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## Generation and Characterization of a Conditional Allele of *Interferon regulatory factor 6*

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### Abstract

Interferon Regulatory Factor 6 (IRF6) is a critical regulator of differentiation, proliferation and migration of keratinocytes. Mutations in *IRF6* cause two autosomal dominant disorders characterized by cleft lip with or without cleft palate. In addition, DNA variation in *IRF6* confers significant risk for non-syndromic cleft lip and palate. *IRF6* is also implicated in adult onset development and disease processes, including mammary gland development and squamous cell carcinoma. Mice homozygous for a null allele of *Irf6* die shortly after birth due to severe skin, limb, and craniofacial defects, thus impeding the study of gene function after birth. To circumvent this, a conditional allele of *Irf6* was generated. To validate the functionality of the conditional allele, we used three “deleter” Cre strains: *Gdf9-Cre*, *CAG-Cre*, and *Ella-Cre*. When Cre expression was driven by the *Gdf9-Cre* or *CAG-Cre* transgenes, 100% recombination was observed as indicated by DNA genotyping and phenotyping. In contrast, use of the *Ella-Cre* transgenic line resulted in incomplete recombination, despite expression at the one-cell stage. In sum, we generated a novel tool to delete *Irf6* in a tissue specific fashion, allowing for study of gene function past perinatal stages. However, recombination efficiency of this allele was dictated by the *Cre*-driver used.

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## Keywords

IRF6; conditional allele; cleft lip and palate; Van der Woude Syndrome; epithelium; Cre/lox

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## Introduction

Interferon Regulatory Factor 6 (IRF6) is a member of the Interferon Regulatory Factor family of transcription factors. This nine-member family contains a highly conserved DNA binding domain and a less conserved protein association domain. With the exception of *IRF6*, other members of the *IRF* family have been implicated in immune response (Tamura *et al.*, 2008). Alternatively, mutations in *IRF6* cause two autosomal dominant orofacial clefting disorders, Van der Woude syndrome (VWS) and Popliteal Pterygium syndrome (PPS) (Kondo *et al.*, 2002). In addition, a DNA variant in *IRF6*, present in 30% of the population worldwide, confers significant risk for non-syndromic cleft lip and palate, one of the most common congenital defects (Rahimov *et al.*, 2008; Zuccherro *et al.*, 2004).

*Irf6* regulates the development of multiples tissues during embryogenesis. Specifically, *Irf6* is expressed in embryonic skin and other ectodermally-derived tissues (Ingraham *et al.*, 2006; Kondo *et al.*, 2002; Richardson *et al.*, 2006). More recent reports also indicated *Irf6* expression and function in mesoderm derived tissues, including the tongue (Goudy *et al.*, 2013). Knockout murine models using conventional gene targeting strategies were generated to study *Irf6* function during embryogenesis. Mice homozygous for a null allele of *Irf6* (*Irf6<sup>gt/gt</sup>*) displayed severe skin, limb, and craniofacial abnormalities (Ingraham *et al.*, 2006; Richardson *et al.*, 2006). Skin from *Irf6<sup>gt/gt</sup>* embryos at embryonic day 17.5 (E17.5) had increased proliferation of the epidermis. In addition, these embryos failed to develop a stratified epidermis, lacking both the granular and cornified layers. Thus, *Irf6* is an essential regulator of proliferation and differentiation in keratinocytes (Ingraham *et al.*, 2006; Richardson *et al.*, 2006).

Other studies indicate a role for *Irf6* in adult development and disease. In the mouse, *Irf6* is expressed throughout development of the mammary gland, reaching maximal expression during lactation (Bailey *et al.*, 2009). Interestingly, IRF6 expression is reduced or absent in breast carcinomas (Bailey *et al.*, 2005). Re-introduction of *Irf6*, along with its binding partner Maspin, into breast cancer cells resulted in cell cycle arrest (Bailey and Hendrix, 2008; Bailey *et al.*, 2005). In addition, down-regulation of *IRF6* was associated with squamous cell carcinoma (Botti *et al.*, 2011; Restivo *et al.*, 2011; Stransky *et al.*, 2011). In squamous cell carcinoma biopsies, down-regulation of *IRF6* was strongly associated with hypermethylation of CpG islands near the *IRF6* promoter (Botti *et al.*, 2011). Children with VWS have increased risk of wound complications following surgical repair of orofacial clefts, suggesting a role for *IRF6* in wound healing (Jones *et al.*, 2010). Studies of wound closure in *Irf6<sup>gt/gt</sup>* embryos indicate a role for *Irf6* in the regulation of cell migration (Biggs *et al.*, 2012). In sum, *Irf6* has been implicated in both congenital and adult disease processes of significant clinical impact. However, studies of *Irf6* function post embryogenesis are precluded by current mouse models because the loss of *Irf6* in the mouse results in perinatal

lethality. The aim of this work was to develop a conditional allele of *Irf6* to allow investigation of gene function post-embryogenesis in a spatial and temporal fashion.

## Results and Discussion

To generate a conditional allele of *Irf6* (*Irf6<sup>fl</sup>*), we utilized a three *LoxP* site strategy (Fig 1a). Two *LoxP* sites flanked a *Pgk-Neo* selectable marker cassette in intron four, and a third *LoxP* site was located in intron two (*Irf6<sup>neo</sup>*). Recombination between *LoxP* sites flanking the selectable marker produced a floxed allele with exons three and four being flanked by *LoxP* sites. Recombination between the remaining *LoxP* sites would delete exons three and four (*Irf6<sup>fl</sup>*). This allele is predicted to be a null allele, since exon three encodes a critical region of the DNA Binding Domain, and excision of these exons results in frameshift and the introduction of a premature stop codon.

