KDM6B interacts with TFDP1 to activate P53 signalling in regulating mouse palatogenesis

Tingwei Guo, Xia Han, Jinzhi He, Jifan Feng, Junjun Jing, Eva Janečková, Jie Lei, Thach-Vu Ho, Jian Xu and Yang Chai*

Center for Craniofacial Molecular Biology, Herman Ostrow School of Dentistry, University of Southern California, Los Angeles, CA 90033, USA

Corresponding author:

Yang Chai
University Professor
George and MaryLou Boone Chair in Craniofacial Biology
Center for Craniofacial Molecular Biology
University of Southern California
2250 Alcazar Street – CSA 103
Los Angeles, CA 90033
Phone number: 323-442-3480
ychai@usc.edu

Key words: *Kdm6b*, epigenetic regulation, *Trp53*, H3K27me3, *Tfdp1*, palatogenesis

33 Abstract

34 Epigenetic regulation plays extensive roles in diseases and development. Disruption of epigenetic
35 regulation not only increases the risk of cancer, but can also cause various developmental defects.
36 However, the question of how epigenetic changes lead to tissue-specific responses during neural crest
37 fate determination and differentiation remains understudied. Using palatogenesis as a model, we reveal
38 the functional significance of *Kdm6b*, a H3K27me3 demethylase, in regulating mouse embryonic
39 development. Our study shows that *Kdm6b* plays an essential role in cranial neural crest development,
40 and loss of *Kdm6b* disturbs P53 pathway-mediated activity, leading to complete cleft palate along with
41 cell proliferation and differentiation defects in mice. Furthermore, activity of H3K27me3 on the
42 promoter of *Trp53* is antagonistically controlled by *Kdm6b*, and *Ezh2* in cranial neural crest cells. More
43 importantly, without *Kdm6b*, the transcription factor TFDP1, which normally binds to the promoter of
44 *Trp53*, cannot activate *Trp53* expression in palatal mesenchymal cells. Furthermore, the function of
45 *Kdm6b* in activating *Trp53* in these cells cannot be compensated for by the closely related histone
46 demethylase *Kdm6a*. Collectively, our results highlight the important role of the epigenetic regulator
47 KDM6B and how it specifically interacts with TFDP1 to achieve its functional specificity in regulating
48 *Trp53* expression, and further provide mechanistic insights into the epigenetic regulatory network during
49 organogenesis.

50

51

52 **Introduction**

53 Embryonic development is a highly complex self-assembly process during which precursor cells are
54 coordinated to generate appropriate cell types and assemble them into well-defined structures, tissues,
55 and organs (Shahbazi et al. 2016). During this process, precursor cells undergo extensive and rapid cell
56 proliferation until they reach the point of exit from the cell cycle to differentiate into various cell
57 lineages (Ruijtenberg and van den Heuvel 2016; Miermont et al. 2019). How these precursor cells
58 modulate expression of different genes and proceed through diverse proliferation and differentiation
59 processes is a very complex and interesting question. Growing evidence shows that epigenetic regulation,
60 which includes mechanisms such as DNA methylation, histone modifications, chromatin accessibility,
61 and higher-order organization of chromatin, provides the ability to modify gene expression and
62 associated protein production in a cell type-specific manner, thus playing an essential role in achieving
63 signaling specificity and in regulating cell fate during embryonic development (Hanna et al. 2018).

64 Among these various layers of epigenetic regulation, DNA methylation and histone methylation are the
65 best-characterized and known to be key regulators of diverse cellular events (Bannister and Kouzarides
66 2011; Smith and Meissner 2013; Molina-Serrano et al. 2019). For example, methylation of lysine 27 on
67 histone H3 (H3K27me) by methyltransferases is a feature of heterochromatin that renders it inaccessible
68 to transcription factors, thus maintaining transcriptional repression, across many species (Wiles and
69 Selker 2017). On the other hand, methylation of H3K4me3 found near the promoter region can couple
70 with the NURF complex to increase chromatin accessibility for gene activation (Wysocka et al. 2006;
71 Soares et al. 2017). Demethylation, which results from removing a methyl group, also plays important
72 roles during development. For instance, demethylation of H3K4 is required for maintaining pluripotency
73 in embryonic stem cells, and demethylases KDM6A and KDM6B are required for proper gene
74 expression in mature T cells (Lessard and Crabtree 2010; Jambhekar et al. 2019). These studies clearly

75 show that failure to maintain epigenomic integrity can cause deleterious consequences for embryonic
76 development and adult tissue homeostasis (Henckel et al. 2007; Kim et al. 2009; Kang et al. 2019).

77 Palatogenesis is a complex process known to be regulated by multiple genetic regulatory mechanisms,
78 including several signaling pathways (BMP, SHH, WNT, FGF, and TGF β) and different transcription
79 factors (such as *Msx1*, *Sox9*, *Lhx6/8*, *Dlx5*, *Shox2* and more) (Satokata and Maas 1994; Yu et al. 2005;
80 Chai and Maxson 2006; Levi et al. 2006; Cobourne et al. 2009; Lee and Saint-Jeannet 2011; Nakamura
81 et al. 2011; Bush and Jiang 2012; He and Chen 2012; Parada and Chai 2012; Xu et al. 2016; Reynolds et
82 al. 2019). However, environmental effects can also contribute to orofacial defects, which lends further
83 support to the notion that genetic factors are not sufficient to fully explain the etiology of many birth
84 defects (Dixon et al. 2011; Roessler et al. 2012; Seelan et al. 2012). Furthermore, case studies have
85 revealed that heterozygous mutation of a chromatin-remodeling factor, *SATB2*, and variation in DNA
86 methylation can cause cleft palate in patients (Leoyklang et al. 2007; Chandrasekharan and Ramanathan
87 2014; Young et al. 2021). These cases have drawn our attention to the function of epigenetic regulation
88 in palatogenesis.

89 The contribution of cranial neural crest cells (CNCCs) is critical to palate mesenchyme formation.
90 Recently, studies have begun to address the role of epigenetic regulation in neural crest cell fates
91 determination during development. For instance, homozygous loss of *Arid1a*, a subunit of SWI/SNF
92 chromatin remodeling complex, in neural crest cells results in lethality in mice, associated with severe
93 defects in the heart and craniofacial bones (Chandler and Magnuson 2016). In addition, both lysine
94 methyltransferase *Kmt2a* and demethylase *Kdm6a* are essential for cardiac and neural crest development
95 (Shpargel et al. 2017; Sen et al. 2020). However, how these epigenetic changes lead to tissue-specific
96 response during neural crest fate determination remain to be elucidated.

97 In this study, using palatogenesis as a model we investigated the functional significance of the
98 demethylase *Kdm6b* in regulating the fate of CNCCs during palatogenesis. We have discovered that loss
99 of *Kdm6b* in CNC-derived cells results in complete cleft palate along with soft palate muscle defects.
100 We also found cell proliferation and differentiation defects of CNC-derived cells in *Kdm6b* mutant mice.
101 More importantly, our study shows that the level of H3K27me3 on the promoter of *Trp53* (also known
102 as P53 in human) is antagonistically controlled by *Kdm6b* and *Ezh2*. Furthermore, without *Kdm6b*, the
103 transcription factor TFDP1, which binds to the promoter of *Trp53*, cannot activate expression of *Trp53*
104 in palatal mesenchymal cells. More importantly, the function of *Kdm6b* in activating *Trp53* in these cells
105 cannot be compensated for by the closely related histone demethylase *Kdm6a*. Our study highlights the
106 importance of epigenetic regulation on cell fate decision and its function in regulating activity of *Trp53*
107 in CNC-derived cells during organogenesis.

