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- **1 Neural crest and periderm-specific requirements of** *Irf6* **during neural tube and<br>2 craniofacial development.<br>3 Shannon H. Carroll<sup>1</sup>, Sogand Schafer<sup>1</sup>, Eileen Dalessandro<sup>1</sup>, Thach-Vu Ho<sup>2</sup>, Yang Cl craniofacial development.**<br>
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4 Shannon H. Carroll<sup>1</sup>, Sogan<br>
5 C. Liao<sup>1,3,4</sup> 4<br>5<br>6<br>7 Shannon H. Carroll<sup>1</sup>, Sogand Schafer<sup>1</sup>, Eileen Dalessandro<sup>1</sup>, Thach-Vu Ho<sup>2</sup>, Yang Chai<sup>2</sup>
- C. Liao $1,3,4$
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16 **Abstract**<br>17 *IRF6* is a<br>18 ability to i<br>19 ablation i IT IRF6 is a key genetic determinant of syndromic and non-syndromic cleft lip and palate. The<br>ability to interrogate post-embryonic requirements of *Irf6* has been hindered, as global *Irf6*<br>ablation in the mouse causes ne ability to interrogate post-embryonic requirements of *Irf6* has been hindered, as global *Irf6*<br>ablation in the mouse causes neonatal lethality. Prior work analyzing *Irf6* in mouse models<br>defined its role in the embryoni ablation in the mouse causes neonatal lethality. Prior work analyzing *Irf6* in mouse models<br>defined its role in the embryonic surface epithelium and periderm where it is required to reg<br>cell proliferation and differentiat defined its role in the embryonic surface epithelium and periderm where it is required to regulate<br>
21 cell proliferation and differentiation. Several reports have also described *Irf6* gene expression in<br>
22 other cell ty cell proliferation and differentiation. Several reports have also described *Irf6* gene expression in<br>
22 other cell types, such as muscle, and neuroectoderm. However, analysis of a functional role in<br>
23 non-epithelial ce other cell types, such as muscle, and neuroectoderm. However, analysis of a functional role in<br>
23 non-epithelial cell lineages has been incomplete due to the severity and lethality of the *Irf6*<br>
24 knockout model and the non-epithelial cell lineages has been incomplete due to the severity and lethality of the *Irf6*<br>knockout model and the paucity of work with a conditional *Irf6* allele. Here we describe the<br>generation and characterization knockout model and the paucity of work with a conditional *Irf6* allele. Here we describe the<br>25 generation and characterization of a new *Irf6* floxed mouse model and analysis of *Irf6* ablat<br>26 periderm and neural crest generation and characterization of a new *Irf6* floxed mouse model and analysis of *Irf6* ablation in<br>
26 periderm and neural crest lineages. This work found that loss of *Irf6* in periderm recapitulates a<br>
27 mild *Irf6* periderm and neural crest lineages. This work found that loss of *Irf6* in periderm recapitulates a<br>27 mild *Irf6* null phenotype, suggesting that *Irf6*-mediated signaling in periderm plays a crucial role<br>28 in regulating mild *Irf6* null phenotype, suggesting that *Irf6*-mediated signaling in periderm plays a crucial role<br>28 in regulating embryonic development. Further, conditional ablation of *Irf6* in neural crest cells<br>29 resulted in an in regulating embryonic development. Further, conditional ablation of *Irf6* in neural crest cells<br>resulted in an anterior neural tube defect of variable penetrance. The generation of this<br>conditional *Irf6* allele allows resulted in an anterior neural tube defect of variable penetrance. The generation of this<br>
20 conditional *Irf6* allele allows for new insights into craniofacial development and new exp<br>
21 into the post-natal role of *Irf* 30 conditional *Irf6* allele allows for new insights into craniofacial development and new exploration<br>31 into the post-natal role of *Irf6*.<br>32 Keywords<br>24 I<sup>Ff</sup>6 alst polste pourel tube pourel areat *New der Weude Sundre* 31 into the post-natal role of *Irf6*.<br>32<br>33 Keywords<br>34 Irf6, cleft palate, neural tube, r  $33$ <br> $34$ <br> $35$ <br> $36$ 33 Keywords<br>34 Irf6, cleft p<br>35<br>36 34 Irf6, cleft palate, neural tube, neural crest, Van der Woude Syndrome, periderm<br>35<br>36

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37 **Introduction**<br>38 *IRF6* was one<br>39 uncovered fro<br>40 popliteal ptery *IRF6* was one of the first genetic determinants of syndromic cleft lip and palate malformation,<br>39 uncovered from genome-wide association studies of Van der Woude syndrome (VWS) and<br>40 popliteal pterygium syndromic (PPS) 39 uncovered from genome-wide association studies of Van der Woude syndrome (VWS) and<br>30 popliteal pterygium syndrome (PPS) (Kondo et al., 2002). *IRF6* gene variants are also majo<br>31 contributors to non-syndromic cleft li popliteal pterygium syndrome (PPS) (Kondo et al., 2002). *IRF6* gene variants are also major<br>contributors to non-syndromic cleft lip with or without cleft palate (Leslie et al., 2013; Park et a<br>2007; Rahimov et al., 2008; contributors to non-syndromic cleft lip with or without cleft palate (Leslie et al., 2013; Park et al., 2007; Rahimov et al., 2008; Zucchero et al., 2004). Multiple studies using mouse and zebrafish models have shown that 2007; Rahimov et al., 2008; Zucchero et al., 2004). Multiple studies using mouse and zebrafish<br>
43 models have shown that *Irf6* is expressed in the basal epithelium and periderm during<br>
44 embryonic development with dynam models have shown that *Irf6* is expressed in the basal epithelium and periderm during<br>embryonic development with dynamic expression in the oral epithelium during palatoge<br>(Carroll et al., 2020; de la Garza et al., 2013; D embryonic development with dynamic expression in the oral epithelium during palatogenesis<br>
(Carroll et al., 2020; de la Garza et al., 2013; Dougherty et al., 2013; Ferretti et al., 2011;<br>
Ingraham et al., 2006; Iwata et al (Carroll et al., 2020; de la Garza et al., 2013; Dougherty et al., 2013; Ferretti et al., 2011;<br>
Ingraham et al., 2006; Iwata et al., 2013; Knight et al., 2006; Kousa et al., 2017; Richards<br>
al., 2006; Xu et al., 2006). *I* Ingraham et al., 2006; Iwata et al., 2013; Knight et al., 2006; Kousa et al., 2017; Richardson et al., 2006; Xu et al., 2006). *Irf6* is necessary for keratinocyte differentiation (Biggs et al., 2012; Ingraham et al., 2006 al., 2006; Xu et al., 2006). *Irf6* is necessary for keratinocyte differentiation (Biggs et al., 2012;<br>Ingraham et al., 2006; Restivo et al., 2011; Richardson et al., 2006) and for the development of<br>the periderm (de la Ga Ingraham et al., 2006; Restivo et al., 2011; Richardson et al., 2006) and for the development of<br>the periderm (de la Garza et al., 2013; Li et al., 2017; Richardson et al., 2009; Richardson et al.<br>2014; Sabel et al., 2009) the periderm (de la Garza et al., 2013; Li et al., 2017; Richardson et al., 2009; Richardson et al.,<br>
2014; Sabel et al., 2009). Ablation of *Irf6* in mice resulted in severe epithelial adhesions that<br>
caused "cocooning" o 2014; Sabel et al., 2009). Ablation of *Irf6* in mice resulted in severe epithelial adhesions that<br>51 caused "cocooning" of the embryo and caused adherence of palatal shelves to the tongue in<br>52 vertical orientation preclu caused "cocooning" of the embryo and caused adherence of palatal shelves to the tongue in the<br>
state of the secondary palate (Ingraham et al.,<br>
2006; Richardson et al., 2009; Richardson et al., 2014) and fuse (Iwata et al. vertical orientation precluding elevation and fusion of the secondary palate (Ingraham et al.,<br>
2006; Richardson et al., 2009; Richardson et al., 2014) and fuse (Iwata et al., 2013).<br>
While most studies have examined the r 2006; Richardson et al., 2009; Richardson et al., 2014) and fuse (Iwata et al., 2013).<br>While most studies have examined the requirement of *Irf6* in epithelial differer<br>several studies have described *Irf6* function in no 54 While most studies have examined the requirement of *Irf6* in epithelial differentiation,<br>55 several studies have described *Irf6* function in non-epithelial tissue, in either autonomous or<br>56 non-cell-autonomous fashio several studies have described *Irf6* function in non-epithelial tissue, in either autonomous or<br>non-cell-autonomous fashion (Goudy et al., 2013; Thompson et al., 2019). *Irf6* is expressed i<br>cell types not restricted to s non-cell-autonomous fashion (Goudy et al., 2013; Thompson et al., 2019). *Irf6* is expressed in<br>
cell types not restricted to surface epithelium during early development, including the<br>
craniofacial mesenchyme and neuroect s cell types not restricted to surface epithelium during early development, including the<br>
s craniofacial mesenchyme and neuroectoderm (Carroll et al., 2020; Fakhouri et al., 20<br>
et al., 2013; Sabel et al., 2009; Thompson craniofacial mesenchyme and neuroectoderm (Carroll et al., 2020; Fakhouri et al., 2017; Goudy<br>
et al., 2013; Sabel et al., 2009; Thompson et al., 2019). Further, analysis of murine *MCS9.7*<br>
enhancer element activity, whic et al., 2013; Sabel et al., 2009; Thompson et al., 2019). Further, analysis of murine *MCS9.7*<br>
enhancer element activity, which replicates endogenous *Irf6* expression in most tissues, yie<br>
expression in developing somite enhancer element activity, which replicates endogenous *Irf6* expression in most tissues, yielded<br>61 expression in developing somites, tongue, axial cartilage, and muscle (Fakhouri et al., 2012).<br>62 We and others have desc expression in developing somites, tongue, axial cartilage, and muscle (Fakhouri et al., 2012).<br>
62 We and others have described that mesenchymal-derived craniofacial tissue, such as muscle<br>
62 We and others have described 62 We and others have described that mesenchymal-derived craniofacial tissue, such as muscle<br>
Solution of the muscle of that mesenchymal-derived craniofacial tissue, such as muscle<br>
That muscle is that the muscle of the mu