Embryonic stem cells containing the recombinant clones were acquired by G418 selection followed by PCR-based screening (Fig 1b). Excision of the floxed *Pgk-Neo* cassette in ES cells (SV129) was conducted by transfection with a Cre-expression vector (de Greef *et al.*, 2016). PCR-based genotyping identified clones with the wild type, *Irf6<sup>neo</sup>*, *Irf6<sup>fl</sup>* and the *Irf6<sup>nl</sup>* alleles. Southern blot analysis confirmed correct targeting and subsequent recombination to generate the *Irf6<sup>nl</sup>* allele (Fig 1c). Chimeric mice were generated by injection of ES cells containing the *Irf6<sup>fl</sup>* allele into C57Bl/6J blastocysts. Mice homozygous for the *Irf6* conditional allele (*Irf6<sup>fl/fl</sup>*) were viable and born at the expected Mendelian ratio (data not shown). Also, they showed no developmental or reproductive defects when compared to *Irf6<sup>+/+</sup>* or *Irf6<sup>fl/+</sup>* littermates, suggesting that this allele does not affect normal gene function.

To test the functionality of the *Irf6<sup>fl</sup>* allele, we employed the *Gdf9-Cre* transgenic mouse line (*tg<sup>Gdf9-Cre/+</sup>*). The *Gdf9* promoter is sufficient to drive expression of Cre recombinase in the oocyte beginning at postnatal day three (Lan *et al.*, 2004). Thus, recombination at the *Irf6* locus could only occur in females carrying the *Cre* transgene and the conditional *Irf6* allele (*tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>fl/+</sup>*). In *tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>fl/+</sup>* females, we expected a recombination event in the oocyte to produce *Irf6<sup>+</sup>* or *Irf6<sup>nl</sup>* gametes. To test this, *tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>fl/+</sup>* females were mated to *Irf6<sup>gt/fl</sup>* males. If recombination of the conditional allele was complete, we expect 25% of embryos to phenocopy embryos deficient for *Irf6* (Ingraham *et al.*, 2006). From these matings, six litters were collected at weaning (N = 43 pups). We observed a loss of two expected genotypes, *tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>gt/nl</sup>* and *tg<sup>+/+</sup>; Irf6<sup>gt/nl</sup>* ( $\chi^2$ -test; p=0.002; Table 1). These data were consistent with the perinatal lethality observed in *Irf6<sup>gt/gt</sup>*. Additionally, we note the presence of *Irf6<sup>fl/nl</sup>* and *Irf6<sup>fl/+</sup>* animals and the absence of *Irf6<sup>nl/nl</sup>* or *Irf6<sup>nl/+</sup>* animals, suggesting that the conditional allele from the sire did not recombine.

To confirm if the observed underrepresentation of genotypes was due to peri-natal lethality of mutant pups, we performed timed matings between *tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>fl/+</sup>* females and *Irf6<sup>gt/+</sup>* males. Pregnant females were sacrificed at E17.5 and three litters were collected (N = 34 embryos). We observed nine embryos that phenocopied *Irf6<sup>gt/gt</sup>* embryos (Fig 2a,b) and were referred to as conditional knockouts (cKO). In addition to severe skin, limb, and craniofacial abnormalities, cKO embryos had impaired barrier function (Fig 2c,d) and severe

oral adhesions (Fig 2e,f). These embryos lacked the *Irf6<sup>fl</sup>* allele and were positive for the *Irf6<sup>fl</sup>* and *Irf6<sup>gt</sup>* alleles (Fig 2e). Notably, the mutant phenotype was independent of the presence of the *Gdf9-Cre* transgene, indicating that the *Gdf9-Cre* mediated recombination of the *Irf6<sup>fl</sup>* allele occurred prior to completion of meiosis I. Furthermore, these data demonstrate that the *Irf6<sup>fl</sup>* allele is capable of efficient recombination, resulting in a null allele.

Histological analysis of embryonic skin from wildtype, *Irf6<sup>gt/gt</sup>* and cKO embryos showed an expanded epidermis (Fig 3a–c). To confirm the molecular profile, wildtype, *Irf6<sup>gt/gt</sup>*, and cKO embryos were immunostained at E17.5. In wildtype embryos, Irf6 expression was seen throughout the spinous and basal layers of the epidermis. As expected, this expression was lost in *Irf6<sup>gt/gt</sup>* and cKO embryos (Fig 3d–f). In wildtype skin, Keratin14 (K14) was restricted to the basal layer. K14 expression was expanded throughout the epidermis in *Irf6<sup>gt/gt</sup>* and cKO embryos (Fig 3g–i). A similar pattern of expression was observed for p63, another marker of the basal layer and a pro-proliferation gene (Fig 3k–l). Lastly, expression of loricrin, a marker of terminally differentiated keratinocytes, was completely lost in cKO embryos (Fig. 3m–o). These results are consistent with those observed in *Irf6*-deficient skin (Ingraham *et al.*, 2006).