108

109 **Results**

110 **Loss of *Kdm6b* in CNC-derived cells results in craniofacial malformations**

111 Previous research has shown that the X-chromosome-linked H3K27 demethylase KDM6A is
112 indispensable for neural crest cell differentiation and viability as it establishes appropriate chromatin
113 structure (Schwarz et al. 2014; Shpargel et al. 2017). However, we do not yet have a comprehensive
114 understanding of the roles of two other members of the KDM6 family, *Kdm6b* and *Uty*, in regulating
115 CNCCs during craniofacial development. More importantly, we have yet to understand how
116 demethylase achieves its functional specificity in regulating downstream target genes. In order to
117 elucidate the functions of *Kdm6b* and *Uty*, we first evaluated the expression patterns of KDM6 family
118 members in the palatal region (Figure 1-figure supplement 1A-F). We found that, of these, *Kdm6b* is
119 more abundantly expressed than *Kdm6a* and *Uty* in both palate mesenchymal and epithelial cells, which
120 indicated it might play a critical role in regulating palatogenesis.

121 To investigate the tissue-specific function of *Kdm6b* during craniofacial development, we generated
122 *Wnt1^{Cre};Kdm6b^{fl/fl}* and *Krt14^{Cre};Kdm6b^{fl/fl}* mice to specifically target the deletion of *Kdm6b* in CNC-
123 derived and epithelial cells, respectively. Loss of *Kdm6b* in CNC-derived cells resulted in complete cleft
124 palate in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (90% phenotype penetrance, N=10) and postnatal lethality at newborn
125 stage (100% phenotype penetrance, N=10) without interrupting expression of other KDM6 family
126 members (Figure 1-figure supplement 1A-N and Figure 1A-B). To evaluate when *Kdm6b* was
127 inactivated in the CNC-derived cells, we also investigated the expression of *Kdm6b* at E9.5, well prior to
128 the formation of the palate primordium, and found that *Kdm6b* was efficiently inactivated in the CNC-
129 derived cells at this stage (Figure 1-figure supplement 1O-P). Interestingly, loss of *Kdm6b* in epithelial
130 cells did not lead to obvious defects in the craniofacial region in *Krt14^{Cre};Kdm6b^{fl/fl}* mice (Figure 1-

figure supplement 2A-H). These results emphasized that *Kdm6b* is specifically required in CNC-derived cells during palatogenesis. CT images also confirmed the complete cleft palate phenotype and revealed that the most severe defects in the palatal region of *Wnt1^{Cre};Kdm6b^{fl/fl}* mice were hypoplastic palatine processes of the maxilla and palatine bones (Figure 1C-D). Except for a minor flattened skull, other CNC-derived bones did not show significant differences between control and *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 1-figure supplement 2I-N). To evaluate the phenotype in more detail, we performed histological analysis and found that although the palatal shelves were able to elevate, the maxilla and palatine bones, as well as the palate stromal mesenchyme and soft palate muscles, failed to grow towards the midline in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 1G-R and Figure 1-figure supplement 3A-P). Furthermore, in the posterior soft palate region, *Wnt1^{Cre};Kdm6b^{fl/fl}* mice also showed morphological defects related to the orientation of muscle fibers and the pterygoid plate (Figure 1-figure supplement 3A-X). However, since the soft palate forms subsequent to the hard palate, it is difficult to identify whether the soft palatal muscle phenotype is a primary defect or a consequence resulting from an anterior cleft. Therefore, we focused on the anterior hard palate for further investigation. Collectively, these data indicate that mesenchymal *Kdm6b* is indispensable for craniofacial development and plays an essential role during palatogenesis.

147

***Kdm6b* is critical for proliferation and differentiation of CNC-derived palatal mesenchymal cells.**

During craniofacial development, CNCCs migrate ventro-laterally and populate the branchial arches to give rise to distinct mesenchymal structures in the head and neck, such as the palate. Failure of CNCCs to populate pharyngeal arches causes craniofacial defects (Noden 1983; Noden 1991; Trainor and Krumlauf 2000; Cordero et al. 2011). To determine whether *Kdm6b* mutant CNCCs successfully populate the first pharyngeal arch, which gives rise to the palatal shelves, we generated tdTomato

154 reporter mice and collected samples at E10.5. The results showed that CNCCs migration was not
155 adversely affected in *Kdm6b* mutant mice (Figure 2-figure supplement 1A-B). Then we evaluated the
156 process of palatogenesis at different embryonic stages, and found that the cleft palate phenotype
157 emerged as early as E14.5 in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 2-figure supplement 1C-D). These data
158 established that *Kdm6b* is not essential for CNCCs entering the pharyngeal arch but is specifically
159 required in regulating post-migratory CNC-derived cells during palatogenesis.

160 Because cell proliferation defects in CNC-derived cells frequently lead to craniofacial defects, we tested
161 whether loss of *Kdm6b* can affect cell proliferation using EdU labeling. After 2 hours of EdU labeling,
162 we found that the number of cells positively stained with EdU were significantly increased in the CNC-
163 derived palatal mesenchyme in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice compared to controls (Figure 2A-C). In addition,
164 after 48 hours of EdU labeling, we found that the number of Ki67 and EdU double-positive cells was
165 significantly increased in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 2D-H). These results indicated that loss of
166 *Kdm6b* in CNC-derived cells resulted in more cells remaining in the cell cycle and actively proliferating,
167 which further led to hyperproliferation of mesenchymal cells in the palatal region of *Wnt1^{Cre};Kdm6b^{fl/fl}*
168 mice. Meanwhile, palatal mesenchymal cells of *Wnt1^{Cre};Kdm6b^{fl/fl}* mice showed more expression of β -
169 Galactosidase, which suggested increased cellular senescence in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 2-
170 figure supplement 1E-G). To evaluate cellular senescence *in vivo*, we stained Lamin B1 in EdU-labeled
171 samples. After 48 hours of EdU labeling, we found there was less expression of Lamin B1 in the palatal
172 mesenchyme of *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. More importantly, fewer EdU+ cells express Lamin B1 were
173 observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 2-figure supplement 1H-L). These data suggested that
174 hyperproliferation may cause increased cellular senescence in palatal mesenchymal cells of
175 *Wnt1^{Cre};Kdm6b^{fl/fl}* mice.

Typically, cell proliferation and differentiation are inversely correlated. Differentiation of precursor cells is generally associated with arrested proliferation and permanently exiting the cell cycle (Ruijtenberg and van den Heuvel 2016). To test whether cell differentiation was affected in the CNC-derived palatal mesenchyme in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice, we examined the distribution of the early osteogenesis marker RUNX2 and the later osteogenesis marker SP7 in the palatal region from E13.5 to E15.5 (Figure 2I-R). There was a decrease in the number of RUNX2+ cells in the palatal mesenchyme at both E13.5 and E14.5 in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice in comparison to the control (Figure 2I-L and Q). In addition, SP7+ cells were also decreased in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice at both E14.5 and E15.5 (Figure 2M-P and R). Furthermore, when we induced osteogenic differentiation in palatal mesenchymal cells from E13.5 embryos for three weeks, we found that cells from *Wnt1^{Cre};Kdm6b^{fl/fl}* mice showed much less calcium deposition than cells from control mice, indicating a reduction in osteogenic potential in cells from *Kdm6b* mutant mice (Figure 2S-W). These results indicated that *Kdm6b* was indispensable for maintaining normal proliferation and differentiation of CNC-derived cells.

189

190 **Loss of *Kdm6b* in CNC-derived cells disturbs P53 pathway-mediated activity**

In order to identify the downstream targets of *Kdm6b* in the palatal mesenchyme, we performed RNA-seq analysis of palatal tissue at E12.5. The results showed that more genes were downregulated than upregulated in the palatal mesenchyme in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 3A), which is consistent with the function of *Kdm6b* in removing the repressive mark H3K27me3. We further used Ingenuity Pathway Analysis (IPA) and Gene Ontology (GO) analysis to analyze the pathways that were most disturbed in the palatal mesenchyme in *Kdm6b* mutant mice. Surprisingly, both analyses indicated that pathways involving *Trp53* might be disturbed in the palatal mesenchyme in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 3B-C).

