the brain. All animals injected exhibited a stiffening of their tail within 30-40 seconds after injection. Nine out of twenty four animals injected progressed into full-blown seizures characterized by violent, uncontrollable spastic convulsions of their entire body. When the genotype of the animals was revealed, we determined that all nine of the animals that had seizures were knockout animals. None of the wild type animals had seizures.

Just prior to the onset of the seizure, the animals exhibited staccato like movement and began to rear. While the seizures were occurring, all of the animals exhibited a flagellar-like motion in their tail. Furthermore, their front limbs seemed to become immobile. The tonic-colonic-like seizures lasted for 30 to 45 seconds, at which time the animals ceased to move. All animals were euthanized within 3 minutes of becoming inactive.

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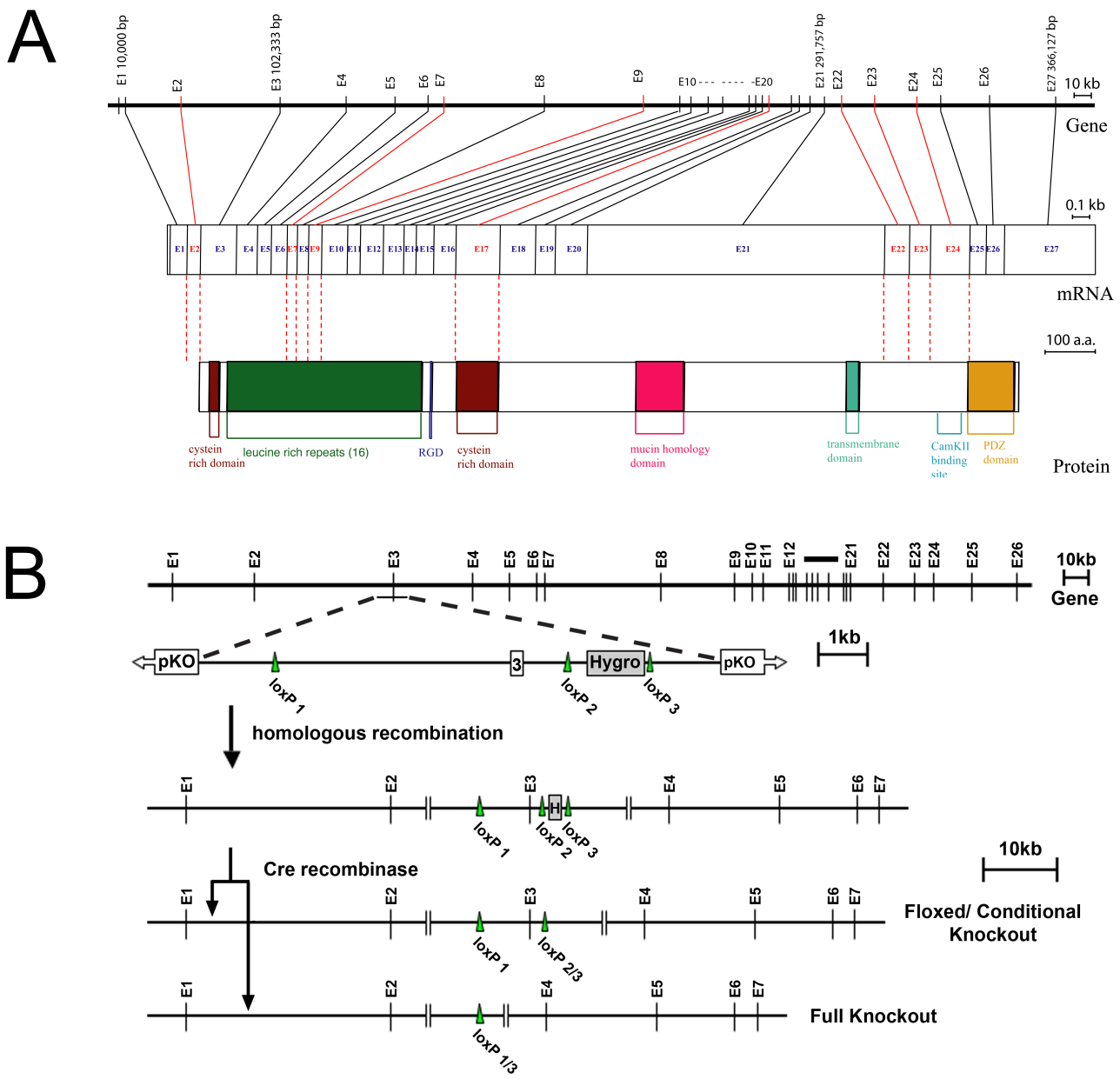