To further confirm the functionality of the *Irf6<sup>fl</sup>* allele, we utilized two additional *Cre* transgenic lines. First, we used *CAG-Cre*, where *Cre* expression is driven by the cytomegalovirus immediate early enhancer-chicken beta-actin promoter in mature oocytes. In this line, *Cre* mRNA or protein was stored in the oocyte and can facilitate recombination following fertilization (Sakai and Miyazaki, 1997). *CAG-Cre* transgene positive (*tg<sup>CAG-Cre/+</sup>; Irf6<sup>+/+</sup>*) animals were mated to *Irf6<sup>fl/fl</sup>* animals (N=30 offspring). Genotypic analysis indicated that ten animals were *tg<sup>CAG-Cre/+</sup>*. These animals showed complete recombination of the *Irf6<sup>fl</sup>* allele, as evidenced by the presence of only the *Irf6<sup>fl</sup>* allele (supplementary Table 1).

We also used the classic deleter strain, *Ella-Cre*, which targets expression of the recombinase to the stages of embryogenesis preceding implantation. We mated *tg<sup>Ella-Cre/+</sup>; Irf6<sup>gt/+</sup>* animals with *Irf6<sup>gt/fl</sup>* animals (N=124 embryos). This allowed us to produce wildtype or mutant embryos carrying two genotypes: 1) *Irf6<sup>gt/gt</sup>* or 2) *tg<sup>Ella-Cre/+</sup>; Irf6<sup>gt/fl</sup>*. Sixty-five embryos were *tg<sup>Ella-Cre/+</sup>*. Of these embryos, nineteen were also positive for the *Irf6<sup>fl</sup>* allele but showed no evidence of recombination. We observed eight *tg<sup>Ella-Cre/+</sup>* embryos that showed incomplete recombination, as indicated by the presence of both the *Irf6<sup>fl</sup>* and *Irf6<sup>fl</sup>* alleles (Fig 4). These embryos were grossly normal. Nine *tg<sup>Ella-Cre/+</sup>* embryos showed complete recombination in tail tissues, as indicated by presence of the *Irf6<sup>fl</sup>* only (Table 2). Strikingly, only one embryo phenocopied *Irf6<sup>gt/gt</sup>* embryos (*tg<sup>Ella-Cre/+</sup>; Irf6<sup>gt/fl</sup>*), even though three embryos had the correct genotype to generate the mutant phenotype in the event of recombination (*tg<sup>Ella-Cre/+</sup>; Irf6<sup>gt/fl</sup>*). This finding was in accordance with published results where complete recombination using the *Ella-cre* allele was only observed in 50% of animals (Lakso *et al.*, 1996).

We created a conditional allele for *Irf6*. While the allele is capable of recombination, we observed variable efficiency that appeared to be dependent on the cell type. While this could

simply reflect variable expression or activity of individual Cre transgenes or recombinase, respectively (McLellan *et al.*, 2017) (Bao *et al.*, 2013), we hypothesize that a *cis* effect at the *Irf6* locus contributes to the variable efficiency. This hypothesis is based on the following: 1) In the current study, we observed no or incomplete recombination with the *Ella-Cre* “deleter strain, and with two other tissue specific Cre transgenes, including *K14-Cre-ER* (Vasioukhin *et al.*, 1999) and *Tgfb3-Cre* (Yang *et al.*, 2008) (data not shown). And 2), A previous study used this *Irf6<sup>fl</sup>* allele to examine the role of *Irf6* in dental epithelium, using the *Pitx2-Cre* transgene (Chu *et al.*, 2016). While the *Pitx2-Cre* driver was previously shown to be competent to knockout other genes in oral epithelium that were required for palatogenesis (Xiong *et al.*, 2009) (He *et al.*, 2011), and *Irf6* function in oral epithelium is required for palatogenesis (Ingraham *et al.*, 2006; Richardson *et al.*, 2009), no pathology was observed during palatogenesis (Chu *et al.*, 2016). Thus, the failure of multiple Cre drivers to recombine the *Irf6<sup>fl</sup>* allele is consistent with the hypothesis that a *cis* effect at the *Irf6* locus is inhibiting Cre recombination.

While, we do not know the mechanism underlying the tissue dependence of Cre recombination at the *Irf6* locus, one possible explanation is variation in chromatin structure at the *Irf6* locus. In support of this hypothesis, a recent study showed that hypermethylation of the *IRF6* promoter region was associated with down-regulation of expression in squamous cell carcinoma (Botti *et al.*, 2011; Rotondo *et al.*, 2016; Stransky *et al.*, 2011). DNA methylation is associated with nucleosome compaction, rendering the DNA inaccessible to transcription factors required to facilitate gene expression. As a result, in tissue types where *Irf6* is methylated, we hypothesize that the DNA was inaccessible to Cre recombinase. Long and Rossi (2009) showed that methylation of promoter elements upstream of reporter genes activated by Cre expression (Z/AP and Z/EG strains) inhibited Cre-mediated recombination (Long and Rossi, 2009). Further studies to determine the methylation state of *Irf6* in different cell types must be done to confirm this hypothesis.