and cartilage are dysmorphic in the *Irf6* null mice (Carroll et al., 2020; Chu et al., 2016;<br>
164 Thompson et al., 2019). However, it remains unclear whether there is a cell-autonomour<br>
165 *Irf6* in non-epithelial cell t Frompson et al., 2019). However, it remains unclear whether there is a cell-autonomous role of<br>
formation in these dysmorphologies are associated consequences of the severe epithelial adhesions caused<br>
by a dysfunctional e *Irf6* in non-epithelial cell types, a non-cell-autonomous role caused by loss of epithelial *Irf6*, or if<br>66 these dysmorphologies are associated consequences of the severe epithelial adhesions caused<br>67 by a dysfunctiona

these dysmorphologies are associated consequences of the severe epithelial adhesions caused<br>by a dysfunctional epithelium.<br>In addition to orofacial and epithelial development, *Irf6* has been found to have a role in<br>neurul 67 by a dysfunctional epithelium.<br>
68 In addition to orofacial<br>
69 neurulation. *Irf6* is expressed in Tfap2a, a known regulator of n In addition to orofacial and epithelial development, *Irf6* has been found to have a role in<br>neurulation. *Irf6* is expressed in the neuroectoderm of the neural folds and is co-expressed with<br>Tfap2a, a known regulator of n neurulation. *Irf6* is expressed in the neuroectoderm of the neural folds and is co-expressed with<br>
Tfap2a, a known regulator of neural tube closure (Kousa et al., 2019). Although neural tube<br>
defects are not apparent in *Tfap2a,* a known regulator of neural tube closure (Kousa et al., 2019). Although neural tube<br>defects are not apparent in *Irf6* null mice, ablation of other genes in the Irf6 regulatory pathw<br>i.e. *Tfap2A* and *Grhl3*, le defects are not apparent in *Irf6* null mice, ablation of other genes in the Irf6 regulatory pathway,<br>i.e. *Tfap2A* and *Grhl3*, leads to rostral and caudal neural tube defects (Schorle et al., 1996; Ting<br>et al., 2003; Zha i.e. *Tfap2A* and *Grhl3*, leads to rostral and caudal neural tube defects (Schorle et al., 1996; Ting<br>et al., 2003; Zhang et al., 1996). Utilizing an *Irf6* hypomorph allele and a *Krt4:Irf6* transgenic<br>mouse to titrate et al., 2003; Zhang et al., 1996). Utilizing an *Irf6* hypomorph allele and a *Krt4:Irf6* transgenic<br>74 mouse to titrate *Irf6* expression levels, Kousa et al. found homeostasis of *Irf6* to be required to<br>75 neurulation (

mouse to titrate *Irf6* expression levels, Kousa et al. found homeostasis of *Irf6* to be required for<br>
The ability to interrogate non-epithelial and post-natal functions of *Irf6* has been impaired<br>
by the severe and leth 75 neurulation (Kousa et al., 2019).<br>76 The ability to interrogate r<br>77 by the severe and lethal phenoty<br>78 floxed mouse model has given so The ability to interrogate non-epithelial and post-natal functions of *Irf6* has been impaired<br>by the severe and lethal phenotype of the *Irf6* null mouse models. A previously generated *Irf6*<br>floxed mouse model has given by the severe and lethal phenotype of the *Irf6* null mouse models. A previously generated *Irf6*<br>floxed mouse model has given some insight (Smith et al., 2017). Conditional ablation of *Irf6* ir<br>oral epithelium via a *Pit* floxed mouse model has given some insight (Smith et al., 2017). Conditional ablation of *Irf6* in<br>oral epithelium via a *Pitx2*-Cre driver line resulted in tooth development and maturation defects<br>(Chu et al., 2016). Since oral epithelium via a *Pitx2*-Cre driver line resulted in tooth development and maturation defects<br>
(Chu et al., 2016). Since the previously generated *Irf6* floxed allele was reported to show<br>
variable recombination effic 80 (Chu et al., 2016). Since the previously generated *Irf6* floxed allele was reported to show<br>81 variable recombination efficiency and we remained unsuccessful in acquiring it (Smith et<br>82 2017), we generated a new condi variable recombination efficiency and we remained unsuccessful in acquiring it (Smith et al.,<br>
2017), we generated a new conditional *Irf6* floxed mouse allele for this work. This *Irf6*<br>
conditional allele demonstrated co 2017), we generated a new conditional *Irf6* floxed mouse allele for this work. This *Irf6*<br>conditional allele demonstrated complete recombination efficiency with every ubiquite<br>tissue-restricted Cre drivers we have tested

conditional allele demonstrated complete recombination efficiency with every ubiquitous and<br>84 tissue-restricted Cre drivers we have tested.<br>85 In this study, we describe the generation of a new conditional *Irf6* mouse al tissue-restricted Cre drivers we have tested.<br>85 In this study, we describe the generat<br>86 analyze *Wnt1*-Cre2-mediated disruption of *In*<br>87 the *Krt6ai*-Cre driver line to ablate *Irf6* functic In this study, we describe the generation of a new conditional *Irf6* mouse allele and<br>analyze *Wnt1*-Cre2-mediated disruption of *Irf6* in the neural crest cells (NCCs). We also uti<br>the *Krt6ai*-Cre driver line to ablate analyze *Wnt1-*Cre2-mediated disruption of *Irf6* in the neural crest cells (NCCs). We also utilized<br>87 the *Krt6ai-*Cre driver line to ablate *Irf6* function in periderm. These results demonstrate for the<br>4 87 the *Krt6ai*-Cre driver line to ablate *Irf6* function in periderm. These results demonstrate for the

- first time a cell-autonomous role for Irf6 in the neural crest as well as corroborate the functional<br>
role of Irf6 in the periderm during orofacial development.<br>
90<br> **Materials and Methods**<br>
83<br> **Concration of a new condit**
- role of Irf6 in the periderm during orofacial development.<br>90<br>**91 Materials and Methods<br>92 Generation of a new conditional Irf6 mouse allele**
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- 91<br>92<br>93<br>04 91 **Materials and Methods<br>92 <b>Generation of a new co**<br>93 All procedures were appr<br>94 University where the initia Generation of a new conditional *Irf6* mouse allele<br>93 All procedures were approved by IACUCs for Massac<br>94 University where the initial work was carried out. The<br>95 introduce loxP sites (Miura et al., 2018) flanking exon All procedures were approved by IACUCs for Massachusetts General Hospital and Harvard<br>
94 University where the initial work was carried out. The *Easi*-CRISPR protocol was utilized to<br>
95 introduce loxP sites (Miura et al. University where the initial work was carried out. The *Easi*-CRISPR protocol was utilized to<br>
95 introduce loxP sites (Miura et al., 2018) flanking exons 3 and 4 of *Irf6*. As these exons conta<br>
96 the DNA binding region introduce loxP sites (Miura et al., 2018) flanking exons 3 and 4 of *Irf6*. As these exons contain<br>196 the DNA binding region (Kondo et al., 2002), they are predicted to be required for Irf6<br>197 transcriptional function an the DNA binding region (Kondo et al., 2002), they are predicted to be required for Irf6<br>197 transcriptional function and have been previously targeted for conditional ablation of Ir<br>198 et al., 2017). Guide RNAs (gRNA) wer transcriptional function and have been previously targeted for conditional ablation of Irf6 (Smith<br>et al., 2017). Guide RNAs (gRNA) were designed within the intronic regions flanking exons 3<br>and 4 using the CRISPR gRNA des et al., 2017). Guide RNAs (gRNA) were designed within the intronic regions flanking exons 3<br>and 4 using the CRISPR gRNA design tool from Benchling and were ordered from Synthego.<br>Single-stranded DNA (ssDNA) donor sequences and 4 using the CRISPR gRNA design tool from Benchling and were ordered from Synthego.<br>
00 Single-stranded DNA (ssDNA) donor sequences were designed to contain the loxp sequence<br>
101 flanked by homologous arms and were ord 100 Single-stranded DNA (ssDNA) donor sequences were designed to contain the loxp sequence<br>101 flanked by homologous arms and were ordered from IDT. Cas9, gRNA and donor ssDNA were<br>102 injected into mouse zygotes by the Ha 101 flanked by homologous arms and were ordered from IDT. Cas9, gRNA and donor ssDNA were<br>102 injected into mouse zygotes by the Harvard Genome Modification Facility. Resulting viable pups<br>103 were genotyped by PCR as well injected into mouse zygotes by the Harvard Genome Modification Facility. Resulting viable pups<br>103 were genotyped by PCR as well as sequenced to ensure the insertion of the loxP sequences<br>104 within the same DNA strand. A were genotyped by PCR as well as sequenced to ensure the insertion of the loxP sequences<br>104 within the same DNA strand. A female mouse was identified with the correct genome<br>105 modifications, was phenotypically normal, a within the same DNA strand. A female mouse was identified with the correct genome<br>105 modifications, was phenotypically normal, and was designated F0. Breeding with a wi<br>106 C57BL/6J mouse generated F1s, which were in-cros modifications, was phenotypically normal, and was designated F0. Breeding with a wildtype<br>
106 C57BL/6J mouse generated F1s, which were in-crossed to generate mice homozygous for the<br>
107 floxed *Irf6* allele (*Irf6*<sup>f//f)</sup> 106 C57BL/6J mouse generated F1s, which were in-crossed to generate mice homozygous for the<br>
107 floxed *Irf6* allele (*Irf6<sup>f//fl</sup>*).<br>
108 **Mouse lines**<br>
110 To validate efficient Cro recombination and to confirm recombin floxed *Irf6* allele (*Irf6*<sup>f/fl</sup>).