198 The tumor suppressor P53 plays prominent roles in regulating DNA damage response, including
199 arresting cell growth for DNA repair, directing cellular senescence, and activating apoptosis (Mijit et al.
200 2020). Mutation of *Trp53* is a major cause of cancer development (Williams and Schumacher 2016).
201 Previous research has shown that some homozygous *Trp53* mutant mice exhibit craniofacial defects
202 including cleft palate, while inappropriate activation of *Trp53* during embryogenesis also causes
203 developmental defects including craniofacial abnormalities (Tateossian et al. 2015; Bowen et al. 2019).
204 These results suggest that precise dosage of *Trp53* is indispensable for craniofacial development. We
205 analyzed expression of *Trp53* in our samples and found that it significantly decreased in the palatal
206 region of the *Kdm6b* mutant mice (Figure 3D-H). These results indicate that *Kdm6b* plays an important
207 role in regulating the P53 pathway in the CNC-derived mesenchyme during palatogenesis. To further
208 evaluate the consequence of downregulated *Trp53* in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice, we assessed DNA damage,
209 which are tightly related to the function of *Trp53*, in our study. We found that DNA damage increased,
210 as indicated by γ H2AX expression, in the palatal mesenchyme in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 3I-M).
211 More importantly, we observed significantly increased γ H2AX foci in the EdU+ cells of
212 *Wnt1^{Cre};Kdm6b^{fl/fl}* palatal mesenchyme (Figure 3N-R). These data indicated that actively proliferating
213 cells in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice experienced more severe DNA damage compared to those in the control
214 mice, which might be the result of replication stress caused by the hyperproliferation we observed in
215 *Wnt1^{Cre};Kdm6b^{fl/fl}* mice.

216

217 **Altered *Trp53* expression is responsible for the developmental defects in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice**

218 To further test whether downregulated expression of *Trp53* is a key factor in the developmental defects
219 we observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice, we transfected palatal mesenchymal cells from control mice
220 with siRNA to knock down *Trp53*. qPCR revealed that expression of *Trp53* was significantly decreased

221 in the cells treated with siRNA after three days (Figure 4-figure supplement 1A). At the same time, the
222 group transfected with siRNA for *Trp53* showed a significant increase in EdU+ cells (Figure 4A-C). In
223 addition, significantly increased γ H2AX+ cells were also observed in the group transfected with siRNA
224 for *Trp53* (Figure 4D-F). These data suggested that downregulated expression of *Trp53* in the palatal
225 mesenchymal cells is a key factor that led to the hyperproliferation and increased DNA damage we
226 observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Furthermore, both expression of *Runx2* and *Sp7* were also
227 significantly reduced in the palatal mesenchymal cells transfected with siRNA for *Trp53* (Figure 4-
228 figure supplement 1B-C), which indicated that downregulated expression of *Trp53* in the palatal
229 mesenchymal cells resulted in differentiation defects, which were also observed in *Wnt1^{Cre};Kdm6b^{fl/fl}*
230 mice.

231 To further investigate the function of *Trp53* in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice, we tried to increase P53 in
232 *Kdm6b* mutant mice using available small molecules. Previous research showed that MDM2, a ubiquitin
233 ligase, specifically targets P53 for degradation and there is increased P53 activity in *Mdm2* mutant mice,
234 which exhibit a range of developmental defects (Arya et al. 2010; Bowen and Attardi 2019; Bowen et al.
235 2019). Nutlin-3, an MDM2 inhibitor that can specifically interrupt interaction between MDM2 and P53,
236 increases P53 in mouse primary neural stem progenitor cells and rescues neurogenic deficits in *Fmr1*
237 KO mice (Li et al. 2016). We treated pregnant mice with Nutlin-3 at a dosage based on their body
238 weight at E10.5, E12.5 and E14.5 of pregnancy and then collected samples at E16.5 for analysis. To
239 assess the potential influence of the solvent used to dissolve Nutlin-3 (10% DMSO in corn oil), we also
240 treated mice with 10% DMSO in corn oil at the same embryonic stages. None of the *Kdm6b* mutant
241 mice were rescued after this treatment (N=3) (Figure 4-figure supplement 1D-E). In contrast, Nutlin-3
242 treatment successfully rescued the cleft palate in three out of five *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 4G-R).
243 The remaining two showed a normal hard palate, but presented with posterior soft palate defects.

Western blot analysis showed that the protein level of P53 was successfully restored in the Nutlin-3-treated group (Figure 4-figure supplement 1F-G). This result further revealed that downregulation of *Trp53* in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice plays an essential role in the palatal defects. The genetic interaction between *Kdm6b* and *Trp53* is important for the development of post-migratory CNCCs.

248

Level of H3K27me3 is antagonistically regulated by *Kdm6b* and *Ezh2* during palatogenesis

The lysine-specific demethylase KDM6B is able to activate gene expression via removing the H3K27me3 repressive mark (Jiang et al. 2013). To investigate whether *Kdm6b* regulates the expression of *Trp53* through modifying the level of H3K27me3, we first examined the status of H3K27me3 in our samples and found that loss of *Kdm6b* in CNC-derived cells resulted in accumulation of H3K27me3 in the nucleus of CNC-derived palatal mesenchymal cells (Figure 5A-D). Furthermore, immunoblotting revealed that the level of H3K27me3 was increased in the palatal region of *Kdm6b* mutant mice (Figure 5E). Since the level of H3K27me3 can also be modified by the methyltransferases EZH1 and EZH2, we further evaluated whether expression of EZH1 and EZH2 was affected in the palatal region. We found no obvious differences in either the distribution of *Ezh1*⁺ cells or the EZH1 protein level between control and *Kdm6b* mutant mice (Figure 5F-J). Similarly, no dramatic changes were observed in either the distribution of EZH2⁺ cells or its protein level between control and *Kdm6b* mutant mice (Figure 5K-O). These results indicated that increased H3K27me3 in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice was mainly caused by loss of *Kdm6b* in CNC-derived cells. However, we did notice a broader contribution and stronger signal of EZH2 than EZH1 in the CNC-derived palatal mesenchyme. To investigate whether an increase of H3K27me3 in the CNC-derived cells caused the cleft phenotype we observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice, we generated *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* mice and assessed the level of H3K27me3 in this model. In *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* mice, we observed a rescue of the abnormal accumulation of H3K27me3

(Figure 5P-V). More importantly, haploinsufficiency of *Ezh2* in this model successfully rescued the cleft palate phenotype observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 6A-O) with 70% efficiency (N = 10). CT scanning showed that both the palatine processes of the maxilla and palatine bone were restored in the *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* mice (Figure 6A-C). Both bone and palatal mesenchymal tissue were rescued in *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* mice (Figure 6D-O). Furthermore, both EdU+ and RUNX2+ cells were restored to normal levels in *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* mice (Figure 7A-H). These results suggested that an antagonistic interaction between the histone demethylase KDM6B and methyltransferase EZH2 that modulates H3K27me3 is essential for palatogenesis.