Finally, we note that *Ella-Cre* was not an effective transgene for assessing the functionality of newly derived conditional alleles. Instead, we propose the usage of oocyte specific Cre-drivers, such as *Gdf9-Cre*, or other more efficient one-cell stage drivers, such as *CAG-Cre*, to validate the functionality of conditional alleles. However, it is important to note that the mating strategy employed for these studies may have promoted the observed recombination inefficiency. It would be beneficial to address recombination with *tg<sup>Ella-Cre/+</sup>; Irf6<sup>fl/+</sup>* females.

## Methods

### Generation of a conditional allele for *Irf6*

Mouse BAC clone (RPCI22-516G1) was digested with restriction enzymes. A 1.8 kb *KpnI*/*Bam*HI fragment for the 5′-arm and a 3.9 kb of *Bam*HI/*Hind*III fragment for the 3′-arm were cloned into pBluescript II SK(-) (Agilent Technologies). Three kilobases (kb) of the *Bam*HI fragment, containing exons three and four, was cloned into the ploxP3-Neo-pA vector (kind gift from Professor Takeshi Yagi, Osaka University). 5.8 kb of the *Xba*I fragment which contains floxed exons and *Pgk-Neo* cassette was subcloned into the *Bam*HI site between 5′- and 3′-arms (Fig. 1a). The resulting targeting construct was digested with

*NotI* and electroporated into mouse R1 ES cells. After G418 selection, ES cells were screened by PCR. Primer set of 5'-GAGAAATAGGGCCTTCACGGTG-3' (sense) and 5'-TGTGCCCTCTGATGCTGGAACAG-3' (antisense) for 5'-side, 5'-TCGCCCTTCTTGACGAGTTCTTCTG-3' (sense, in *Pgk-Neo* cassette) and 5'-GCTCAACTCCCTTTGTGACTGTCC-3' (antisense) for 3' side were used (Fig 1b). Recombinant ES clones were used for establishment of *Irf6* hypomorphic mouse (*Irf6<sup>neo</sup>*). To establish floxed exons (*fl*) and null (*nl*) strains, the *Pgk-Neo* cassette and the floxed exons, respectively, were removed in the recombinant ES cells by transfection with a *Cre* expression vector (de Greef *et al.*, 2016). Resultant clones were then screened by Southern hybridization. Genomic DNA was digested with *ApaI* or *NheI* and hybridized to a 5' or 3' probe, respectively (Fig 1c). The 5' probe corresponded to a region in intron two. The 3' probe corresponded to a region within exon and intron seven. Hybridization with the 5' probe following restriction enzyme digest resulted in two products, 11.3kb (wildtype allele) and 8.3kb (null). Hybridization with the 3' probe also produced two fragments, 14.3kb (wildtype) and 11.3kb (null). The 5' hybridization probe was amplified with primer set 5'-AGTTGTGACTGACTGTAGGATCAGG-3' (forward) and 5'-ACCAAACTTCACCAGGAGTATAGGA-3' (reverse). The 3' hybridization probe was amplified with primer set of 5'-AGAGTAAAGAATGGTTGTCAGTGGAG-3' (forward) and 5'-GACACCAGTATTCAAGAGGATTGAG-3' (reverse) (Fig 1c). To generate a conditional mouse line for *Irf6*, embryonic stem cells carrying the *Irf6<sup>fl</sup>* allele were injected into C57Bl/6J blastocyst and inserted into pseudo-pregnant dams. Chimeric males were mated to C57Bl/6J females and germline transmission was determined by Polymerase Chain Reaction (PCR)-based genotyping of progeny. The *Irf6<sup>fl/fl</sup>* mice reported here are available to the scientific community.

### Mouse and embryo genotyping

To attain genomic DNA, tail tissue was digested in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 0.1% SDS) with Proteinase-K (20mg/ml, Roche) at 55°C, overnight, followed by ethanol precipitation of DNA. PCR-based genotyping was used to identify alleles (see below). All PCR was conducted using JumpStart REDTaq Ready Mix (Sigma-Aldrich, St. Louis, MO) and separated and visualized by electrophoresis on 1.5% agarose gels.

### Detection of the *Irf6<sup>neo</sup>*, *Irf6<sup>fl</sup>*, and *Irf6<sup>nl</sup>* alleles

Primer set of 5'-GCAGAGTGGAGCACACTTCA-3' and 5'-AAGCATGTCTATTTGGGGGTT-3' was used to determine the absence of the 5' *LoxP* site in the *Irf6<sup>+</sup>* (221 bp) but its presence (570 bp) in the *Irf6<sup>fl</sup>* and *Irf6<sup>neo</sup>* alleles. Primer set of 5'-TGGCAAAATCTATTTGAGTGG-3' and 5'-CACACTGACCTCAATGTCCAA-3' was used to determine the absence of the 3' *LoxP* site in the *Irf6<sup>+</sup>* (222 bp) but its presence (379 bp) in the *Irf6<sup>fl</sup>* alleles. This primer set also distinguished the *Irf6<sup>fl</sup>* and *Irf6<sup>neo</sup>* alleles, as these primers do not amplify a product from the *Irf6<sup>neo</sup>* allele under these conditions because the expected product is too large. Primer set of 5'-GCAGAGTGGAGCACACTTCA-3' and 5'-CACACTGACCTCAATGTCCAA-3' was used to detect the *Irf6<sup>nl</sup>* allele (499 bp). PCR was performed as follows: 1) 95°C for two



minutes, 2) 95°C for 15 seconds, 3) 55°C for 15 seconds, 4) 72°C for 45 seconds, 5) repeat steps 2–4 35 times, 6) 72°C for five minutes.