Figure 2.1 Structure of the Densin gene and design of the targeting construct. (a) The Densin gene contains 27 exons, spanning 358,959 nucleotides. Exons flanked by alternative splicing consensus sequences are marked red. cDNAs demonstrating alternative splicing of these exons have been characterized (Jiao et al., 2008). The translation start codon is contained in Exon 3, along with the first cysteine-rich domain, and the beginning of the LRR. The region encoded by exons 22, 23, and 24, and known to bind β-catenin (Heikkila et al., 2007), Shank (Quitsch et al., 2005), and CaMKII (Walikonis et al., 2001 and Strack, et al., 2000), can be alternatively spiced (Jiao et al., 2008 and Strack et al., 2000). The last two exons encode the PDZ domain. (b) The exon 3 targeting construct included a LoxP site inserted into intron 2 and a hygromycin selection cassette flanked by LoxP sites, inserted into intron 3. Expression of Cre-recombinase *in utero* results in either the deletion of the hygromycin selection cassette, resulting in a floxed exon 3/ conditional knockout, or in the deletion of both exon 3 and the hygromycin cassette, resulting in a full knockout.

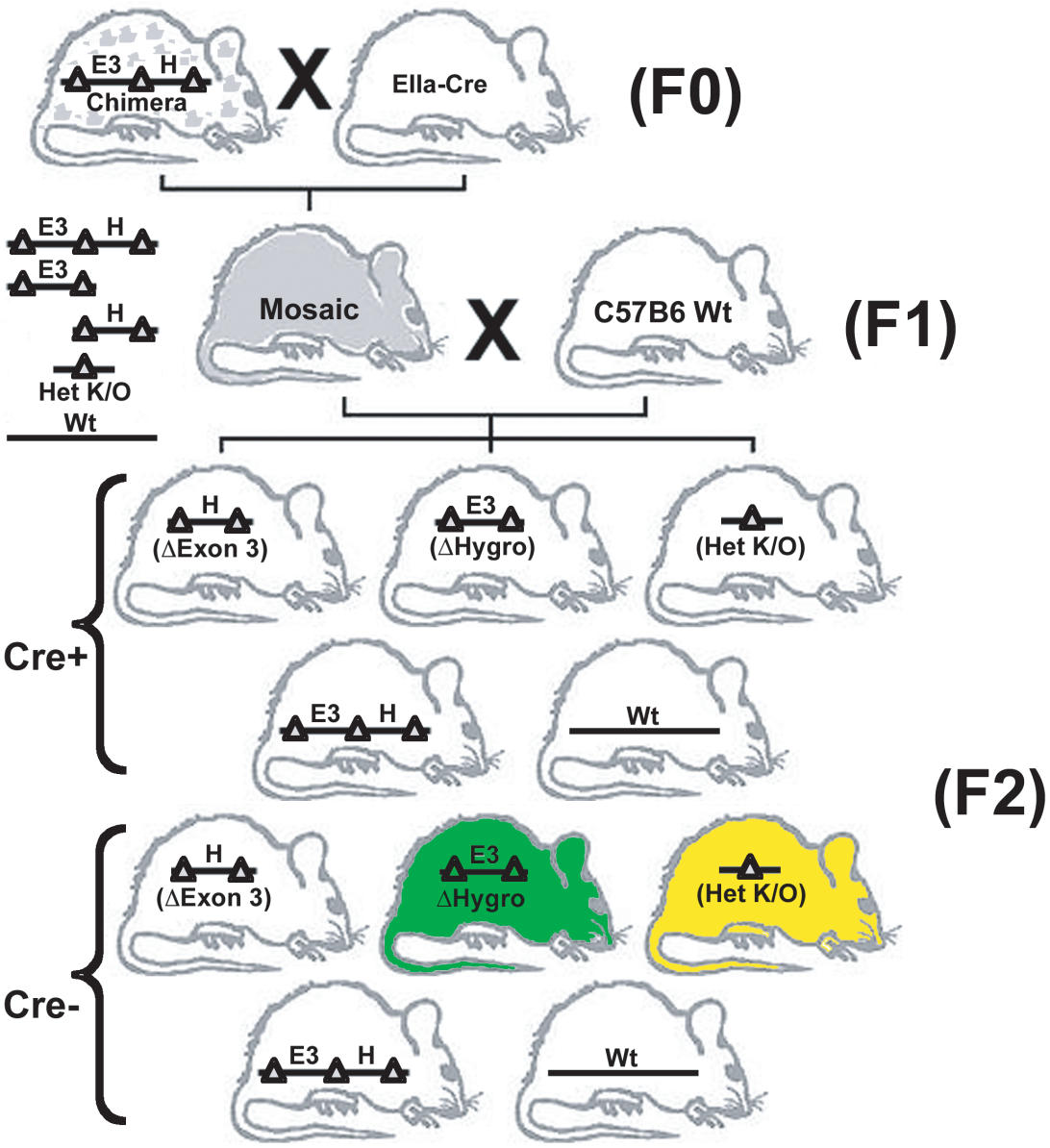


Figure 2.2: Cre- mediated germline mosaicism and breeding strategy for the generation of floxed conditional ko and full knockout animals. Chimeric males produced from implanted embryos are mated with Cre-recombinase expressing females. F1 generation males exhibiting genomic mosaicism are bred to wt C57B6 females to segregate Cre+ alleles from floxed and exon 3 deleted alleles. F2 generation ΔHygro (heterozygous floxed exon 3, green) and total Δ (heterozygous exon 3 deletion, yellow) founder animals are continuously bred to wt C57B5 animals. F3 generation exon 3 deleted heterozygous animals are interbred for subsequent experimentation.