107 floxed *Irf6* allele (*Irf6*<sup>1/11</sup>).<br>108<br>109 **Mouse lines**<br>110 To validate efficient Cre 109<br>110<br>111<br>112 109 **Mouse lines**<br>110 To validate ef<br>111 mice were bre<br>112 (Jackson Lab To validate efficient Cre recombination and to confirm recombination ablates *Irf6* function, *Irf6*fl/fl 111<br>112<br>113 111 mice were bred to the Cre deleter lines CMV-Cre (Jackson Labs stock# 006054) and Ella-Cre<br>
112 (Jackson Labs stock# 003724). The resulting pups (viable and non-viable) were phenotyped<br>
113 and genotyped. Wnt1Cre2 and S 112 (Jackson Labs stock# 003724). The resulting pups (viable and non-viable) were phenotyped<br>113 and genotyped. Wnt1Cre2 and Sox10Cre were obtained from Jackson Labs (stock# 022501<br>113 and genotyped. Wnt1Cre2 and Sox10Cre 113 and genotyped. *Wnt1*Cre2 and *Sox10*Cre were obtained from Jackson Labs (stock# 022501

- 114 and 025807, respectively). *Krt6ai*-Cre came from Vesa Kaartinen. Crect line came from Russ<br>115 Carstens but originated from Trevor Williams. For timed pregnancies, E0.5 was determined<br>116 upon observation of a copulat Carstens but originated from Trevor Williams. For timed pregnancies, E0.5 was determined<br>116 upon observation of a copulatory plug.<br>117 **Histology and in situ hybridization**<br>118 Mise were fixed with 4% fermeldebyde followe
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116 upon observation of a copulatory plug.<br>117<br>**118 Histology and** *in situ* **hybridization**<br>119 Mice were fixed with 4% formaldehyd<br>120 Tiesues were embedded in OCT and 117<br>118<br>119<br>120<br>121 **Histology and** *in situ* **hybridization<br>119 Mice were fixed with 4% formaldeh<br>120 Tissues were embedded in OCT are<br>121 Staining was performed according to** Mice were fixed with 4% formaldehyde followed by cryoprotection in 15 and 30% sucrose.<br>
120 Tissues were embedded in OCT and 10  $\mu$ m sections were made. Hematoxylin and Eosin<br>
121 staining was performed according to a sta Tissues were embedded in OCT and 10 μm sections were made. Hematoxylin and Eosin<br>121 staining was performed according to a standard protocol (Fischer et al., 2008) and slides were<br>122 imaged with a Leica DM6 upright micro

staining was performed according to a standard protocol (Fischer et al., 2008) and slides were<br>122 imaged with a Leica DM6 upright microscope and LAS X software.<br>123 RNAscope probes for mouse *Irf6, Wnt1*, and Sox10 were d imaged with a Leica DM6 upright microscope and LAS X software.<br>
123 RNAscope probes for mouse *Irf6*, *Wnt1*, and *Sox10* were des<br>
124 Advanced Cell Diagnostics. RNAscope *in situ* hybridization was<br>
125 manufacturer's pr RNAscope probes for mouse *Irf6*, *Wnt1*, and *Sox10* were designed and manufactured by<br>124 Advanced Cell Diagnostics. RNAscope *in situ* hybridization was performed according to the<br>125 manufacturer's protocol (Advanced C 124 Advanced Cell Diagnostics. RNAscope *in situ* hybridization was performed according to the<br>
125 manufacturer's protocol (Advanced Cell Diagnostics). Slides were imaged using a confocal laser<br>
126 scanning microscope (L manufacturer's protocol (Advanced Cell Diagnostics). Slides were imaged using a confocal laser<br>126 scanning microscope (Leica SP8) and image processing was performed using ImageJ version<br>127 2.0 (2018).<br>128 MicroCT analysi scanning microscope (Leica SP8) and image processing was performed using ImageJ version<br>127 2.0 (2018).<br>128 MicroCT analysis and measurements<br>129 Seepe were performed using a uCT40 benefited accounts (Seepee Medical AC Prü

127 2.0 (2018).<br>128<br>129 **MicroCT ar**<br>130 Scans were 128<br>129<br>130<br>131<br>131 **MicroCT analysis and measurements**<br>130 Scans were performed using a µCT40 be<br>131 Switzerland). Scans were acquired with a<br>132 potential, 114 mA intensity, and 300 ms 130 Scans were performed using a  $\mu$ CT40 benchtop scanner (Scanco Medical AG, Brüttisellen,<br>131 Switzerland). Scans were acquired with a 15  $\mu$ m<sup>3</sup> isotropic voxel size, 70 kVP peak x-ray tub<br>132 potential, 114 mA inten

Switzerland). Scans were acquired with a 15  $\mu$ m<sup>3</sup>

Switzerland). Scans were acquired with a 15  $\mu$ m<sup>3</sup> isotropic voxel size, 70 kVP peak x-ray tube<br>
potential, 114 mA intensity, and 300 ms integration time. Morphometric landmarks were chose<br>
as previously described (Ho e potential, 114 mA intensity, and 300 ms integration time. Morphometric landmarks were chosen<br>as previously described (Ho et al., 2015) and measurements were made using Avizo software.<br>134<br>**Results**<br>135 **Results** 

- 133 as previously described (Ho et al., 2015) and measurements were made using Avizo software.<br>134<br>**Results**<br>136 Irf6 is expressed in Wnt1+ neuroectoderm and neural crest cell-derived cranial<br>137 measurehyme
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- 134<br>135<br>136<br>137<br>138

135 **Results<br>136** *Irf6* **is ex<br>137 mesencl<br>138** *Irf6* **null r** 138 *Irf6* null mice exhibit a foreshortened midface as well as malformation of neural crest-derived<br>139 maxilla (Fakhouri et al., 2017; Richardson et al., 2006; Thompson et al., 2019). *Irf6* gene

*Irf6* **is expressed in** *Wnt1***+ neuroectoderm and neural crest cell-derived cranial<br>
mesenchyme<br>** *Iff6* **null mice exhibit a foreshortened midface as well as malformation of neural crest-<br>
maxilla (Fakhouri et al., 2017; Ri** 137 **mesenchyme**<br>138 *Irf6* null mice ex<br>139 maxilla (Fakho 139 maxilla (Fakhouri et al., 2017; Richardson et al., 2006; Thompson et al., 2019). *Irf6* gene 140 dosage has also been found to impact neural tube closure (Kousa et al., 2019). It was<br>
141 previously reported that *Irf6* is expressed in the neuroectoderm and neural folds of mot<br>
142 embryos (Bertol et al., 2022; K 141 previously reported that *Irf6* is expressed in the neuroectoderm and neural folds of mouse<br>
142 embryos (Bertol et al., 2022; Kousa et al., 2019). To examine whether cranial neural crest<br>
143 express *Irf6*, we analyz embryos (Bertol et al., 2022; Kousa et al., 2019). To examine whether cranial neural crest cells<br>
express *Irf6*, we analyzed *Irf6* mRNA expression by RNAscope *in situ* hybridization in mouse<br>
embryos during early cranio express *Irf6*, we analyzed *Irf6* mRNA expression by RNAscope *in situ* hybridization in mouse<br>
144 embryos during early craniofacial development. At E8 and E9, we found *Irf6* mRNA co-<br>
145 expressed with *Wnt1* in the n embryos during early craniofacial development. At E8 and E9, we found *Irf6* mRNA co-<br>expressed with *Wnt1* in the neuroectoderm. *Irf6* mRNA expression was also found co-<br>expressed with *Sox10*, demonstrating *Irf6* expre expressed with *Wnt1* in the neuroectoderm. *Irf6* mRNA expression was also found co-<br>expressed with Sox10, demonstrating *Irf6* expression in migratory neural crest cells (F<br>Further, *Irf6* mRNA is expressed within the ne expressed with *Sox10*, demonstrating *Irf6* expression in migratory neural crest cells (Fig 1b).<br>147 Further, *Irf6* mRNA is expressed within the neural crest-derived craniofacial mesenchyme at 148 (Fig 1c) and E13.5 (Fig 147 Further, *Irf6* mRNA is expressed within the neural crest-derived craniofacial mesenchyme at E9 (Fig 1c) and E13.5 (Fig 1d). Based on these detailed gene expression findings, as well as previously reported expression o (Fig 1c) and E13.5 (Fig 1d). Based on these detailed gene expression findings, as well as<br>149 previously reported expression of *Irf6* in the neuroectoderm (Bertol et al., 2022; Kousa et a<br>150 2019), we posited that *Irf6* previously reported expression of *Irf6* in the neuroectoderm (Bertol et al., 2022; Kousa et al.,<br>150 2019), we posited that *Irf6* contributes to craniofacial development beyond its established role<br>151 the surface epithe 2019), we posited that *Irf6* contributes to craniofacial development beyond its established role in<br>151 the surface epithelium and periderm.<br>152 **Generation and validation of an** *Irf6* conditional allele<br>154 Severe epith

151 the surface epithelium and periderm.<br>152<br>**153 Generation and validation of an Irfl<br>154** Severe epithelial adhesions and perin 152<br>153<br>154<br>155<br>156 **Generation and validation of an** *Irf6* **conditional allele<br>154 Severe epithelial adhesions and perinatal lethality in the** *Inf***<br>155 (Ingraham et al., 2006) and the** *Irf6* **R84C single nucleotid<br>156 al., 2006) impeded full a** 154 Severe epithelial adhesions and perinatal lethality in the *Irf6* complete knockout embryos<br>155 (Ingraham et al., 2006) and the *Irf6* R84C single nucleotide substitution mouse (Richardson)<br>156 al., 2006) impeded full 155 (Ingraham et al., 2006) and the *Irf6* R84C single nucleotide substitution mouse (Richardson et al., 2006) impeded full analysis of *Irf6* function. The previously reported *Irf6* floxed mouse allele<br>157 was reported t 156 al., 2006) impeded full analysis of *Irf6* function. The previously reported *Irf6* floxed mouse allele<br>157 was reported to exhibit inconsistent recombination depending on the Cre driver used,<br>158 confounding analysis was reported to exhibit inconsistent recombination depending on the Cre driver used,<br>158 confounding analysis of *Irf6* requirement in the multiple tissue types (Smith et al., 2017<br>159 the complexity of *Irf6* gene express 158 confounding analysis of *Irf6* requirement in the multiple tissue types (Smith et al., 2017). Given<br>159 the complexity of *Irf6* gene expression in neuroectoderm and neural crest during early<br>160 embryogenesis, we gene the complexity of *Irf6* gene expression in neuroectoderm and neural crest during early<br>
160 embryogenesis, we generated a new *Irf6* floxed allele to better understand *Irf6* function<br>
161 utilized an CRISPReasi technique 160 embryogenesis, we generated a new *Irf6* floxed allele to better understand *Irf6* function. We utilized an CRISPReasi technique (Miura et al., 2018) to insert loxP sequences flanking exor and 4 of the *Irf6* gene (Fig utilized an CRISPReasi technique (Miura et al., 2018) to insert loxP sequences flanking exon 3<br>
and 4 of the *Irf6* gene (Fig 2a). Insertion of the (22bp) loxP sequence was verified by PCR<br>
genotyping of potential founders and 4 of the *Irf6* gene (Fig 2a). Insertion of the (22bp) loxP sequence was verified by PCR<br>163 genotyping of potential founders (Fig 2b), followed by Sanger sequencing to confirm loxP<br>164 insertion without disruption of 163 genotyping of potential founders (Fig 2b), followed by Sanger sequencing to confirm loxP<br>164 insertion without disruption of exonic sequences.<br>164 164 insertion without disruption of exonic sequences.<br>
164 insertion without disruption of exonic sequences.