### Detection of the *Irf6<sup>gt</sup>* allele

Primer set of 5′-*GACCAGACCGTGCAGGGGCTGTGG-3′* and 5′-*GAGAGGCTAGGGTGGAAAGGGATTC-3′* identifies the *Irf6<sup>gt</sup>* allele (283bp). PCR conditions were: 1) 95°C for three minutes, 2) 95°C for 38 seconds, 3) 60°C for 50 seconds, 4) 72°C for 15 seconds, 5) repeat steps 2–4 40 times, 6) 72°C for five minutes. PCR Master Mix was supplemented with 5M Betaine monohydrate to aid in amplification of GC-rich regions.

### Detection of Cre transgenes

The *Gdf9-Cre* transgene was detected as described by Lan *et al* (2004) using primer set of 5′-*TCTGATGAAGTCAGGAAGAACC-3′* and 5′-*GAGATGTCCTTCACTCTGATT-3′*. PCR conditions were: 1) 95°C for five minutes, 2) 95°C for one minute, 3) 58°C for two minutes, 4) 72°C for one minute, 5) repeat steps 2–4 35 times, 6) 72°C for five minutes. Master mix was supplemented with 5 Molar (5M) Betaine monohydrate to aid in amplification of GC-rich regions.

The *Ella-Cre* transgene was detected using primer set of 5′-*GCGGTCTGGCAGTAAAACTATC-3′* and 5′-*GTGAAACAGCATTGCTGTCACCT-3′*. PCR conditions were: 1) 94°C for three minutes, 2) 94°C for 30 seconds, 3) 51.7°C for one minute, 4) 72°C for one minute, 5) repeat steps 2–4 35 times, 6) 72°C for five minutes.

The CAG-Cre transgene was detected using primer set of 5′-*CCTACAGCTCCTGGGCAACGTGC-3′* and 5′-*CTAATCGCCATCTTCCAGCAGG-3′*. PCR conditions were: 1) 94°C for three minutes, 2) 94°C for 30 seconds, 3) 60°C for 30 seconds, 4) 72°C for two minutes, 5) repeat steps 2–4 30 times, 6) 72°C for five minutes.

### Mating Strategies

**Gdf9-Cre**—A male hemizygous for the *Gdf9-iCre* transgene (*tg<sup>Gdf9-Cre/+</sup>*) was purchased from Jackson Labs ([www.jax.org](http://www.jax.org)) and mated to *Irf6<sup>fl/fl</sup>* females to generate females carrying both the *Cre* transgene and one copy of the *Irf6<sup>fl</sup>* allele (*tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>fl/+</sup>*). These females were mated to compound heterozygous males for *Irf6* (*Irf6<sup>gt/fl</sup>*). Tail snips were collected from pups upon weaning and genotyped. A chi-squared test was used to determine deviations from expected Mendelian ratios.

*Tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>fl/+</sup>* females were then placed into timed matings with males heterozygous for a null allele of *Irf6* (*Irf6<sup>gt/+</sup>*). The presence of a copulation plug was denoted as embryonic day 0.5 (E0.5) and embryos were collected on E17.5.

**Ella-Cre**—A homozygous *Ella-Cre* male (*tg<sup>Ella-Cre/Ella-Cre</sup>*) was purchased from Jackson Labs ([www.jax.org](http://www.jax.org)) and mated to *Irf6<sup>gt/+</sup>* females to produce double heterozygous *tg<sup>Ella-Cre/+</sup>; Irf6<sup>gt/+</sup>* mice. Timed matings were set up between *tg<sup>Ella-Cre/+</sup>; Irf6<sup>gt/+</sup>* animals and *Irf6<sup>gt/fl</sup>* animals.

**CAG-Cre**—Validation of the *Irf6<sup>fl</sup>* allele using *CAG-Cre* was conducted multiple ways. 1) Females homozygous (*tg<sup>CAG-Cre/CAG-Cre</sup>*) or heterozygous (*tg<sup>CAG-Cre/+</sup>*) for the *CAG-Cre* transgene were mated to an *Irf6<sup>fl/fl</sup>* males. 2) *Tg<sup>CAG-Cre/+</sup>* males were mated to *Irf6<sup>fl/fl</sup>* females. Litters were born and tail snips were collected upon weaning and subjected to PCR based genotyping. A chi-squared test was used to verify Mendelian ratios.

**Embryo Collection and processing**—On E17.5, pregnant females were sacrificed using isoflurane induced comatosis followed by cervical dislocation. Embryos were decapitated. Heads and bodies were fixed overnight in 10% neutral buffered formalin at 4°C. Following fixation, embryos were processed and embedded in paraffin by the Histopathology Laboratory at Michigan State University using standard protocols. All animals were used in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Michigan State University Institutional Animal Use and Care Committee.

**Histological analysis**—Whole heads from embryos at E17.5 were sectioned sagittally at 7µm. Sections were then stained with hematoxylin and eosin (H&E). Briefly, for deparaffinization and rehydration, sections were passed through three changes of xylene followed by washes through reducing grades of ethanol. Slides were then incubated in Gill's Hematoxylin No. III (Sigma-Aldrich, St. Louis, MO) for 1.5 minutes, washed briefly in tap water, and incubated in a 1% Eosin solution (Eosin Y-VWR, West Chester, PA) for 1.5 minutes. To dehydrate, slides were passed through increasing grades of ethanol. Lastly, slides were mounted using Permount mounting media (VWR, Radnor, PA). Stained sections were imaged using a Nikon 90i upright microscope.