275

***Kdm6b* activates expression of *Trp53* through removing H3K27me3 at the promoter of *Trp53* and interacts with transcription factor TFDP1 in regulating P53 signaling pathway**

Chromatin accessibility represents the degree to which chromatinized DNA is able to physically interact with nuclear macromolecules such as transcription factors for gene regulation (Klemm et al. 2019). The repressive mark H3K27me3 is usually associated with facultative heterochromatin and results in transcriptional repression due to decreased chromatin accessibility (Wiles and Selker 2017; Moller et al. 2019; den Broeder et al. 2020). The methyltransferase EZH2 and demethylases KDM6A/KDM6B can regulate the methylation status of H3K27 to affect gene expression (Pediconi et al. 2019). To test whether *Kdm6b* and *Ezh2* can regulate expression of *Trp53* via H3K27me3, we first examined whether deposition of H3K27me3 changes at the promoter of *Trp53* in our models using ChIP-qPCR. A primer set was designed at 1127 bp upstream of *Trp53* exon1 and the results showed that deposition of H3K27me3 significantly increased at the promoter of *Trp53* in the palatal region of *Kdm6b* mutant mice, while this increase was dampened in the *Ezh2* haploinsufficiency model (Figure 8A). Meanwhile, haploinsufficiency of *Ezh2* in *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* mice was able to restore the decreased expression

290 of *Trp53* observed in the CNC-derived palatal mesenchyme of *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 8B-H).
291 These data suggested that *Kdm6b* and *Ezh2* co-regulate expression of *Trp53* through H3K27me3. To
292 further reveal whether KDM6B regulates the expression of *Trp53* directly, we performed ChIP-qPCR
293 using KDM6B antibody and found that deposition of KDM6B significantly increased at the promoter of
294 *Trp53* in the palatal region (Figure 8I). In addition, to test whether KDM6B has a unique role in
295 activating *Trp53* during palatogenesis, we transfected palatal mesenchymal cells from *Kdm6b* mutant
296 mice with either a plasmid overexpressing *Kdm6b* or a plasmid overexpressing another histone
297 demethylase, *Kdm6a*. Increased expression of *Trp53* could be detected only in the group transfected
298 with *Kdm6b*-overexpressing plasmid (Figure 8J-K and Figure 8-figure supplement 1A-B). These results
299 indicated that *Kdm6b* has an essential and unique role in activating *Trp53* during palatogenesis.

300 As a H3K27me3 demethylase, KDM6B is important for the regulation of chromatin structure for gene
301 expression. To target a specific sequence in genome, a histone demethylase needs to interact with DNA
302 binding proteins such as transcription factors or lncRNAs (Dimitrova et al. 2015; Gurrion et al. 2017).
303 To identify a transcription factor that can interact with KDM6B, we performed ATAC-seq analysis of
304 palate tissue at E13.5. Through motif analysis we found that the promoter region of *Trp53* was
305 accessible to members of the E2F transcription factor family (E2F4 and E2F6) and transcription factor
306 TFDP1 (also known as Dp1), a binding partner of E2F family members (Figure 8L and Figure 8-figure
307 supplement 1C). We noticed that this open region was also located at the TSS of *Wrap53*, which was
308 previously reported to regulate endogenous *Trp53* mRNA levels and P53 protein levels ((Mahmoudi et
309 al. 2009). To evaluate whether the decrease of *Trp53* was caused by altered expression of *Wrap53* in our
310 model, we examined expression of *Wrap53* in the palatal region at E13.5. Almost no expression of
311 *Wrap53* was detected in the palatal region (Figure 8-figure supplement 1D-E). Previous research
312 reported that inactivation of E2Fs resulted in milder phenotypes than those associated with loss of *Tfdp1*,

313 which leads to early embryonic lethality (Kohn et al. 2003). This result suggested that TFDP1 may play
314 a more critical role than E2Fs during embryonic development. A motif of TFDP1 was detected 1011 bp
315 upstream of *Trp53* exon 1, which is very close to the H3K27me3 deposition site, by ATAC-seq analysis.
316 ChIP-qPCR using palate tissue at E13.5 also revealed that binding of TFDP1 to the promoter region of
317 *Trp53* decreased in the *Kdm6b* mutant mice (Figure 8M). Immunohistochemistry analysis showed that
318 TFDP1+ cells were distributed in the palatal region and co-expressed with *Trp53* and *Kdm6b* (Figure
319 8N-O). We also confirmed co-expression of *Kdm6b* and *Tfdp1* in the palatal region (especially enriched
320 in *Pax9*+, *Aldh1a2*+ and *Twist1*+ cells) using our previously published scRNA-seq data (Figure 8-figure
321 supplement 1F) (Han et al. 2021). Meanwhile, the expression level and distribution of TFDP1 were not
322 affected in the palatal mesenchyme of *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure 8P and Figure 8-figure
323 supplement 1G-H). These data indicated that *Tfdp1* is not a downstream target of *Kdm6b*. To further test
324 whether *Tfdp1* regulated expression of *Trp53* in the palatal mesenchymal cells, we transfected palatal
325 mesenchymal cells from control mice at E13.5 using siRNA to knock down *Tfdp1*. qPCR revealed that
326 the expression of *Trp53* was decreased in cells treated with siRNA for *Tfdp1* (Figure 8-figure
327 supplement 1I and Figure 8Q). This data further indicated that *Trp53* is a direct downstream target of
328 *Tfdp1*.

329 To reveal the function of *Kdm6b-Tfdp1* interaction in the regulation of *Trp53* during palatogenesis, we
330 transfected palatal mesenchymal cells with *Tfdp1*-overexpressing plasmid and found that expression of
331 *Trp53* increased in the cells from control mice but not in the cells from *Wnt1^{Cre};Kdm6b^{fl/fl}* mice (Figure
332 8-figure supplement 1J-K and Figure 8R-S). This result suggested that *Kdm6b* is specifically required
333 and plays an essential role in activation of *Trp53* through interaction with *Tfdp1* during palatogenesis.
334 We performed Co-IP experiments and found that KDM6B and TFDP1 were indeed involved in the same
335 complex (Figure 8T). Collectively, these data suggested that KDM6B and TFDP1 work together to

336 activate *Trp53* expression in the palatal mesenchyme and play an important role in regulating
337 palatogenesis (Figure 9).

338 **Discussion**

339 The development of an organism from a single cell to multiple different cell types requires tightly
340 regulated gene expression (Bruneau et al. 2019). Transcription factors, which are among the key
341 regulators of this process, are intimately involved in cell fate commitment (Nelms and Labosky 2010;
342 Soldatov et al. 2019). However, a transcription factor by itself cannot act on densely packed DNA in
343 chromatin form. Thus, transcription factors must work in coordination with epigenetic regulatory
344 mechanisms such as histone modifications, DNA methylation, chromatin remodeling and others to
345 dynamically regulate chromatin states for gene expression (Wilson and Filipp 2018; Gokbuget and
346 Blelloch 2019). Insults to the epigenetic landscape due to genetic, environmental or metabolic factors
347 can lead to diverse developmental defects and diseases (Hobbs et al. 2014; Zoghbi and Beaudet 2016;
348 Flavahan et al. 2017). Cleft palate comprises 30% of orofacial clefts, and can result from genetic
349 mutations, environmental effects, or a combination thereof (Seelan et al. 2012). Much progress has been
350 made in taking inventory of the gene mutations associated with craniofacial defects in recent years, and
351 growing evidence has shown that epigenetic regulation plays an important role during neural crest
352 development. For example, haploinsufficiency of *KDM6A* in humans causes severe psychomotor
353 developmental delay, global growth restriction, seizures and cleft palate (Lindgren et al. 2013).
354 Furthermore, studies have shown that *Kdm6a* and *Arid1a* are both indispensable during neural crest
355 development (Chandler and Magnuson 2016; Shpargel et al. 2017). DNA methyltransferase3A
356 (DNMT3A) plays a critical role in mediating the transition from neural tube to neural crest fate (Hu et al.
357 2012). Meanwhile, loss of *Ezh2*, a component of PRC2, in CNC-derived cells completely prevents
358 craniofacial bone and cartilage formation (Schwarz et al. 2014). These studies have clearly shown that

359 epigenetic regulation is crucial for neural crest development. In this study, we further demonstrate the
360 important role of epigenetic regulation during the neural crest contribution to palate development using
361 *Wnt1^{Cre};Kdm6b^{fl/fl}* mice as a model. We show that the demethylase KDM6B is not only required for
362 normal CNC-derived palatal mesenchymal cell proliferation, but also for maintaining cell differentiation.