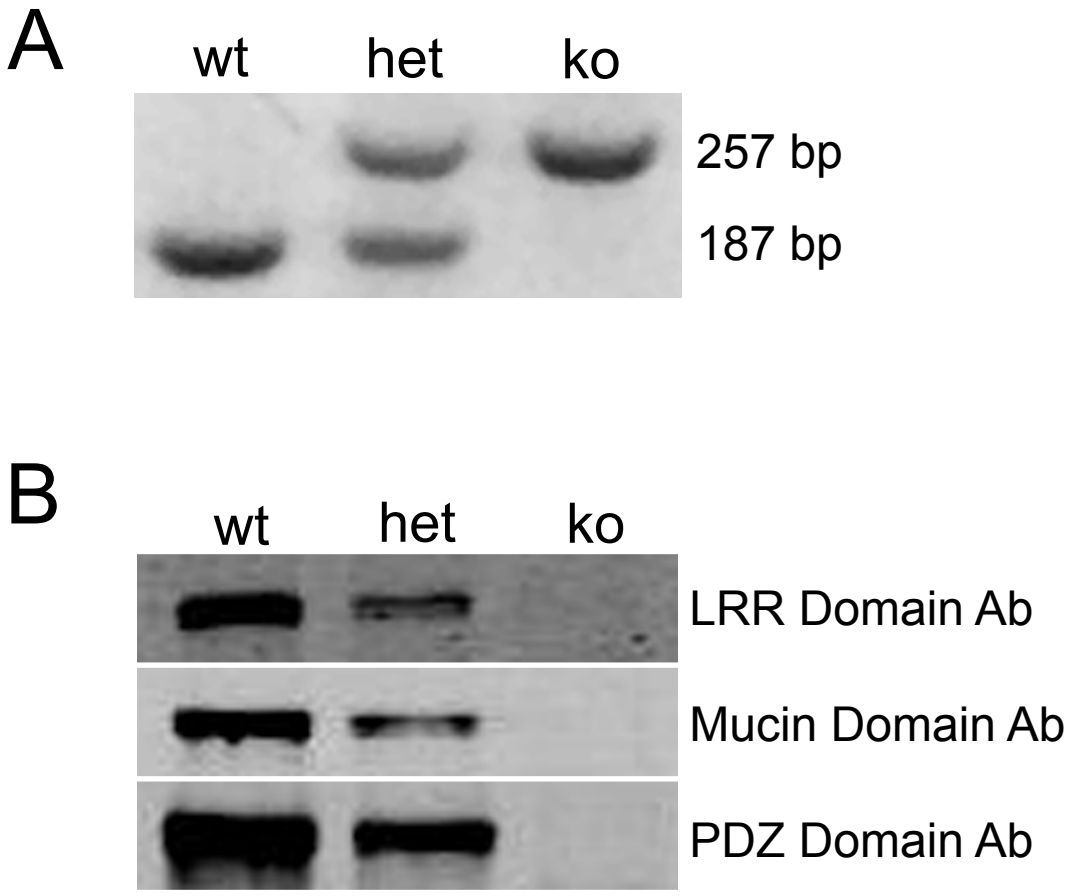


Figure 2.3 Genomic PCR and immunoblot confirmation of knockout. (a) Examples of PCR products from DNA of wildtype (wt), heterozygous (het), and knockout (ko) litter mates. (b) Immunoblot analysis of Densin protein expression in forebrain homogenates of 6-week-old animals. Antibodies to three distinct regions of Densin were used to detect expression levels of the protein.