**Dye Exclusion Assay**—The dye exclusion assay was carried out as described by Ingraham, et al (2006). Briefly, whole embryos were collected at E17.5 and fixed in 100% methanol for five minutes. Following fixation, embryos were rinsed briefly in 1X PBS and stained in 0.1% toluidine blue for one minute. Staining was followed by washing with 1X PBS.

**Immunofluorescence**—For immunofluorescent detection of markers of the epidermis, sections were deparaffinized and rehydrated in reducing concentrations of ethanol. Sections were subjected to antigen retrieval by boiling in 10mM Sodium citrate (pH6.0) for 30 minutes. Tissues sections were permeabilized using 0.5% Triton X-100 followed by blocking in blocking solution (10% normal goat serum, 0.1% Bovine Serum Albumin in 1X Phosphate Buffered Saline (PBS)) for one hour at room temperature. Sections were then incubated in anti-mouse F' (ab) fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) for five minutes to reduce non-specific binding of anti-mouse secondary. Sections were incubated with 1:100 rabbit anti-Irf6 (Sigma-Aldrich; St. Louis, MO), 1:250 rabbit anti-Keratin 14 (Covance), 1:150 mouse anti-p63 (Santa Cruz), and 1:250 rabbit anti-loricrin (Covance) overnight at 4°C, followed by incubation with either goat anti-mouse AlexaFluor 488 or goat anti-rabbit AlexaFluor 555 (Life Technologies, Grand Island, NY). Nuclei were counterstained for 10 minutes in a 1:10,000 dilution of 4' 6-diaminidino-2-phenylindole (DAPI; Life Technologies, Grand Island, NY). Slides were then mounted in