165 To test whether Cre expression resulted in recombination and loss of function, the<br>166 confirmed founder mouse was bred to two different deleter strains; CMV-Cre and Ella-Cre<br>167 found that pups that were homozygous fo 166 confirmed founder mouse was bred to two different deleter strains; *CMV*-Cre and *Ella*-Cre. We<br>167 found that pups that were homozygous for loxP but negative for Cre were phenotypically norma<br>168 and healthy. Pups tha 167 found that pups that were homozygous for loxP but negative for Cre were phenotypically normal<br>168 and healthy. Pups that were homozygous for loxP and positive for *CMV*-Cre or *Ella*-Cre<br>169 recapitulated the epithelia and healthy. Pups that were homozygous for loxP and positive for *CMV*-Cre or *Ella*-Cre<br>
169 recapitulated the epithelial adhesions, limb abnormalities, and cleft palate displayed by *li*<br>
170 knockout mice (Fig 2c,d). Fu 169 recapitulated the epithelial adhesions, limb abnormalities, and cleft palate displayed by *Irf6* total<br>170 knockout mice (Fig 2c,d). Further, we found this phenotype to be completely penetrant. Based<br>171 on these resul knockout mice (Fig 2c,d). Further, we found this phenotype to be completely penetrant. Based<br>171 on these results, we determined faithful recombination of the *Irf6* floxed allele leading to<br>172 functional Irf6 ablation.<br>1 171 on these results, we determined faithful recombination of the *Irf6* floxed allele leading to<br>172 functional Irf6 ablation.<br>173 **Ablation of** *Irf6* in the *Wnt1* lineage leads to a cranial defect and increased perinat 172 functional Irf6 ablation.<br>173<br>174 **Ablation of** *Irf6* **in the**<br>175 **lethality** 

173<br>174<br>175<br>176<br>177 **Ablation of** *Irf6* **in the** *Wnt1* **lineage leads to a cranial defect and increased perinatal<br>175 lethality<br>176 With recombination of the floxed** *Irf6* **allele validated, we tested the effect of** *Irf6* **ablation in<br>177 NCC lin** 175 **lethality**<br>176 With reco<br>177 NCC line<br>178 recombin With recombination of the floxed *Irf6* allele validated, we tested the effect of *Irf6* ablation in the<br>177 NCC lineage. We utilized the *Wnt1*-Cre2 and the Sox10-Cre mouse lines to drive the<br>178 recombination of the flox 177 NCC lineage. We utilized the *Wnt1*-Cre2 and the *Sox10*-Cre mouse lines to drive the<br>178 recombination of the floxed genome sequence. *Wnt*1 is expressed in the neural folds<br>179 migratory NCCs (Lewis et al., 2013; Sch 178 recombination of the floxed genome sequence. *Wnt*1 is expressed in the neural folds and pre-<br>179 migratory NCCs (Lewis et al., 2013; Schock et al., 2017) whereas *Sox10* is expressed in<br>180 migratory NCCs (Matsuoka et migratory NCCs (Lewis et al., 2013; Schock et al., 2017) whereas *Sox10* is expressed in<br>180 migratory NCCs (Matsuoka et al., 2005). Analysis of *Sox10*-Cre *Irf6* cKO pups revealed n<br>181 phenotypic effect of *Irf6* ablati migratory NCCs (Matsuoka et al., 2005). Analysis of *Sox10*-Cre *Irf6* cKO pups revealed no<br>181 phenotypic effect of *Irf6* ablation in migratory and post-migratory NCCs (data not shown). T<br>182 finding suggests that althou 181 phenotypic effect of *Irf6* ablation in migratory and post-migratory NCCs (data not shown). This<br>182 finding suggests that although *Irf6* mRNA can be found in NCC-derived mesenchymal tissue, it<br>183 expression is not n finding suggests that although *Irf6* mRNA can be found in NCC-derived mesenchymal tissue, its<br>
183 expression is not necessary for craniofacial development. In contrast to Sox10-Cre driven<br>
184 ablation, analysis of *Wnt1* expression is not necessary for craniofacial development. In contrast to *Sox10*-Cre driven<br>184 ablation, analysis of *Wnt1*-Cre2 *Irf6* cKO pups revealed a range of phenotype severity with<br>185 some pups phenotypically nor ablation, analysis of *Wnt1*-Cre2 *Irf6* cKO pups revealed a range of phenotype severity with<br>185 some pups phenotypically normal and viable. We also observed P0 pups that were largely<br>186 normal but exhibited skin lesions 185 some pups phenotypically normal and viable. We also observed P0 pups that were largely<br>186 normal but exhibited skin lesions overlying the nasal and frontal bones (Fig 3a). These skin<br>187 lesions resolved but led to de 186 normal but exhibited skin lesions overlying the nasal and frontal bones (Fig 3a). These skin<br>187 lesions resolved but led to delayed fur growth (Fig 3b). To detect whether *Wnt1*-Cre2 cKO o<br>188 resulted in differences 187 lesions resolved but led to delayed fur growth (Fig 3b). To detect whether *Wnt1*-Cre2 cKO of *Irf6*<br>188 resulted in differences in pup survival, litter size at P0 was recorded and compared to genotype<br>189 ratios at 3 188 resulted in differences in pup survival, litter size at P0 was recorded and compared to genotype<br>189 ratios at 3 weeks of age. Based on total pup numbers and expected ratio (based on parent<br>190 genotypes), we expected 189 ratios at 3 weeks of age. Based on total pup numbers and expected ratio (based on parent<br>190 genotypes), we expected approximately 6 *Wnt1*-Cre2 *Irf6* cKO at weaning. Instead, 1 *Irf6* cl 190 genotypes), we expected approximately 6 *Wnt1*-Cre2 *Irf6* cKO at weaning. Instead, 1 *Irf6* cKO

191 pup was identified at 3 weeks of age, suggesting perinatal lethality between birth and weaning.<br>
192 The numbers of wild-type and heterozygous pups were as expected. We did not find the<br>
193 lethality of the *Wnt1*-Cre 192 The numbers of wild-type and heterozygous pups were as expected. We did not find the<br>193 lethality of the *Wnt1*-Cre2 *Irf6* cKO pups to be due to cleft palate, as histological examina<br>194 P0 dead or moribund pups show 193 lethality of the *Wnt1*-Cre2 *Irf6* cKO pups to be due to cleft palate, as histological examination of<br>194 P0 dead or moribund pups showed palatogenesis to be normal (Fig. 3c).<br>195 To examine whether the underlying cal 194 P0 dead or moribund pups showed palatogenesis to be normal (Fig. 3c).<br>
195 To examine whether the underlying calvarial development was aff<br>
196 Cre2 *Irf6* cKO before parturition, we performed histology on coronal sect 195 To examine whether the underlying calvarial development was affected in the *Wnt1*-<br>196 Cre2 *Irf6* cKO before parturition, we performed histology on coronal sections taken through<br>197 nasal-frontal bone junction of *W* 196 Cre2 *Irf6* cKO before parturition, we performed histology on coronal sections taken through the<br>197 nasal-frontal bone junction of *Wnt1*-Cre2 *Irf6* cKO E16 pups and littermate controls. We found<br>198 that control mic nasal-frontal bone junction of *Wnt1*-Cre2 *Irf6* cKO E16 pups and littermate controls. We found<br>that control mice had bone tissue at the midline, forming a suture between right and left calvari<br>In contrast, the *Wnt1*-Cre that control mice had bone tissue at the midline, forming a suture between right and left calvaria.<br>
199 In contrast, the *Wnt1*-Cre2<sup>+</sup>;Irf6<sup>ft/ft</sup> cKO mice exhibited a large gap devoid of bone tissue that<br>
199 spanned th In contrast, the *Wnt1*-Cre2<sup>+</sup>;Irf6<sup>fl/fl</sup> In contrast, the *Wnt1*-Cre2<sup>+</sup>;Irf6<sup>n/n</sup> cKO mice exhibited a large gap devoid of bone tissue that<br>200 spanned the midline (Fig 3d). These findings of a midline cranial defect and partial lethality in<br>201 *Wnt1*-Cre2 *Irf* 200 spanned the midline (Fig 3d). These findings of a midline cranial defect and partial lethality in<br>
201 Wnt1-Cre2 Irf6 cKO mice suggest that Irf6 expression in the pre-migratory NCCs is functional<br>
202 required for cran *Wnt1*-Cre2 *Irf6* cKO mice suggest that *Irf6* expression in the pre-migratory NCCs is functionally<br>
202 required for craniofacial development.<br>
203 **Wnt1-Cre2** *Irf6* **cKO mice exhibited incomplete frontal and parietal bon** 