363 Epigenetic regulators, transcription factors, and lineage-specific genes work together to achieve
364 spatiotemporally restricted, tissue-specific gene regulation (Hu et al. 2014). In this study, we reveal that
365 *Kdm6b* works with the transcription factor *Tfdp1* to specifically regulate the expression of *Trp53* during
366 palatogenesis. The molecular mechanisms underlying the function of *Trp53* in genomic stability and
367 tumor suppression have been studied extensively. However, the role of *Trp53* in regulating the
368 development of CNC-derived cells still remains largely unclear, although several studies have been
369 conducted recently on certain aspects of this topic. For instance, it has been shown that *Trp53* is able to
370 coordinate CNC cell growth and epithelial-mesenchymal transition/delamination processes by
371 modulating cell cycle genes and proliferation (Rinon et al. 2011). It has also been established that both
372 deletion and overexpression of *Trp53* result in craniofacial defects (Tateossian et al. 2015; Bowen et al.
373 2019). Furthermore, nuclear stabilization of P53 protein in *Tcof1^{+/-}* mice induces neural crest cell
374 progenitors to undergo cell-cycle arrest and caspase3-mediated apoptosis in the neuroepithelium.
375 Inhibition of P53 function successfully rescues the neurocristopathy in an animal model of Treacher
376 Collins syndrome, which results from mutation in *Tcof1* (Jones et al. 2008). These studies have clearly
377 shown that appropriate function of *Trp53* is indispensable in CNCCs. However, none of these studies
378 have addressed upstream regulation of *Trp53* in CNCCs.

379 Here, we show that proper function of *Trp53* during the differentiation and proliferation of CNCCs is
380 orchestrated by *Kdm6b* and *Ezh2* through H3K27me3. Altering the balance between *Ezh2* and *Kdm6b*
381 can cause abnormal H3K27me3 function, which further affects the downstream transcription factor

382 *Trp53*. In addition, we have detected spontaneous DNA damage in the developing palate and increased
 383 accumulation of DNA damage in the *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. These findings further demonstrate the
 384 critical function of *Trp53* in protecting embryonic cells from DNA damage during development.
 385 Furthermore, the ability of cells to proliferate is limited by the length of the telomeres, which gradually
 386 shorten during each cell replication (Blagoev 2009). Once the telomeres are too short for DNA
 387 replication, the result is cellular senescence, which induces an irreversible inability to proliferate
 388 (Bernadotte et al. 2016). In this study we show that downregulated expression of *Trp53* in
 389 *Wnt1^{Cre};Kdm6b^{fl/fl}* mice results in hyperproliferation and increased DNA damage in the proliferative
 390 cells, which might further lead to increased cell senescence. Previous studies reported that few *Trp53^{-/-}*
 391 mice exhibit cleft palate or other craniofacial abnormalities, which suggests that loss of *Trp53* alone is
 392 not powerful enough to cause defects during craniofacial development (Rinon et al. 2011; Tateossian et
 393 al. 2015) and that there might be other factors which can compensate for loss of the function of *Trp53*.
 394 In our study, *Wnt1^{Cre};Kdm6b^{fl/fl}* mice showed a cleft palate phenotype with high penetrance, and using
 395 MDM2 inhibitor Nutlin-3 we successfully rescued cleft palate in *Kdm6b* mutant mice. Our results
 396 suggested that in the *Kdm6b* mutant mice, the decrease of *Trp53* cannot be compensated for by other
 397 factors, and disturbed P53 signaling is the key factor causing cleft palate in *Kdm6b* mutant mice.

398 Previous research has shown that KDM3A functions as a cofactor of STAT3 to activate the JAK2-
 399 STAT3 signaling pathway (Kim et al. 2018) and that KDM2A coordinates with c-Fos in regulating
 400 COX-2 (Lu et al. 2015). Our study shows that KDM6B coordinates with the transcription factor TFDP1
 401 to activate expression of *Trp53* in CNCCs, and that *Ezh2* and *Kdm6b* co-regulate H3K27 methylation
 402 status, which may affect the ability of TFDP1 to bind to the chromatin during palatogenesis. It has been
 403 reported that *Tfdp1* is crucial for embryonic development and for regulating Wnt/ β -catenin signaling
 404 (Kohn et al. 2003; Kim et al. 2012). Interaction between KDM6B and TFDP1 discovered in this study

405 further increases our knowledge of the coordination between epigenetic regulators and transcription
 406 factors during organogenesis. As environmental insults can adversely affect the function of epigenetic
 407 regulators, our findings provide a better understanding of the epigenetic regulation and transcription
 408 factors involved in regulating the fate of CNC cells and craniofacial development, which can provide
 409 important clues about human development, as well as potential therapeutic approaches for craniofacial
 410 birth defects.

411

412 **Materials and Methods**

413 **Key resource table**

414

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain, strain background (M. musculus)	<i>Kdm6b</i> ^{flox/flox}	Manna et al. 2015, Jackson Laboratory	Stock No. 029615 RRID:IMSR_JAX:029615	
Strain, strain background (M. musculus)	<i>Ezh2</i> ^{flox/flox}	Bildsoe et al., 2009, Jackson Laboratory	Stock No. 022616; RRID: IMSR_JAX:022616	
Strain, strain background (M. musculus)	<i>Trp53</i> ^{flox/flox}	Jackson Laboratory	Stock No. 008462 RRID:IMSR_JAX:008462	
Strain, strain background (M. musculus)	<i>Wnt1</i> ^{Cre}	Zhao et al. 2008		
Strain, strain background (M. musculus)	<i>Krt14</i> ^{cre}	Jackson Laboratory	Stock No. 018964; RRID: IMSR_JAX:018964	

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain, strain background (M. musculus)	<i>ROSA26loxP-STOP-loxP-tdTomato</i>	Jackson Laboratory	Stock No. 007905; RRID:IMSR_JAX:007905	
Sequence-based reagent	Mm- <i>Kdm6a</i> probe	Advanced Cell Diagnostics	Cat# 456961	
Sequence-based reagent	Mm- <i>Kdm6b</i> probe	Advanced Cell Diagnostics	Cat# 477971	
Sequence-based reagent	Mm- <i>Kdm6b</i> -01 probe	Advanced Cell Diagnostics	Cat# 501231	
Sequence-based reagent	Mm- <i>Uty</i> probe	Advanced Cell Diagnostics	Cat# 451741	
Sequence-based reagent	Mm- <i>Trp53</i> probe	Advanced Cell Diagnostics	Cat# 402331	
Sequence-based reagent	Mm- <i>Trp53</i> -C2 probe	Advanced Cell Diagnostics	Cat# 402331-C2	
Sequence-based reagent	Mm- <i>Ezh1</i> probe	Advanced Cell Diagnostics	Cat# 415501	
Sequence-based reagent	Mm- <i>Wrap53</i> probe	Advanced Cell Diagnostics	Cat# 1143201-C1	
Antibody	Mouse monoclonal, Myosin heavy chain (MHC)	DSHB	Cat# P13538	(1:10)
Antibody	Rabbit monoclonal, Histone H3 tri methyl K27	Cell Signaling Technology	Cat# 9733s	(1:200) (1:1000)
Antibody	Rabbit monoclonal, Phospho-Histone	Cell Signaling Technology	Cat# 9718s	(1:200)