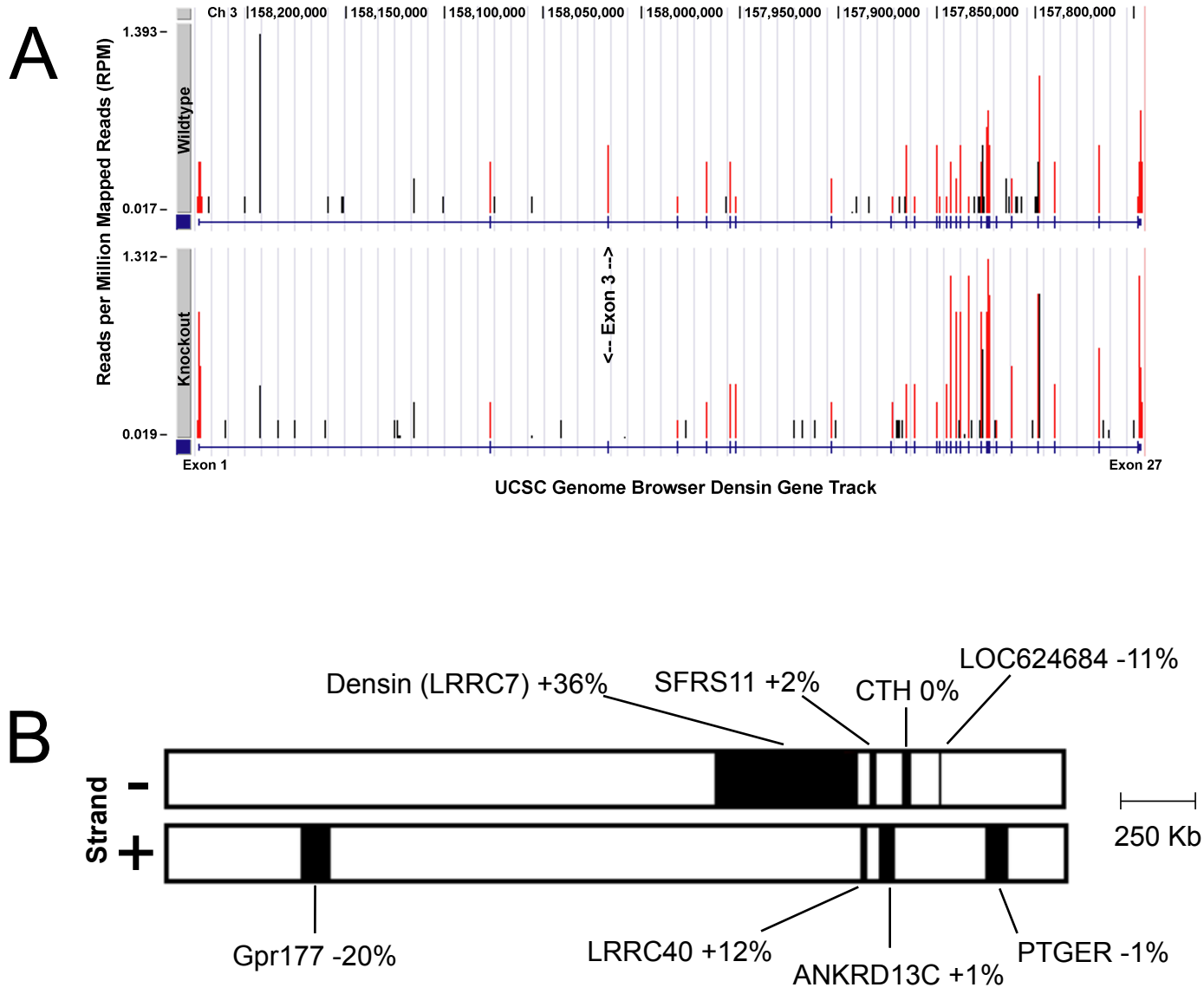