202 required for craniofacial development.<br>203<br>204 *Wnt1-Cre2 Irf6 cKO mice exhibited i*<br>205 The variable severity of the cra 203<br>204<br>205<br>206<br>207 **Wnt1-Cre2** *Irf6* **cKO mice exhibited incomplete frontal and parietal bone development<br>
205 The variable severity of the cranial defect in** *Wnt1***-Cre** *Irf6* **cKO mice spurred us to<br>
206 examine the cranial bone development** The variable severity of the cranial defect in *Wnt1*-Cre *Irf6* cKO mice spurred us to<br>206 examine the cranial bone development of the mice more precisely with microCT analysis.<br>207 Cre cKO pups and sex-matched littermate examine the cranial bone development of the mice more precisely with microCT analysis. *Wnt1*<br>207 Cre cKO pups and sex-matched littermate controls were collected at 10 days of age for microCT<br>208 scanning. For controls, we 207 Cre cKO pups and sex-matched littermate controls were collected at 10 days of age for microCT<br>
208 scanning. For controls, we analyzed both *Wnt1*-Cre negative, *Irf6*<sup>*liff*</sup> and *Wnt1*-Cre positive,<br>
209 *Irf6<sup>wtfi</sup>* scanning. For controls, we analyzed both *Wnt1*-Cre negative, *Inf6<sup>fI/fl</sup>* and *Wnt1*-Cre positive, scanning. For controls, we analyzed both *Wnt1*-Cre negative, *Irf6*<sup>I/I1</sup> and *Wnt1*-Cre positive,<br>209 *Irf6*<sup>W/fl</sup> pups to account for potential differences caused by the *Wnt1*-Cre transgene. *Wnt1*-<br>210 Cre<sup>+</sup>;*Irf6*<sup></sup> *Irf6<sup>wt/fl</sup>* pups to account for potential differences caused by the *Wnt1*-Cre transgene. Wnt1-209 *Irf6<sup>wtm</sup>* pups to account for potential differences caused by the *Wnt1*-Cre transgene. *Wnt1*-210 Cre<sup>+</sup>;*Irf6<sup>ft/ft</sup>* pups exhibited decreased mineralization of the frontal and parietal bones at the midline, althou Cre*<sup>+</sup> ;Irf6fl/fl* 210 Cre<sup>+</sup>;*Irf6<sup>ft/11</sup>* pups exhibited decreased mineralization of the frontal and parietal bones at the<br>211 midline, although the degree of this defect was variable between individuals (Fig 4a). Thes<br>212 observations ar midline, although the degree of this defect was variable between individuals (Fig 4a). These<br>212 observations are similar to previously published microCT analysis of the *Irf6* null mouse<br>213 (Thompson et al., 2019). To qu observations are similar to previously published microCT analysis of the *Irf6* null mouse<br>
213 (Thompson et al., 2019). To quantify potential changes in cranial development in the cK<br>
214 we performed a series of measurem 213 (Thompson et al., 2019). To quantify potential changes in cranial development in the cKO mice,<br>214 we performed a series of measurements based on established anatomical landmarks in cKO<br>215 versus sex-matched littermat we performed a series of measurements based on established anatomical landmarks in cKO<br>215 versus sex-matched littermate *Wnt1*-Cre negative controls (Fig 4a). Overall, we did not detect<br>216 significant differences in leng 215 versus sex-matched littermate *Wnt1*-Cre negative controls (Fig 4a). Overall, we did not detect<br>216 significant differences in length or width measurements of the frontal, maxillary, or nasal bones<br><sup>216</sup> significant di 216 significant differences in length or width measurements of the frontal, maxillary, or nasal bones,



243 and it was noted that the *Krt6ai*-Cre<sup>+</sup>;*Irf6*<sup>fl/fl</sup> genotype was not found at 3 weeks of age. As *Irf6*<br>and it was noted that the *Krt6ai*-Cre<sup>+</sup>;*Irf6*<sup>fl/fl</sup> genotype was not found at 3 weeks of age. As *Irf6*<br>and it

244 global null mice die shortly after birth, we observed neonates at P0 and found that a few lacked<br>
245 a milk spot and appeared to be failing to thrive. Pups were collected and genotyping determined<br>
246 these unhealth 245 a milk spot and appeared to be failing to thrive. Pups were collected and genotyping determined<br>
246 these unhealthy pups to be *Krt6ai*-Cre<sup>+</sup>;*Irf6*<sup>*I/II*</sup> whereas healthy pups were negative for Cre or<br>
247 were *I* these unhealthy pups to be *Krt6ai*-Cre<sup>+</sup>;*Irf6*f<sup>I/fl</sup> 246 these unhealthy pups to be *Krt6ai*-Cre<sup>+</sup>; *Irf6*<sup>*tm*</sup> whereas healthy pups were negative for Cre or<br>247 were *Irf6*<sup>*wtfl*</sup>. Closer examination of P0 neonates revealed shiny skin as has been previously<br>248 noted fo were *Irf6*wt/fl. were *Irf6*<sup>wtri</sup>. Closer examination of P0 neonates revealed shiny skin as has been previously<br>
248 noted for KO (Ingraham et al., 2006). *Krt6ai-*Cre<sup>+</sup>;*Irf6*<sup>t/fl</sup> pups also exhibited pterygium of the<br>
250 null mouse noted for KO (Ingraham et al., 2006). *Krt6ai-*Cre<sup>+</sup>;*Irf6*f<sup>//fl</sup> noted for KO (Ingraham et al., 2006). *Krt6ai-*Cre<sup>+</sup>;*Irf6*f<sup>*in*</sup> pups also exhibited pterygium of the<br>fore and hind limbs consistent with a milder form of the cocooning observed in the global *Irf6*<br>null mouse (Ingraha fore and hind limbs consistent with a milder form of the cocooning observed in the global *Irf6*<br>250 null mouse (Ingraham et al., 2006) (Fig. 6A,B). *Krt6ai*:Cre<sup>+</sup>; *Irf6*<sup>f/ff</sup> neonates exhibited simple<br>251 syndactyly di null mouse (Ingraham et al., 2006) (Fig. 6A,B). *Krt6ai*:Cre<sup>+</sup>;*Irf6<sup>f\/fl</sup>* 

250 null mouse (Ingraham et al., 2006) (Fig. 6A,B). *Krt6ai*:Cre<sup>+</sup>;*Irf6*<sup>1/11</sup> neonates exhibited simple<br>251 syndactyly digits of the fore and hind limbs (Fig. 6B).<br>252 The lack of a milk spot in the *Krt6ai*-Cre<sup>+</sup>;*Ir* 251 syndactyly digits of the fore and hind limbs (Fig. 6B).<br>252 The lack of a milk spot in the *Krt6ai*-Cre<sup>+</sup>; *Irf6<sup>t/fl</sup>* neona<br>253 possible palate defects and oral adhesions as occur v<br>254 examination revealed that *K* The lack of a milk spot in the *Krt6ai*-Cre<sup>+</sup>;*Irf6*f<sup>//fl</sup> 252 The lack of a milk spot in the *Krt6ai*-Cre<sup>+</sup>;*Irf6t<sup>i/n</sup>* neonates suggested impaired feeding and<br>253 possible palate defects and oral adhesions as occur with global *Irf6* ablation. Histological<br>254 examination rev possible palate defects and oral adhesions as occur with global *Irf6* ablation. Histological<br>254 examination revealed that *Krt6ai*-Cre<sup>+</sup>; *Irf6*<sup>1/f1</sup> mice present with lateral adhesions of the ton<br>255 the oral cavity a examination revealed that *Krt6ai*-Cre<sup>+</sup>;*Irf6*<sup>f/fl</sup> 254 examination revealed that *Krt6ai*-Cre<sup>+</sup>;*Irf6*<sup>1/m</sup> mice present with lateral adhesions of the tongue to<br>255 the oral cavity and a cleft of the secondary palate of variable penetrance (Fig. 6C). In some<br>256 *Krt6ai* the oral cavity and a cleft of the secondary palate of variable penetrance (Fig. 6C). In some<br>256 *Krt6ai*-Cre<sup>+</sup>;*Irf6*<sup>f//f</sup> individuals we found sublingual fluid accumulation that we presume<br>257 be caused by the oral ad *Krt6ai*-Cre+ ;*Irf6*fl/fl 256 *Krt6ai*-Cre<sup>+</sup>;*Irf6*<sup>ti/it</sup> individuals we found sublingual fluid accumulation that we presume to<br>257 be caused by the oral adhesions. No differences were observed in the lip or primary palate.<br>258 To compare *Krt6a* be caused by the oral adhesions. No differences were observed in the lip or primary palate.<br>
258 To compare *Krt6ai* periderm-specific *Irf6* ablation findings to pan-epithelial ablation, we<br>
259 utilized the *Crect* drive To compare *Krt6ai* periderm-specific *Irf6* ablation findings to pan-epithelial ablation, we<br>
259 utilized the *Crect* driver line. The *Crect* mouse has been previously utilized to conditionally<br>
260 ablate gene expressi utilized the *Crect* driver line. The *Crect* mouse has been previously utilized to conditionally<br>
260 ablate gene expression in the ectoderm, including the oral and cranial epithelium (Reid et a<br>
261 2011; Schock et al., ablate gene expression in the ectoderm, including the oral and cranial epithelium (Reid et al.,<br>
261 2011; Schock et al., 2017). Crect<sup>+</sup>; Irfo<sup>f/If1</sup> embryos were examined at approximately E17 and<br>
262 were found to recap 2011; Schock et al., 2017). *Crect*<sup>+</sup> ;*Irf6*fl/fl 261 2011; Schock et al., 2017). *Crect<sup>+</sup>*;*Irf6*<sup>t/m</sup> embryos were examined at approximately E17 and<br>262 were found to recapitulate the *Irf6* knockout phenotype with abnormal skin, foreshortened lir<br>263 and deficient de were found to recapitulate the *Irf6* knockout phenotype with abnormal skin, foreshortened limbs,<br>
263 and deficient development of the maxilla and mandible (Fig. S1). Histology of these mice<br>
264 showed adhesion of the to and deficient development of the maxilla and mandible (Fig. S1). Histology of these mice<br>264 showed adhesion of the tongue to the palate, similar to *Irf6* global null mice (Fig. S1). This<br>265 finding suggests that *Crect* 264 showed adhesion of the tongue to the palate, similar to *Irf6* global null mice (Fig. S1). This<br>265 finding suggests that *Crect* expression largely overlaps with the expression of endogenous<br>266 gene expression, leadi 265 finding suggests that *Crect* expression largely overlaps with the expression of endogenous *Irf6*<br>266 gene expression, leading to complete *Irf6* ablation in the *Crect*<sup>+</sup>; *Irf6*<sup>1/f1</sup> cKO mouse.<br>267 gene expression, leading to complete *Irf6* ablation in the *Crect*<sup>+</sup>;*Irf6*<sup>f//fl</sup> gene expression, leading to complete *Irf6* ablation in the *Crect<sup>+</sup>;Irf6<sup>tm</sup>* cKO mouse.<br>267<br>Sides of the distribution of the creck<sup>+</sup>,Irf6<sup>tm</sup> cKO mouse.