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
	H2A.X			
Antibody	Rabbit monoclonal, DP1	Abcam	Cat# ab124678	(1:100) (1:1000)
Antibody	Rabbit monoclonal, EZH2	Cell Signaling Technology	Cat# 5246s	(1:200) (1:2000)
Antibody	Rabbit monoclonal, RUNX2	Cell Signaling Technology	Cat# 12556s	(1:200)
Antibody	Rabbit polyclonal, SP7	Abcam	Cat# ab22552	(1:200)
Antibody	Rabbit monoclonal, Lamin B1	Cell Signaling Technology	Cat# 17416s	(1:100)
Antibody	Goat polyclonal anti- Mouse Alexa Fluor 488	Life Technologies	Cat# A11001	(1:200)
Antibody	Goat polyclonal anti- Mouse Alexa Fluor 568	Life Technologies	Cat# A-11004	(1:200)
Antibody	Goat polyclonal anti- Rat Alexa Fluor 488	Life Technologies	Cat# A-11006	(1:200)
Antibody	Goat polyclonal anti- Rabbit Alexa Fluor 488	Life Technologies	Cat# A-11008	(1:200)
Antibody	Goat polyclonal anti- Rabbit Alexa Fluor 568	Life Technologies	Cat# A-11036	(1:200)
Antibody	Rabbit polyclonal, EZH1	Abcam	Cat# ab189833	(1:1000)
Antibody	Rabbit polyclonal, KDM6A	Abcam	Cat# ab36938	(1:1000)
Antibody	Rabbit polyclonal, KDM6B (C-term)	AbCEPTA	Cat# AP1022b	(1:1000)
Antibody	Rabbit polyclonal,	AbCEPTA	Cat# AP1022a	(1:1000)

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
	KDM6B (N-term)			
Antibody	Mouse monoclonal, P53	Santa Cruz	Cat# sc-126	(1:1000)
Antibody	Rabbit monoclonal, Histone H3	Cell Signaling Technology	Cat# 4499s	(1:1000)
Antibody	Mouse monoclonal, Beta Actin	Abcam	Cat# Ab20272	(1:2000)
Antibody	Goat polyclonal Rabbit IgG HRP- conjugated antibody	R&D System	Cat# HAF008	(1:2000)
Antibody	Goat polyclonal Mouse IgG HRP- conjugated antibody	R&D System	Cat# HAF007	(1:2000)
Antibody	Mouse monoclonal HRP, Mouse Anti Rabbit IgG LCS	IPKine	Cat# A25022	(1:2000)
Commercial assay or kit	Goat anti-mouse IgG Alexa Fluor 488 Tyramide SuperBoost Kit	ThermoFisher Scientific	Cat# B40912	(1:200)
Commercial assay or kit	RNAscope Multiplex Fluorescent Kit v2	Advanced Cell Diagnostics	Cat# 323110	
Commercial assay or kit	RNAscope 2.5 HD Assay – RED	Advanced Cell Diagnostics	Cat# 322350	
Commercial assay or kit	TSA Plus Cyanine 3 System	Perkin Elmer	Cat# NEL744001KT	
Commercial assay or kit	TSA Plus Fluoresceine System	Perkin Elmer	Cat# NEL771B001KT	
Commercial assay or kit	RNeasy Micro Kit	QIAGEN	Cat# 74004	
Commercial	DAB Peroxidase (HRP)	Vector	RRID:AB_2336382	

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
assay or kit	Substrate Kit (With Nickel)	Laboratories	Cat# SK4100	
Software, algorithm	ImageJ	NIH	RRID:SCR_003070	
Software, algorithm	Ingenuity Pathway Analysis	Qiagen.Inc	RRID:SCR_008653	
Software, algorithm	GraphPad Prism	GraphPad Software	RRID:SCR_002798	
Software, algorithm	Seurat	Satija lab	RRID:SCR_016341	
Software, algorithm	Cell ranger	10X Genomics.Inc	RRID:SCR_017344	

Animals

To generate *Wnt1^{Cre};Kdm6b^{fl/fl}* mice, we crossed *Wnt1^{Cre};Kdm6b^{fl/+}* mice with *Kdm6b^{fl/fl}* mice (Zhao et al. 2008; Manna et al. 2015). Reporter mice used in this study were tdTomato conditional reporter (JAX#007905) (Madisen et al. 2010). *Ezh2^{fl/fl}* and *Trp53^{fl/fl}* mice were purchased from Jackson Laboratory (JAX#022616, #008462) (Marino et al. 2000; Shen et al. 2008). Genotyping was carried out as previously described (Zhao et al. 2008). Briefly, tail samples were lysed by using DirectPCR tail solution (Viagen 102 T) with overnight incubation at 55°C. After heat inactivation at 85°C for 1 hour, PCR-based genotyping (GoTaq Green MasterMix, Promega, and C1000 Touch Cyclor, Bio-rad) was used to detect the genes. All mouse studies were conducted with protocols approved by the Department of Animal Resources and the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California (Protocols 9320 and 20299).

427 **MicroCT analysis**

428 MicroCT was used to analyze the control, *Kdm6b*, and other mutant samples. Mouse samples were
429 dissected and fixed in 4% paraformaldehyde overnight at 4°C followed by CT scanning (Scanco
430 Medical μ CT50 scanner) at the University of Southern California Molecular Imaging Center as
431 previously described (Grosshans et al. 2006; Sugii et al. 2017). AVIZO 9.1.0 (Visualization Sciences
432 Group) was used for visualization and 3D microCT reconstruction.

433 **Alcian blue-Alizarin red staining**

434 Mouse heads were dissected and fixed in 95% EtOH overnight at room temperature. Staining was
435 performed as previously described (Rigueur and Lyons 2014). Briefly, 95% EtOH was replaced with
436 100% acetone for 2 days and then samples were incubated in Alcian blue solution (80% EtOH, 20%
437 glacial acetic acid, and 0.03% (w/v) Alcian blue 8GX (Sigma, A3157) for 1-3 days. Samples were then
438 de-stained with 70% EtOH and incubated in 95% EtOH overnight. After incubation, samples were pre-
439 cleared with 1% KOH and then incubated in Alizarin red solution (0.005% (w/v) Alizarin red (Sigma,
440 A5533) in 1% (w/v) KOH) for 2-5 days. After clearing samples with 1% KOH, they were stored in 100%
441 glycerol until analysis.

442 **Sample preparation for sectioning**

443 Samples for paraffin sectioning were prepared using the standard protocol in our laboratory. Briefly,
444 samples were fixed in 4% PFA and decalcified with 10% EDTA as needed. Then, samples were
445 dehydrated with serial ethanol solutions (50%, 70%, 80%, 90% and 100%) at room temperature
446 followed by xylene and then embedded in paraffin wax. Sections were cut to 6 μ m on a microtome
447 (Leica) and mounted on SuperFrost Plus slides (Fisher, 48311-703). Cryosectioning samples were fixed
448 and decalcified the same way as samples prepared for paraffin sectioning. Sucrose (15% and 30%) was

449 used to remove water from the samples before embedding them in OCT compound (Tissue-Tek, 4583).
450 Cryosections were cut to 8 μ m on a cryostat (Leica) and mounted on SuperFrost Plus slides (Fisher).

451 **Histological analysis**

452 Paraffin sections prepared as described above were used for histological analysis. Hematoxylin and
453 Eosin staining were performed using the standard protocol (Cardiff et al. 2014).

454 **Immunofluorescence assay**

455 Cryosections and paraffin sections prepared as described above were used for immunofluorescence
456 assays. Sections were dried for 2 hours at 55°C. Paraffin sections were deparaffinized and rehydrated
457 before antigen retrieval. Heat mediated antigen retrieval was used to process sections (Vector, H-3300)
458 and then samples were blocked for 1 hour in blocking buffer at room temperature (PerkinElmer,
459 FP1020). Primary antibodies diluted in blocking buffer were incubated with samples overnight at 4°C.
460 After washing with PBST (0.1% Tween20 in 1xPBS), samples were then incubated with secondary
461 antibodies at room temperature for 2 hours. DAPI (Sigma, D9542) was used for nuclear staining. All
462 images were acquired using Leica DMI 3000B and Keyence BZ-X710/810 microscopes. Detailed
463 information about primary and secondary antibodies is listed in Supplementary File 1.