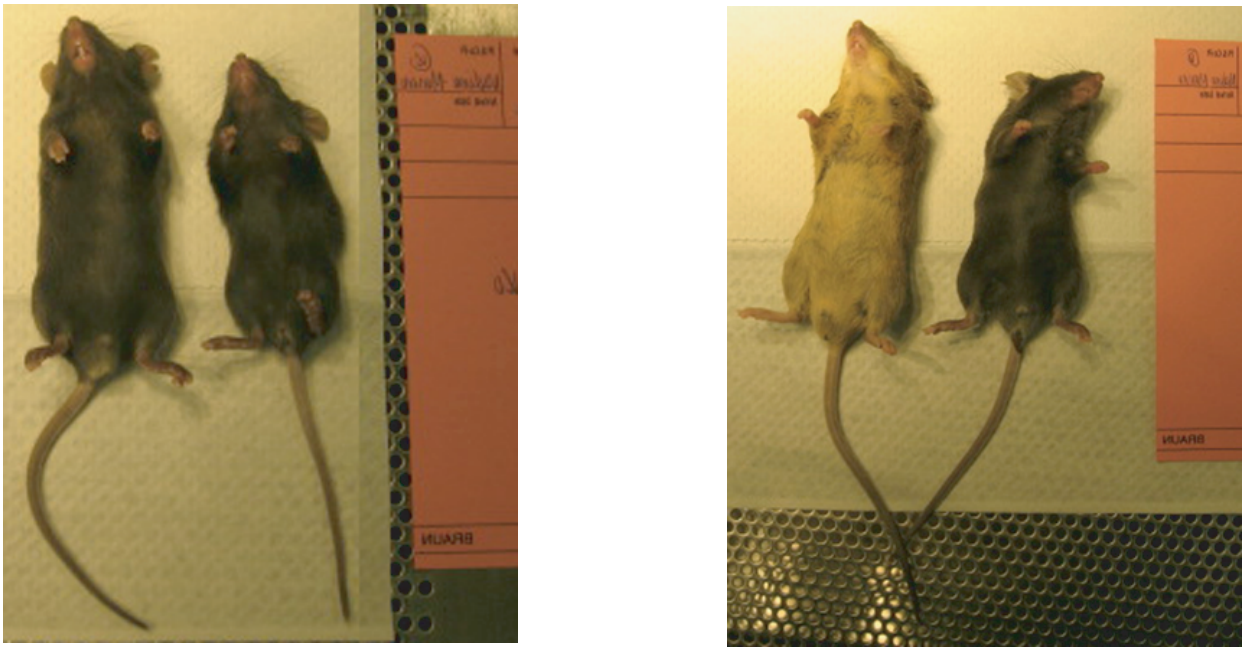