268 **Discussion**<br>269 Mutari<br>270 of cleft lip, clip<br>271 *Irf6*R84C mu 269 Mutations in *IRF6* underlie VWS and PPS, which are characterized by varying degrees<br>270 of cleft lip, cleft palate, lip pits, skin folds, syndactyly, and oral adhesions (REF). *Irf6* null and th<br>271 *Irf6*R84C mutant 270 of cleft lip, cleft palate, lip pits, skin folds, syndactyly, and oral adhesions (REF). *Irf6* null and the<br>271 *Irf6*R84C mutant mouse models recapitulate aspects of these syndromes with severe oral<br>272 adhesions, sur *Irf6*R84C mutant mouse models recapitulate aspects of these syndromes with severe oral<br>
272 adhesions, surface epithelium adhesions, and dysfunctional keratinocytes which cause ne<br>
273 lethality (Ingraham et al., 2006; Ko adhesions, surface epithelium adhesions, and dysfunctional keratinocytes which cause neonatal<br>
273 lethality (Ingraham et al., 2006; Kondo et al., 2002). *IRF6* is also associated with non-syndromic<br>
274 cleft lip and pala lethality (Ingraham et al., 2006; Kondo et al., 2002). *IRF6* is also associated with non-syndromic<br>
274 cleft lip and palate (Leslie et al., 2016), and yet the severe adhesions of the tongue within the<br>
275 oral cavity in cleft lip and palate (Leslie et al., 2016), and yet the severe adhesions of the tongue within the<br>275 oral cavity in the *Irf6* null and *Irf6R84C* mutant mouse models complicate a direct comparison<br>276 the human condition oral cavity in the *Irf6* null and *Irf6R84C* mutant mouse models complicate a direct comparison to<br>
276 the human condition. This study generated a new *Irf6* conditional knockout mouse model and<br>
277 demonstrated reliabl the human condition. This study generated a new *Irf6* conditional knockout mouse model and<br>277 demonstrated reliable recombination of the conditional allele when tested with various Cre driv<br>278 lines. This new conditiona demonstrated reliable recombination of the conditional allele when tested with various Cre driver<br>
278 lines. This new conditional Irf6 allele facilitated the investigation of tissue-specific roles of *Irf6*.<br>
279 *IRF6*, lines. This new conditional Irf6 allele facilitated the investigation of tissue-specific roles of *Irf6*.<br>
279 *IRF6, TFAP2A*, and *GRHL3* share a genetic regulatory pathway and ablation of each of<br>
280 these genes in mice *IRF6, TFAP2A*, and *GRHL3* share a genetic regulatory pathway and ablation of each of<br>280 these genes in mice causes similar cleft, skin, and limb defects (Ingraham et al., 2006; Kousa e<br>281 al., 2019; Richardson et al., these genes in mice causes similar cleft, skin, and limb defects (Ingraham et al., 2006; Kousa et al., 2019; Richardson et al., 2006; Schorle et al., 1996; Siewert et al., 2023; Smith et al., 2017; Ting et al., 2003; Zhang 281 al., 2019; Richardson et al., 2006; Schorle et al., 1996; Siewert et al., 2023; Smith et al., 2017;<br>282 Ting et al., 2003; Zhang et al., 1996). As such, it is intriguing that Tfap2a and Grhl3 are<br>283 associated with ne 282 Ting et al., 2003; Zhang et al., 1996). As such, it is intriguing that Tfap2a and Grhl3 are<br>283 associated with neural tube defects, whereas defects are not observed in the Irf6 ablate<br>284 (Schorle et al., 1996; Ting e associated with neural tube defects, whereas defects are not observed in the Irf6 ablated mice<br>
284 (Schorle et al., 1996; Ting et al., 2003; Zhang et al., 1996). To investigate this phenomenon<br>
285 Kousa et al., developed 284 (Schorle et al., 1996; Ting et al., 2003; Zhang et al., 1996). To investigate this phenomenon<br>285 Kousa et al., developed an *Irf6* loss-of-function and gain-of-function allelic series in mice and<br>286 found rostral neu Kousa et al., developed an *Irf6* loss-of-function and gain-of-function allelic series in mice and<br>286 found rostral neural tube defects associated with *Irf6* overexpression and caudal defects<br>287 associated with *Irf6* l found rostral neural tube defects associated with *Irf6* overexpression and caudal defects<br>
287 associated with *Irf6* loss of function (Kousa et al., 2019). We hypothesized that the sever<br>
288 epithelial adhesions resulti associated with *Irf6* loss of function (Kousa et al., 2019). We hypothesized that the severe<br>
288 epithelial adhesions resulting from periderm dysfunction in the *Irf6* null mouse may mask n<br>
289 tube defects and we there epithelial adhesions resulting from periderm dysfunction in the *Irf6* null mouse may mask neural<br>
289 tube defects and we therefore generated a conditional KO where *Irf6* would be ablated in *Wnt1*<br>
290 expressing neuroe tube defects and we therefore generated a conditional KO where *Irf6* would be ablated in *Wnt1*<br>290 expressing neuroectoderm and neural crest cells, including those in the neural folds. We found<br>291 a rostromedial defect expressing neuroectoderm and neural crest cells, including those in the neural folds. We found<br>
291 a rostromedial defect in these mice of varying severity that affected the skin and calvarial bone.<br>
292 Further, we found 291 a rostromedial defect in these mice of varying severity that affected the skin and calvarial bone.<br>292 Further, we found changes to neural fold morphology and *Wht1* expression patterns in these<br>293 embryos. Together,

292 Further, we found changes to neural fold morphology and *Wnt1* expression patterns in these<br>293 embryos. Together, these data corroborate a role for *Irf6* in the patterning and morphogenesis<br>293 293 embryos. Together, these data corroborate a role for *Irf6* in the patterning and morphogenesis

294 of the rostral neural tube in mice. Differences in phenotype and severity between our results and<br>295 Kousa et al. may be attributed to spatial and temporal differences in the respective<br>296 overexpression and knockout 295 Kousa et al. may be attributed to spatial and temporal differences in the respective<br>296 overexpression and knockout drivers that were utilized (*Krt14* versus *Wnt1*). Furthe<br>297 neural tube phenotypes may become app overexpression and knockout drivers that were utilized (*Krt14* versus *Wnt1*). Further, additional<br>
297 neural tube phenotypes may become apparent in the *Wnt1*-Cre *Irf6* cKO upon combinatorial<br>
298 *Irf6* is widely expr

neural tube phenotypes may become apparent in the *Wnt1*-Cre *Irf6* cKO upon combinatorial<br>298 genetic disruption of *Tfap2a* or *Grhl3*.<br>299 *Irf6* is widely expressed in the pan-epithelium and its specific role in variou genetic disruption of *Tfap2a* or *Grhl3*.<br>
299 *Irf6* is widely expressed in the populations (i.e. basal epithelium vers<br>
301 phenotype have had limited direct inve *Irf6* is widely expressed in the pan-epithelium and its specific role in various epithelial<br>
200 populations (i.e. basal epithelium versus periderm) and those contributions to the mutant<br>
201 perceptibelium by utilizing t 300 populations (i.e. basal epithelium versus periderm) and those contributions to the mutant<br>301 phenotype have had limited direct investigation. Kousa et al. previously investigated the r<br>302 *Irf6* in the basal epithel 301 phenotype have had limited direct investigation. Kousa et al. previously investigated the role of<br>302 *Irf6* in the basal epithelium by utilizing the *Krt14* promoter to express *Irf6* in the basal epithelium<br>303 on an *Irf6* in the basal epithelium by utilizing the *Krt14* promoter to express *Irf6* in the basal epithelium<br>
on an *Irf6* global null background. It was found that *Irf6* expression in the basal epithelium<br>
partially rescue 303 on an *Irf6* global null background. It was found that *Irf6* expression in the basal epithelium<br>304 partially rescued some aspects of the *Irf6* null phenotype, namely the skin adhesions of the<br>305 and appendicular sk 304 partially rescued some aspects of the *Irf6* null phenotype, namely the skin adhesions of the axial<br>305 and appendicular skeleton but did not rescue the cleft palate (Kousa et al., 2017). Utilizing our<br>306 *Irf6* floxe and appendicular skeleton but did not rescue the cleft palate (Kousa et al., 2017). Utilizing our<br>306 *Irf6* floxed mouse and the *Krt6ai*-Cre driver, we found that ablation of *Irf6* in the periderm largel<br>307 phenocopied *Irf6* floxed mouse and the *Krt6ai*-Cre driver, we found that ablation of *Irf6* in the periderm largely<br>
307 phenocopied the *Krt14:Irf6<sup>tg</sup>* rescue. Limb defects were similar in that the limbs were not<br>
308 adhered to t phenocopied the *Krt14:Irf6<sup>tg</sup>* rescue. Limb defects were similar in that the limbs were not phenocopied the *Krt14:Irf6<sup>g</sup>* rescue. Limb defects were similar in that the limbs were not<br>308 adhered to the body yet syndactyly of the digits were observed. Whereas Kousa et al. re<br>309 oral adhesions slightly less seve 308 adhered to the body yet syndactyly of the digits were observed. Whereas Kousa et al. reported<br>309 oral adhesions slightly less severe than the global KO and cleft palate, the periderm-specific *Infl*<br>310 KO mice had re oral adhesions slightly less severe than the global KO and cleft palate, the periderm-specific *Irf6*<br>310 KO mice had relatively mild oral adhesion and cleft of the palate was incompletely penetrant.<br>311 Therefore, our dat 310 KO mice had relatively mild oral adhesion and cleft of the palate was incompletely penetrant.<br>311 Therefore, our data coincide with previous findings, and differences in phenotype and severity<br>312 are likely due to dif

311 Therefore, our data coincide with previous findings, and differences in phenotype and severity<br>312 are likely due to differences in cell specificity and timing of expression.<br>313 Irf6 has a key role in the regulation o are likely due to differences in cell specificity and timing of expression.<br>313 Irf6 has a key role in the regulation of epithelial proliferation and<br>314 al., 2008; Biggs et al., 2012; Girousi et al., 2021; Oberbeck et al. Irf6 has a key role in the regulation of epithelial proliferation and differentiation (Bailey et al., 2008; Biggs et al., 2012; Girousi et al., 2021; Oberbeck et al., 2019). As such, *IRF6* is implicated in epidermal wound al., 2008; Biggs et al., 2012; Girousi et al., 2021; Oberbeck et al., 2019). As such, *IRF6* is<br>315 implicated in epidermal wound healing and children with VWS have an increased risk of w<br>316 complications following surgic implicated in epidermal wound healing and children with VWS have an increased risk of wound<br>316 complications following surgical repair of orofacial clefts (Hixon et al., 2017; Jones et al., 2010;<br>317 Rhea et al., 2020). F 316 complications following surgical repair of orofacial clefts (Hixon et al., 2017; Jones et al., 2010;<br>317 Rhea et al., 2020). Further, loss of Irf6 expression is associated with epidermal malignancy<br>318 (Botti et al., 2 317 Rhea et al., 2020). Further, loss of Irf6 expression is associated with epidermal malignancy<br>318 (Botti et al., 2011; Darido et al., 2016; Parisi et al., 2022; Yan et al., 2023). Investigation into<br>319 these roles of I 318 (Botti et al., 2011; Darido et al., 2016; Parisi et al., 2022; Yan et al., 2023). Investigation into<br>319 these roles of Irf6 have, until now, depended on human patient-derived cells, genetically<br>419 these roles of Irf6 319 these roles of Irf6 have, until now, depended on human patient-derived cells, genetically



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- 331 **Acknowledgments**<br>332 CRISPR design cons<br>333 by The Genome Mod<br>334 MicroCT scanning w CRISPR design consultation, zygote microinjection, and embryo implantation were performed<br>333 by The Genome Modification Facility and Harvard University.<br>334 MicroCT scanning was performed by the Center for Musculoskeletal by The Genome Modification Facility and Harvard University.<br>334 MicroCT scanning was performed by the Center for Musculos<br>335 Biomechanical Testing Core (NIH P30 AR070542).<br>336 MicroCT scanning was performed by the Center for Musculoskeletal Research Imaging and<br>335 Biomechanical Testing Core (NIH P30 AR070542).<br>336 **Funding sources**<br>337 Funding sources
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- 339 of Philadelphia, and research grants from the Shriners Hospitals for Children.