ProLong GOLD Antifade reagent (Life Technologies, Grand Island, NY). Images were taken using a Nikon Eclipse 90i fluorescent microscope.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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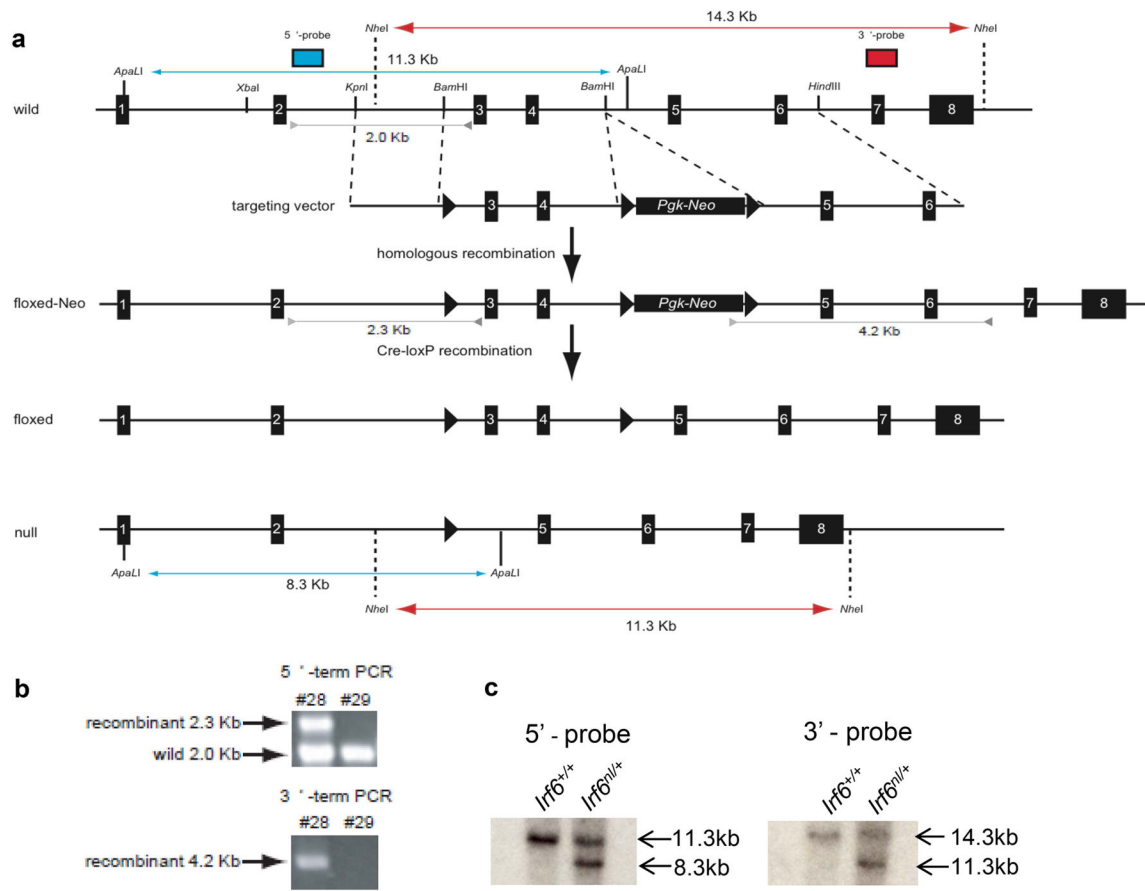
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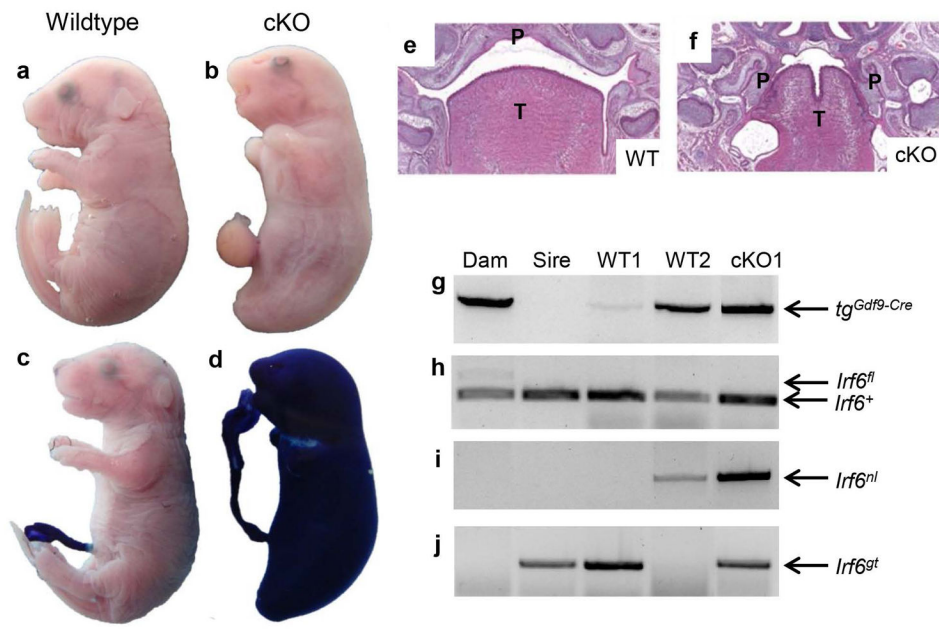
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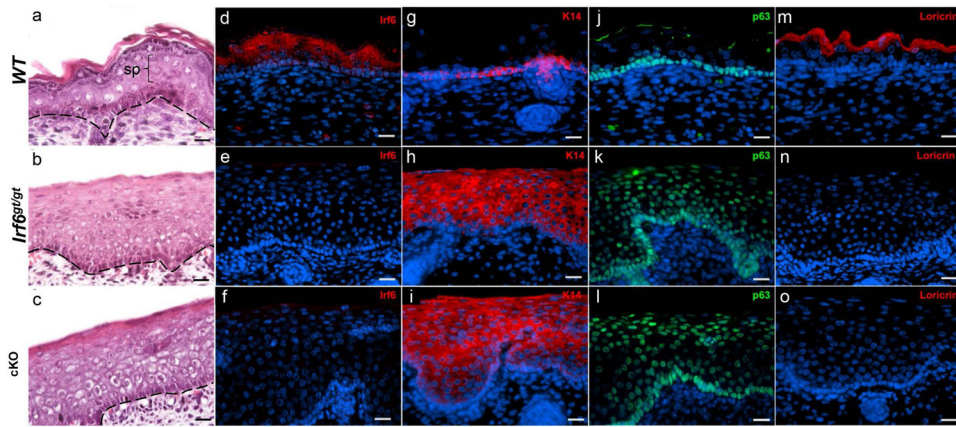
**Figure 1. Construction of a conditional allele of *Irf6***

a) A targeting vector was generated to contain three LoxP sites (triangles). The targeting vector contained a LoxP flanked PGK-neo cassette inserted 3' of exon four. A third LoxP site was incorporated 5' of exon three. b) Cells carrying the recombinant allele were detected via PCR. Regions amplified are indicated by green tracks. c) Southern blot analysis of ES cells. A 5' probe (indicated by blue tracks) and 3' probe (indicated by red tracks) were used. *Irf6*<sup>+</sup> - wildtype allele, *Irf6*<sup>fl</sup> - null allele.