464 **EdU labeling**

465 EdU solution was prepared at 10 mg/mL in PBS, and then pregnant mice at the desired stage were given
466 an intra-peritoneal injection (IP) based on their weight (0.1 mg of EdU/1 g of mouse). Embryos were
467 collected after 2 hours or 48 hours and then prepared for sectioning as above. EdU signal was detected
468 using Click-It EdU cell proliferation kit (Invitrogen, C10337) and images were acquired using Leica
469 DMI 3000B and Keyence BZ-X710/810 microscopes.

470 **RNAscope *in situ* hybridization**

471 RNAscope *in situ* hybridization in this study was performed on cryosections using RNAscope 2.5HD
472 Reagent Kit-RED assay (Advanced Cell Diagnostics, 322350) and RNAscope multiplex fluorescent v2
473 assay (Advanced Cell Diagnostics, 323100) according to the manufacturer's protocol. RNAscope probes
474 used in this study included *Kdm6a*, *Kdm6b*, *Uty*, and *Trp53*. Detailed information about probes is listed
475 in Supplementary File 2.

476 **RNA-sequencing and analysis**

477 Palate samples from control and *Wnt1^{Cre};Kdm6b^{fl/fl}* mice were collected at E12.5 for RNA isolation with
478 RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol. The quality of RNA samples was
479 determined using an Agilent 2100 Bioanalyzer and all samples for sequencing had RNA integrity (RIN)
480 numbers > 9. cDNA library preparation and sequencing were performed at the USC Molecular
481 Genomics Core. Single-end reads with 75 cycles were performed on Illumina Hiseq 4000 equipment and
482 raw reads were trimmed and aligned using TopHat (Version 2.0.8) with the mm10 genome. CPM was
483 used to normalize the data and differential expression was calculated by selecting transcripts that
484 changed with $p < 0.05$.

485 **RNA extraction and real-time qPCR**

486 Palatal tissue used for RNA isolation was dissected at desired stages and an RNeasy Plus Micro Kit
487 (Qiagen, 74034) was used to isolate the total RNA followed by cDNA synthesis using an iScript cDNA
488 synthesis kit (Bio-Rad, 1708891). Real-time qPCR quantification was done on a Bio-Rad CFX96 Real-
489 Time system using SsoFast EvaGreen Supermix (Bio-Rad, 1725201). Detailed information on primers is
490 listed in Supplementary File 3.

491 **ChIP-qPCR**

492 Palate tissue was dissected from control and *Wnt1^{Cre};Kdm6b^{fl/fl}* mice at E13.5. Each replicate contained
493 60-80 mg tissue combined from multiple animals. Samples were prepared following the manufacturer's
494 protocol (Chromatrap, 500191). Briefly, tissue was cut into small pieces and then fixed with 1%
495 formaldehyde at room temperature for 15 minutes, followed by incubating with 0.65 M glycine solution.
496 Then the sample was washed twice with PBS, resuspended in Hypotonic Buffer and incubated at 4°C for
497 10 minutes to obtain nuclei, which were then resuspended in Digestion Buffer. After chromatin was
498 sheared to 100-500 bp fragments using Shearing Cocktail, 10 µg chromatin with H3K27me3 antibody
499 (CST 9733s, 1:50), DP1 antibody (Abcam ab124678, 1:10), KDM6B antibody (Abcepta AP1022b,
500 1:10), or Immunoglobulin G negative control (2 µg) was added to Column Conditioning Buffer to make
501 up the final volume of 1000 µl. Immunoprecipitation (IP) slurry was mixed thoroughly and incubated on
502 an end-to-end rotor for 1 hour at 4 °C. An equivalent amount of chromatin was set as an input. After 1
503 hour incubation, IP slurry was purified using Chromatrap® spin column at room temperature and
504 chromatin was eluted using ChIP-seq elution buffer. Chromatin sample and input were further incubated
505 at 65 °C overnight to reverse cross-linking. DNA was purified with Chromatrap® DNA purification
506 column after proteinase K treatment. ChIP eluates, negative control and input were assayed using real-
507 time qPCR. Primers were designed using the promoter region of *Trp53*. Detailed information is
508 available in Supplementary File 3.

509 **Western blot and Co-Immunoprecipitation**

510 For western blot, palate tissue was dissected from control and *Wnt1^{Cre};Kdm6b^{fl/fl}* mice at E13.5. The
511 tissue sample was lysed using RIPA buffer (Cell Signaling, 9806) with protease inhibitor (Thermo
512 Fisher Scientific, A32929) for 20 minutes on ice followed by centrifugation at 4°C to remove tissue
513 debris. Protein extracts were then mixed with sample buffer (Bio-Rad, 1610747) and boiled at 98°C for
514 10 minutes. Then denatured protein extract was separated in 4%-15% precast polyacrylamide gel (Bio-

Rad, 456-1084) and then transferred to 0.45µm PVDF membrane. Transferred membrane was incubated with 5% milk for 1 hour at room temperature, and incubated with primary antibody at 4°C overnight. After washing with TBST, membrane was incubated with secondary antibody for 2 hours at room temperature and signals were detected using SuperSignal West Femto (Thermo Fisher Scientific, 34094) and Azure 300 (Azure Biosystems).

For CoIP, palate tissue was dissected from control mice at E13.5 and 60-80 mg tissue was combined as one sample for each replicate. After lysing using RIPA buffer, 60 µl of the protein extract was mixed with sample buffer and boiled at 98 °C to serve as input. The remaining protein extract was incubated with primary antibody at 4 °C overnight. Protein G beads from GE Healthcare (GE Healthcare, 10280243) were used to purify the target protein and then the protein sample was analyzed using western blot. Detailed information about primary and secondary antibodies is listed in Supplementary File 4.

siRNA and plasmid transfection

Palatal tissue was dissected from control and *Wnt1^{Cre};Kdm6b^{fl/fl}* mice at E13.5, then cut into small pieces using a scalpel. This minced tissue was then cultured in DMEM medium (Gibco, 2192449) containing 40% MSC FBS (Gibco, 2226685P) and 1% Pen Strep (Gibco, 2145477) at 37°C.

siRNA (Qiagen) and plasmid (OriGene) transfection was performed following the manufacturer's protocol (Qiagen, 301704 and OriGene, TF81001). Briefly, siRNA was transfected into cells in 24-well plates at 10nM for 3 days followed by qPCR and EdU proliferation assay. Plasmid was transfected into cells in 24-well plates using 1µg/µl stock solution for 2 days followed by real-time qPCR. Primers designed for qPCR are listed in Supplementary File 3. siRNA sequence and plasmid information are listed in Supplementary File 5 and Supplementary File 6.

537 **ATAC-seq analysis**

538 Palate tissue of E13.5 control mice was digested using TrypLE express enzyme (Thermo Fisher
539 Scientific, 12605010) and incubated at 37 °C for 20 minutes with shaking at 600 rpm. Single-cell
540 suspension was prepared according to the 10X Genomics sample preparation protocol and processed to
541 generate ATAC-seq libraries according to a published protocol (Buenrostro et al. 2015). Sequencing was
542 performed using the NextSeq 500 platform (Illumina) and ATAC-seq reads were aligned to the UCSC
543 mm10 reference genome using BWA-MEN (Li 2013). Then ATAC-seq peaks were called by MACS2
544 and annotated. Known transcription factor binding motifs were analyzed by HOMER (Zhang et al. 2008;
545 Heinz et al. 2010). Quality files for sequencing is listed in Supplementary File 7.