340 **Figures Legends**<br>341 **Fig. 1.** *Inf6* is expre<br>343 **neural tube during** 341<br>342<br>343<br>344<br>345 **Fig. 1.** *Irf6* is expressed with neural crest cell markers *Wnt1* and *Sox10* in neural folds and<br>neural tube during early embryogenesis. *In situ* hybridization of *Irf6* (yellow), *Wnt1* (red), an<br>*Sox10* (white) RNA t 343 neural tube during early embryogenesis. *In situ* hybridization of *Irf6* (yellow), *Wnt1* (red), and<br>344 Sox10 (white) RNA transcripts. **A.** Coronal section of E8 mouse embryo (dorsal to top) showi<br>345 the neural fold Sox10 (white) RNA transcripts. **A.** Coronal section of E8 mouse embryo (dorsal to top) showing<br>345 the neural fold. *In situ* hybridization shows RNA expression domains of *Irf6*, *Wnt1*, and *Sox10*,<br>346 where *Irf6* and the neural fold. *In situ* hybridization shows RNA expression domains of *Irf6*, *Wnt1*, and *Sox10*,<br>346 where *Irf6* and *Wnt1* transcripts are found in the same regions of the neural tube, highlighted by<br>347 yellow arro where *Irf6* and *Wnt1* transcripts are found in the same regions of the neural tube, highlighted by<br>347 yellow arrow. Box indicates area of higher magnification to the right. **B.** Sagittal section of E9<br>348 mouse embryo ( yellow arrow. Box indicates area of higher magnification to the right. **B.** Sagittal section of E9 mouse embryo (cranial to left). Box indicates a magnified portion of the neural tube. *Irf6* is expressed in the neuroectod mouse embryo (cranial to left). Box indicates a magnified portion of the neural tube. *Irf6* is<br>349 expressed in the neuroectoderm and overlaps with *Wnt1* and *Sox10* expression (yellow and<br>350 **C.** Sagittal section of E9 expressed in the neuroectoderm and overlaps with *Wnt1* and *Sox10* expression (yellow arrows).<br>350 **C.** Sagittal section of E9 mouse embryo (cranial to left). Box indicates a magnified portion of<br>351 frontonasal prominenc **C.** Sagittal section of E9 mouse embryo (cranial to left). Box indicates a magnified portion of frontonasal prominence (FNP). *Irf6* is expressed in the FNP mesenchyme, along with the migratory NCC marker Sox10. **D.** Coro 351 frontonasal prominence (FNP). *Irf6* is expressed in the FNP mesenchyme, along with the<br>352 migratory NCC marker Sox10. **D.** Coronal section of E13.5 embryo (dorsal to top). Box inc<br>353 higher magnification of palate s migratory NCC marker *Sox10*. **D.** Coronal section of E13.5 embryo (dorsal to top). Box indicates<br>353 higher magnification of palate shelf epithelium and mesenchyme. *Irf6* is highly expressed in the<br>354 basal epithelium a higher magnification of palate shelf epithelium and mesenchyme. *Irf6* is highly expressed in the<br>354 basal epithelium and periderm and the palate mesenchyme (yellow arrow). Blue is dapi. Scale:<br>355 100 uM.<br>356 **Fig. 3.** C basal epithelium and periderm and the palate mesenchyme (yellow arrow). Blue is dapi. Scale:<br>355 100 uM.<br>356 **Fig.2.** Generation and validation of a conditional *Irf6* null mouse model. A. Schematic of gene<br>357 **Fig.2.** Ge

355 100 uM.<br>356<br>357 **Fig.2.** Ge<br>358 targeting 356<br>357<br>358<br>359<br>360 **Fig.2.** Generation and validation of a conditional *Irf6* null mouse model. **A.** Schematic of gene<br>
1358 targeting strategy. Introns flanking *Irf6* exons 3 and 4 were targeted for CRISPR-Cas9-directed<br>
1359 homologous re targeting strategy. Introns flanking *Irf6* exons 3 and 4 were targeted for CRISPR-Cas9-directed<br>359 homologous recombination with each donor ssDNA containing loxP sequences (green<br>360 triangles). Insertion of loxP sites i 359 homologous recombination with each donor ssDNA containing loxP sequences (green<br>360 triangles). Insertion of loxP sites into *Irf6* was confirmed by PCR. **B**. and Sanger sequences<br>361 Cre-mediated recombination was va triangles). Insertion of loxP sites into *Irf6* was confirmed by PCR. **B**. and Sanger sequencing. **C.**<br>361 Cre-mediated recombination was validated using the ubiquitous Cre expressing lines *CMV*-Cre<br>362 and *Ella*-Cre. *C* Cre-mediated recombination was validated using the ubiquitous Cre expressing lines *CMV*-Cre<br>
362 and *Ella*-Cre. *CMV*-Cre<sup>+</sup>;*Irf6*<sup>M/fl</sup> and *Ella*-Cre<sup>+</sup>; *Irf6*<sup>M/fl</sup> mice phenocopied the *Irf6* global KO while<br>
363 C and *EIIa*-Cre. *CMV*-Cre<sup>+</sup> ;*Irf6*fl/fl and *EIIa*-Cre<sup>+</sup> ; *Irf6*fl/fl and *Ella*-Cre. *CMV*-Cre<sup>+</sup>;*Irf6*<sup>I/m</sup> and *Ella*-Cre<sup>+</sup>; *Irf6*<sup>I/m</sup> mice phenocopied the *Irf6* global KO while<br>363 Cre<sup>-</sup>;*Irf6*<sup>I/ff</sup> and Cre<sup>+</sup>;*Irf6*<sup>wt/wt</sup> littermates were normal. **D.** Hematoxylin and Eosin stain Cre*- ;Irf6*fl/fl and Cre+ ;*Irf6*wt/wt Solationary Cre<sup>-</sup>; *Irf6*<sup>WM</sup> intermates were normal. **D.** Hematoxylin and Eosin staining of<br>364 coronal sections of E15 CMV-Cre or *Ella*-Cre knockout embryos and littermate controls. T<br>365 row is a relatively anterior s 364 coronal sections of E15 *CMV*-Cre or *Ella*-Cre knockout embryos and littermate controls. Top<br>365 row is a relatively anterior section while the bottom row is relatively posterior. *CMV*-Cre and 365 row is a relatively anterior section while the bottom row is relatively posterior. *CMV*-Cre and

*EIIa*-Cre *Irf6* KO embryos phenocopy the dysmorphic alveolar bone and the cleft palate with oral<br>367 adhesions of the total *Irf6* knockout mouse (arrows).<br>368 **Fig. 3.** Wnt1-Cre-dependent *Irf6* ablation causes cranial

adhesions of the total *Irf6* knockout mouse (arrows).<br>368 **Fig. 3.** *Wnt1*-Cre-dependent *Irf6* ablation causes crancy<br>370 Iittermate control and *Wnt1*-Cre, *Irf6* cKO pups at P0. 368<br>369<br>370<br>371<br>372 **Fig. 3.** *Wnt1*-Cre-dependent *Irf6* ablation causes cranial defects. **A.** Representative images of<br>370 littermate control and *Wnt1*-Cre, *Irf6* cKO pups at P0. At parturition, *Wnt1*-Cre<sup>+</sup>; *Irf6*<sup>ft/ft</sup> cKO mice<br>371 d littermate control and *Wnt1*-Cre, Irf6 cKO pups at P0. At parturition, *Wnt1*-Cre<sup>+</sup>;Irf6<sup>fl/fl</sup> 370 littermate control and *Wnt1*-Cre, *Irf6* cKO pups at P0. At parturition, *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>I/m</sup> cKO mice<br>371 display midline lesions of varying penetrance (arrow). **B.** Representative images of littermate<br>372 cont display midline lesions of varying penetrance (arrow). **B.** Representative images of littermate<br>
control and *Wnt1*-Cre<sup>+</sup>;*Irf6<sup>f/fl</sup>* cKO pups at P6. As the mouse neonate develops, these frontal<br>
lesions resolve but rema control and *Wnt1*-Cre<sup>+</sup>; Irf6<sup>fl/fl</sup> 372 control and *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>1/11</sup> cKO pups at P6. As the mouse neonate develops, these frontal<br>373 lesions resolve but remain evident with deficient or delayed fur growth (arrow). **C.** Hematoxyl<br>374 and eosin sta It is a lesions resolve but remain evident with deficient or delayed fur growth (arrow). **C.** Hematoxylin<br>
374 and eosin staining of coronal sections through the palate of E16 *Wnt1*-Cre<sup>+</sup>; *Irf6*<sup>f/ff</sup> cKO and<br>
375 litte and eosin staining of coronal sections through the palate of E16 *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>fl/fl</sup> and eosin staining of coronal sections through the palate of E16 Wnt1-Cre<sup>+</sup>;Irf6<sup>I/II</sup> cKO and<br>375 littermate control embryos shows normal development (arrow). **D.** Hematoxylin and eosin<br>376 staining of coronal sections Instructure 375 littermate control embryos shows normal development (arrow). **D.** Hematoxylin and eosin<br>376 staining of coronal sections through the nasal and frontal bones of *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>f/fl</sup> cKO a<br>377 littermat staining of coronal sections through the nasal and frontal bones of *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>fl/fl</sup> 376 staining of coronal sections through the nasal and frontal bones of  $Wnt1$ -Cre<sup>+</sup>; Irf6<sup>I/II</sup> cKO and<br>377 littermate control. Sections move anterior to posterior from left to right. Bone tissue is indicate<br>378 with arr littermate control. Sections move anterior to posterior from left to right. Bone tissue is indicated<br>378 with arrows. *Wnt1*-Cre<sup>+</sup>;*Irf6<sup>ft/fl</sup>* cKO mice have a lack of cranial bone development and suture<br>379 formation at with arrows. *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>fl/fl</sup> with arrows. *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>t/n</sup>cKO mice have a lack of cranial bone development and suture<br>379 formation at the midline (bone tissue indicated by arrows). Scale: 100 µM.<br>380 **Fig. 4.** Cranial bone development is imp