**Figure 2. Conditional knockout embryos (cKO) phenocopy *Irf6<sup>gt/gt</sup>* embryos**

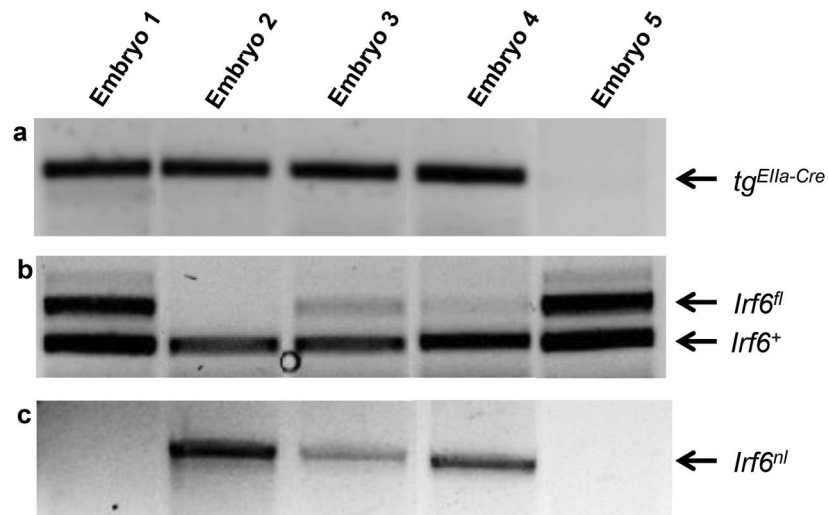
a) Wildtype and b) cKO embryos generated from *Gdf9-Cre* mediated recombination of the *Irf6<sup>fl</sup>* allele. cKO embryos (d; genotype - *Irf6<sup>gt/nl</sup>* with or without the *Gdf9-Cre* transgene) display severe skin, limb, and craniofacial abnormalities and impaired barrier function, as indicated by a dye exclusion assay, when compared to wildtype littermates (c). Cross sections of wildtype (e) and cKO (f) heads indicate severe oral adhesions in cKO embryos (P – palate; T – tongue). PCR-based genotyping for the *Gdf9-Cre* transgene (g), *Irf6<sup>+</sup>* and *Irf6<sup>fl</sup>* (h), *Irf6<sup>nl</sup>* (i), and *Irf6<sup>gt</sup>* (j). Complete recombination of the *Irf6<sup>fl</sup>* is observed irrespective of whether embryos were positive for the *Gdf9-Cre* transgene. cKO embryos were also positive for the *Irf6<sup>gt</sup>* allele. No embryos were positive for the *Irf6<sup>fl</sup>* allele.



**Figure 3. Molecular profile of embryonic skin**

H&E staining of a) Wildtype (WT), b) *Irf6*<sup>gt/gt</sup> and c) cKO skin. Black dashed line indicates the basal layer of the epidermis. The spinous layer (sp) is indicated in a. Immunofluorescent staining for Irf6 (d–f), K14 (g–i), p63 (j–l), and Loricrin (m–o). Images acquired using brightfield fluorescent microscope. Scale bars represent 100 $\mu$ m.





**Figure 4. PCR-based genotyping of *EIIa-Cre* transgenic embryos** (a), *Irf6<sup>+</sup>* and *Irf6<sup>fl</sup>* (b) and *Irf6<sup>nl</sup>* (c) from E17.5 embryos. When using the *EIIa-Cre* transgene was used, we observed embryos with no recombination (Embryo 1), complete recombination (Embryo 2), or incomplete recombination (Embryos 3 and 4) of the *Irf6<sup>fl</sup>* allele. The *Irf6<sup>nl</sup>* was not detected in the absence of the *EIIa-Cre* transgene (Embryo 5).

Table 1

*Gdf9-Cre* mediated recombination of *Irf6<sup>fl</sup>*

♀ <i>tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>fl/+</sup></i> X ♂ <i>tg<sup>+/+</sup>; Irf6<sup>gt/fl</sup></i>		
Genotype	Expected	Observed
<i>tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>gt/nl</sup></i>	5.125	0
<i>tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>fl/nl</sup></i>	5.125	7
<i>tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>gt/+</sup></i>	5.125	3
<i>tg<sup>Gdf9-Cre/+</sup>; Irf6<sup>fl/+</sup></i>	5.125	6
<i>tg<sup>+/+</sup>; Irf6<sup>gt/nl</sup></i>	5.125	0
<i>tg<sup>+/+</sup>; Irf6<sup>fl/nl</sup></i>	5.125	8
<i>tg<sup>+/+</sup>; Irf6<sup>gt/+</sup></i>	5.125	5
<i>tg<sup>+/+</sup>; Irf6<sup>fl/+</sup></i>	5.125	12
CNG		2 (not included)
TOTAL	41	41

Recombination of the *Irf6<sup>fl</sup>* allele in the female results in production of the *Irf6<sup>fl</sup>* allele. Data represent offspring from six litters after weaning. There is a significant underrepresentation of mice with *Irf6<sup>gt/nl</sup>* ( $\chi^2$  test:  $p = 0.0018$ ). CNG – Could Not Genotype.

**Table 2***Ella-Cre* mediated recombination of *Irf6<sup>fl</sup>*

<i>tg<sup>Ella-Cre/+</sup> ; Irf6<sup>g<sup>fl</sup>/+</sup> x tg<sup>+/+</sup> ; Irf6<sup>g<sup>fl</sup>/fl</sup></i>			
<i>Ella-Cre</i> genotype	total	<i>Irf6</i> genotype	total
		<i>Irf6<sup>fl</sup></i>	18
<i>tg<sup>+/+</sup></i>	46	<i>Irf6<sup>fl</sup></i> and <i>Irf6<sup>fl</sup></i>	0
		<i>Irf6<sup>fl</sup></i>	0
		<i>Irf6<sup>fl</sup></i>	19
<i>tg<sup>Ella/+</sup></i>	66	<i>Irf6<sup>fl</sup></i> and <i>Irf6<sup>fl</sup></i>	8
		<i>Irf6<sup>fl</sup></i>	9

Data represent offspring from 15 litters collected at E17.5. Mice lacking the *Ella-Cre* transgene showed no recombination of the *Irf6<sup>fl</sup>* allele. Mice carrying the *Ella-Cre* transgene showed mosaic recombination of *Irf6<sup>fl</sup>*. Some offspring showed no recombination (*Irf6<sup>fl</sup>* only), presence of both the *Irf6<sup>fl</sup>* and *Irf6<sup>fl</sup>* alleles, or complete recombination (*Irf6<sup>fl</sup>* only).

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