Figure 2.4 Validation of exon 3 deletion and confirmation of unaltered gene expression of genes flanking the Densin locus. (a) Comparison of wt and ko animal Densin transcripts. Exon positions (blue dashes along the horizontal axis) are predicted by the University of California, Santa Cruz, genome browser based on Refseq, Uniprot, GenBank, and comparative genomics databases (http://genome.ucsc.edu). Red bars denote RPKM counts mapped to that exon. Black bars represent the RPKM count mapped to positions outside known exon modles. Note the differences in scales for the y-axis. The deletion of exon 3 from the ko transcripts is highlighted. (b) Genes flanking the Densin locus show no significant changes in their level of gene transcription.



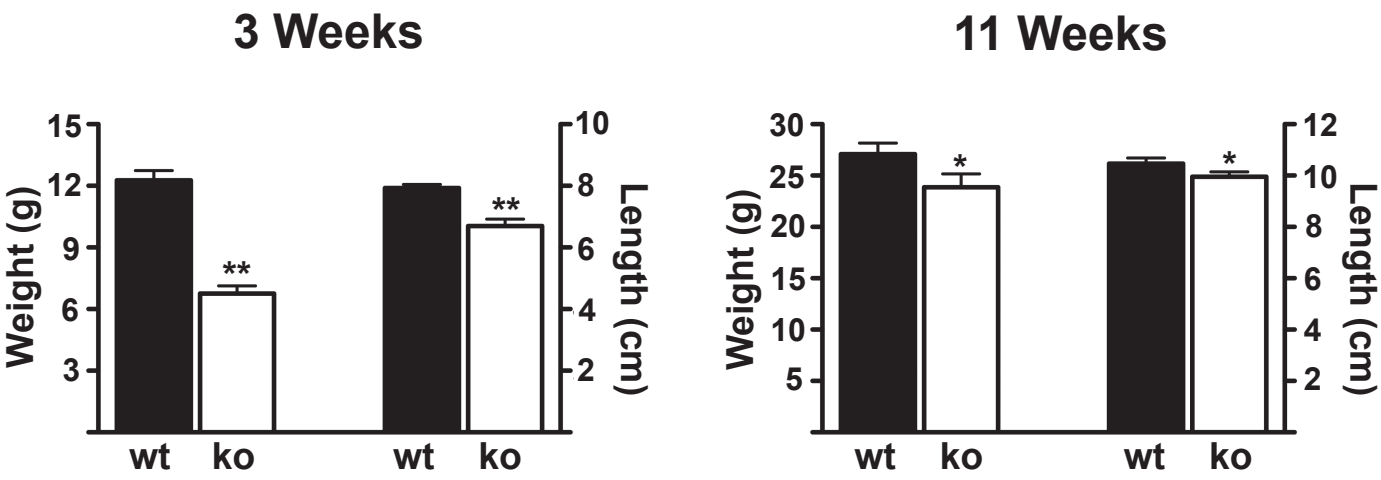


Figure 2.5 The Densin ko mutation causes a runt phenotype (image). At 3 weeks of age, knockout animals are significantly smaller than their wildtype litter mates (ko weight= -44.95%, $p<0.01$, ko length= -15.48%, $p<0.01$); no significant size variation is observed in heterozygous litter mates (data not shown). At 11 weeks of age, animals continue to exhibit a runted phenotype, though the size difference decreases (ko weight= -11.86%, $p<0.05$; ko length= -4.92%, $p<0.05$)