379 formation at the midline (bone tissue indicated by arrows). Scale: 100  $\mu$ M.<br>380 **Fig. 4.** Cranial bone development is impaired in *Wnt1*-Cre *Irf6* cKO mice. A microCT reconstructions of P10 *Wnt1*-Cre<sup>+</sup>; *Irf6*<sup>f/</sup> 380<br>381<br>382<br>383<br>384 **Fig. 4.** Cranial bone development is impaired in *Wnt1*-Cre *Irf6* cKO mice. **A.** Representative microCT reconstructions of P10 *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>f//fl</sup> cKO mice and littermate sex-matched contr *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>f/</sup> microCT reconstructions of P10 *Wnt1*-Cre<sup>+</sup> ;*Irf6*fl/fl microCT reconstructions of P10 *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>tim</sup> cKO mice and littermate sex-matched controls.<br>383 *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>ft/fl</sup> cKO mice have decreased formation or mineralization of the cranial bones at<br>384 the mid Wnt1-Cre<sup>+</sup>; Irf6<sup>fl/fl</sup> 383 *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>t/m</sup> cKO mice have decreased formation or mineralization of the cranial bones at<br>384 the midline with variable penetrance (arrows). Scale: 1 mm. **B.** MicroCT reconstructions were<br>385 utilized for the midline with variable penetrance (arrows). Scale: 1 mm. **B.** MicroCT reconstructions were<br>385 utilized for cranial bone measurements. The space between the left and right frontal bones of<br>386 Wnt1-Cre<sup>+</sup>; Irf6<sup>ft/ff</sup> c 385 utilized for cranial bone measurements. The space between the left and right frontal bones of<br>386 Wnt1-Cre<sup>+</sup>; Irf6<sup>ft/ff</sup> cKO mice was significantly wider than controls (L1-R1, \*p<0.05) and the front<br>387 bones tended Wnt1-Cre<sup>+</sup>; Irf6<sup>fl/fl</sup> 386 *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>1/11</sup> cKO mice was significantly wider than controls (L1-R1, \*p<0.05) and the frontal<br>387 bones tended to have decreased total length (length 1-2). Maxilla of *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>1/11</sup> cKO mice<br>3 bones tended to have decreased total length (length 1-2). Maxilla of *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>f/fl</sup> bones tended to have decreased total length (length 1-2). Maxilla of *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>t/m</sup> cKO mice<br>tended to be smaller (lower length and width measurements) and the frontal bone of *Wnt1*-<br>Cre<sup>+</sup>;*Irf6*<sup>f/m</sup> cKO mice 1388 tended to be smaller (lower length and width measurements) and the frontal bone of *Wnt1*-<br>
239 Cre<sup>+</sup>;*Irf6*<sup>fl/fl</sup>cKO mice tended to be shorter, however, these differences were not significantly<br>
230 different. N=4. Cre<sup>+</sup> ;*Irf6*fl/fl 389 Cre<sup>+</sup>;*Irf6*<sup>f/m</sup> cKO mice tended to be shorter, however, these differences were not significantly<br>390 different. N=4.<br>391 390 different. N=4.

**Fig. 5.** *Irf6* ablation in the neuroectoderm and neural crest changes *Wnt1* expression domains<br>within the neural folds. **A.** RNAscope *in situ* hybridization of transverse sections of *Wnt1*-<br>Cre<sup>+</sup>;*Irf6*<sup>f//fl</sup> cKO an within the neural folds. **A.** RNAscope *in situ* hybridization of transverse sections of *Wnt1*-<br>394 Cre<sup>+</sup>;*Irf6<sup>f/fl</sup>* cKO and littermate control E8 embryos. Rows represent 2 individuals of each<br>395 genotype. Whereas *Wn* Cre<sup>+</sup> ;*Irf6*fl/fl Sequency of the Cre<sup>+</sup>; Irf6<sup>fm</sup> cKO and littermate control E8 embryos. Rows represent 2 individuals of each<br>395 genotype. Whereas *Wnt1* expression (red) is localized to the caudal-dorsal neural folds in<br>396 control embry genotype. Whereas *Wnt1* expression (red) is localized to the caudal-dorsal neural folds in the<br>396 control embryos, *Wnt1* expression in *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>ft/ft</sup> cKO embryos is displaced laterally<br>397 (arrows). Blue is control embryos, *Wnt1* expression in *Wnt1*-Cre<sup>+</sup> ;*Irf6*fl/fl control embryos, *Wnt1* expression in *Wnt1*-Cre<sup>+</sup>;*Irf6*<sup>t//1</sup> cKO embryos is displaced laterally<br>397 (arrows). Blue is dapi. Scale: 100 µM<br>398 **Fig. 6.** Periderm-specific ablation of *Irf6* results in a comparable but m 397 (arrows). Blue is dapi. Scale: 100 µM<br>398 **Fig. 6.** Periderm-specific ablation of *Ir*<br>400 KO phenotype. *Krt6ai*-Cre<sup>+</sup>; *Irf6*<sup>fI/f1</sup> and l 398<br>399<br>400<br>401<br>402 **Fig. 6.** Periderm-specific ablation of *Irf6* results in a comparable but mild form of the global *Irf6*<br>400 KO phenotype. *Krt6ai*-Cre<sup>+</sup>;*Irf6*<sup>f//f|</sup> and littermate control neonates were collected at P1. **A.** Later<br>401 KO phenotype. *Krt6ai*-Cre<sup>+</sup> ;*Irf6*fl/fl 400 KO phenotype. *Krt6ai*-Cre<sup>+</sup>;*Irf6<sup>ft/ft</sup>* and littermate control neonates were collected at P1. **A.** Lateral<br>401 and caudal representation of neonates comparing control *Krt6ai*-Cre<sup>-</sup>;*Irf6<sup>ft/ft</sup>* with *Krt6ai*-<br>4 and caudal representation of neonates comparing control *Krt6ai*-Cre- ;*Irf6*fl/fl and caudal representation of neonates comparing control *Krt6ai*-Cre<sup>-</sup>;*Irf6*<sup>1/11</sup> with *Krt6ai*-Cre<sup>+</sup>;*Irf6*<sup>1/11</sup> cKO. **B.** *Krt6ai*-Cre<sup>-</sup>;*Irf6*<sup>1/11</sup> exhibit normal skin and digits; however *Krt6ai*-Cre<sup>+</sup> reveal Cre<sup>+</sup> ;*Irf6*fl/fl cKO. **B.** *Krt6ai*-Cre- ;*Irf6*fl/fl exhibit normal skin and digits; however *Krt6ai*-Cre<sup>+</sup> ;*Irf6*fl/fl 403<br>404<br>405<br>406 reveal abnormal skin and fused digits phenotype. Scale: 500 μM. **C.** Hematoxylin and Eosin<br>404 staining of coronal sections through vomeronasal and primary palate of neonates. *Krt6ai*-Cre<br>405 ;*Irf6<sup>ft/ft</sup>* mice show nor staining of coronal sections through vomeronasal and primary palate of neonates. *Krt6ai*-Cre- 405<br>406<br>407<br>408 ;*Irf6*fl/fl mice show normal septum and palate. *Krt6ai*-Cre<sup>+</sup> ;*Irf6*fl/fl 105 ; *Irf6*<sup>1/11</sup> mice show normal septum and palate. *Krt6ai*-Cre<sup>+</sup>; *Irf6*<sup>1/11</sup> mice reveal abnormal septum and<br>106 adhesions of the tongue.<br>107 **Fig. S1.** Crect-driven *Irf6* ablation recapitulates the global *Irf6* 406 adhesions of the tongue.<br>407<br>**Fig. S1.** Crect-driven Irf6<br>409 images of littermate contr 407<br>408<br>409<br>410<br>411 Fig. S1. *Crect*-driven *Irf6* ablation recapitulates the global *Irf6* KO phenotype. **A.** Representative<br>
409 images of littermate control and *Crect<sup>t</sup>*-*Irf6<sup>ft/ff</sup>* cKO pups at approximately E17. *Crect<sup>t</sup>*-*Irf6<sup>ft/ff*</sup> images of littermate control and *Crect<sup>+</sup>-Irf6<sup>f//fl</sup>c*KO pups at approximately E17. *Crect<sup>+</sup>-Irf6<sup>fI/fi</sup>* images of littermate control and *Crect<sup>t</sup>*-*Irf6<sup>1071</sup>* cKO pups at approximately E17. *Crect<sup>t</sup>*-*Irf6<sup>1071</sup>* pups<br>exhibit "cocooning" taught skin, abnormal and shortened limbs, and an umbilical hernia that has<br>been des

410 exhibit "cocooning" taught skin, abnormal and shortened limbs, and an umbilical hernia that has<br>411 been described for the *Irf6* global KO. **B.** Hematoxylin and eosin staining of coronal sections of<br>412 approximately 411 been described for the *Irf6* global KO. **B.** Hematoxylin and eosin staining of coronal sections of approximately E17 *Crect<sup>+</sup>*-*Irf6<sup>f//f|</sup>* pup and littermate control. *Crect<sup>+</sup>*-*Irf6<sup>f//f|</sup>* cKO pups exhibit seve

approximately E17 *Crect*<sup>+</sup> -*Irf6fl/fl* pup and littermate control. *Crect*<sup>+</sup> -*Irf6fl/fl* approximately E17 Crect<sup>+</sup>-Irf6<sup>t//1</sup> pup and littermate control. Crect<sup>+</sup>-Irf6<sup>t//1</sup> cKO pups exhibit severe<br>
oral adhesions and cleft palate similar to the global Irf6 KO mouse. Scale: 100 µM.<br>
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413 oral adhesions and cleft palate similar to the global *Irf6* KO mouse. Scale: 100 µM.

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