546 **Cell differentiation assay**

547 Palatal tissue was dissected from control and *Wnt1^{Cre};Kdm6b^{fl/fl}* mice at E13.5 and cultured as previously
548 described. Then the differentiation assay was conducted according to the manufacturer's protocol
549 (Gibco, A1007201) (Chen et al. 2020). Briefly, mesenchymal cells were seeded into cell culture plates at
550 the desired concentration followed by incubation at 36°C in a humidified atmosphere of 5% CO₂ for the
551 required time (a minimum of 2 hours and up to 4 days). Then growth medium was replaced by complete
552 differentiation medium and cells were continuously incubated for 3 weeks under osteogenic conditions.
553 After specific periods of cultivation, cells were stained using 2% Alizarin Red S solution (PH 4.2)
554 solution. Images were acquired using EPSON Scan and Keyence BZ-X710/810 microscopes.
555 Quantification of the Alizarin Red S staining was conducted according to the manufacturer's protocol
556 (ScienCell, 8678).

557 **Senescence β -Galactosidase staining**

558 Palatal tissue was dissected from control mice at E13.5, then cut into small pieces using a scalpel. This
559 minced tissue was then cultured in DMEM medium (Gibco, 2192449) containing 40% MSC FBS
560 (Gibco, 2226685P) and 1% Pen Strep (Gibco, 2145477) at 37°C. A cell monolayer was stained using a
561 senescence β -Galactosidase staining kit (Cell Signaling, 9860) according to the manufacturer's protocol.
562 Images were acquired using a Keyence BZ-X710/810 microscope.

563 **Nutlin-3 treatment**

564 Nutlin-3 (Sigma, N6287) was dissolved in corn oil (Sigma, C8267) with 10% DMSO (Sigma, D2650)
565 and given to pregnant mice on days 10.5, 12.5 and 14.5 of pregnancy at a dosage based on their weight
566 (10 mg/kg) (Li et al. 2016). Then embryos were collected at E16.5 for analysis.

567 **Statistics**

568 Statistical analysis was completed using GraphPad Prism and significance was assessed by independent
569 two-tailed Student's t-test or ANOVA. The chosen level of significance for all statistical tests in this
570 study was $p < 0.05$. Data is presented as mean $\pm$ SEM. N=3 samples were analyzed for each experimental
571 group unless otherwise stated.

572 **Acknowledgements**

573 We thank Bridget Samuels and Linda Hattemer for critical reading of the manuscript and also
574 acknowledge USC Libraries Bioinformatics Service for their assistance with data analysis. We thank the
575 USC Office of Research and the Norris Medical Library for the bioinformatics software and computing
576 resources.

577 **Figure Legends**

578 **Figure 1. Loss of *Kdm6b* in CNC-derived cells results in cleft palate**

579 (A-B) Whole-mount oral view shows complete cleft palate phenotype in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Arrowhead in
580 B indicates the cleft palate. Scale bar: 2 mm. (C-D) CT imaging reveals that the palatine process of the maxilla
581 and palatine bone are missing in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. White arrowheads in C indicates palatine process of
582 maxilla in control mice, and red arrows indicates palatine bone. White asterisk in D indicates missing palatine
583 process of maxilla in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice and red asterisk in D indicates the missing palatine bone in
584 *Kdm6b* mutant mice. Scale bars: 1 mm. (E-F) Sagittal views of CT images demonstrate the locations of HE
585 sections in G-R. Red lines indicate the locations of sections. Yellow arrow in E indicates palatal shelf and
586 yellow asterisk in F indicates cleft. Scale bars: 1 mm. (G-R) Histological analysis of control and
587 *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. H, J, L, N, P, and R are magnified images of boxes in G, I, K, M, O, and Q,
588 respectively. Asterisks in M, O, and Q indicate cleft in *Kdm6b* mutant mice. Scale bar: 200 μ m. Mes:
589 mesenchyme; PPM: palatine process of maxilla; PB: palatine bone.

590 **Figure 2. *Kdm6b* is critical for proliferation and differentiation of CNC-derived palatal mesenchyme cells**

591 (A-B) Immunostaining of EdU at E13.5 after 2 hours of EdU labeling. Dotted lines indicate palatal shelf region.
592 Dashed lines indicate the palatal region used for quantification in C. Scale bar: 50 μ m. (C) Quantification of
593 EdU+ cells represented in A and B. Asterisk indicates $P < 0.05$. (D-G) Co-localization of EdU and Ki67 at
594 E13.5 after 48 hours of EdU labeling. Dotted lines indicate palatal shelf region. Dashed lines indicate the palatal
595 region used for quantification in H. F and G are magnified images of boxes in D and E. Arrows in F and G
596 indicate representative cells that are only EdU+, while arrowheads indicate representative cells that are positive
597 for both EdU and Ki67. Scale bar: 50 μ m. (H) Quantification of EdU and Ki67 double-positive cells represented
598 in D and E. Asterisk indicates $P < 0.05$. (I-L) Immunostaining of RUNX2 at indicated stages. Insets are higher
599 magnification images of boxes in I-L. Asterisks in J and L indicate decreased RUNX2+ cells observed in
600 *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Scale bar: 50 μ m. White dotted lines indicate the palatal region used for quantification
601 in Q. (M-P) Immunostaining of SP7 at indicated stages. Insets are higher magnification images of boxes in O

and P. Asterisk in P indicates decreased SP7+ cells observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Scale bar: 50 μ m.

White dotted lines indicate the palatal region used for quantification in R. (Q-R) Quantification results for RUNX2+ and SP7+ cells represented in I-P. Asterisk indicates $P < 0.05$. (S-W) Osteogenic differentiation assay using Alizarin red S staining. W is the quantification result of Alizarin red S staining represented in S and T. Scale bars: 2 mm in S and T; 200 μ m in U and V. Asterisk indicates $P < 0.05$.

Figure 2-Source data 1 for 2C

Figure 2-Source data 2 for 2H

Figure 2-Source data 3 for 2Q

Figure 2-Source data 4 for 2R

Figure 2-source data 5 for 2W

Figure 3. P53 signaling pathway is disturbed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice

(A) Bulk RNA-seq of palatal tissues collected at E12.5 represented in heatmap. Differentially expressed genes were selected using $P < 0.05$ and fold change < -1.2 or > 1.2 . (B) Top seven signaling pathways disturbed in *Kdm6b* mutant mice, identified by Ingenuity Pathway Analysis. Red box indicates the top upregulated pathway observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* sample. (C) Top ten signaling pathways identified by GO analysis using differentially expressed genes identified by bulk RNA-seq analysis. Red box indicates P53 signaling is one of the top 10 pathways. X-axis shows the percentage of genes hit against total number of pathways hit. (D-G) Expression of *Trp53* at E13.5 using RNAscope *in situ* hybridization. Dotted lines in D and F indicate palatal shelf. E and G are magnified images of boxes in D and F. Asterisk in G indicates decreased expression of *Trp53* observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Scale bar: 50 μ m. (H) RT-qPCR quantification of *Trp53* in palatal tissues collected at E13.5. Asterisk indicates $P < 0.05$. (I-L) Immunostaining of γ H2AX at E13.5. Dotted lines in I and K indicate palatal shelf and dashed lines indicate quantification area. J and L are magnified images of boxes in I and K, respectively. Arrowheads in J and L indicate representative γ H2AX+ cells. Scale bar: 50 μ m. (M) Quantification of γ H2AX+ cells represented in I and K. Asterisk indicates $P < 0.05$. (N-Q) Co-localization of EdU and γ H2AX at E13.5 after 2 hours of EdU labeling. Dotted lines in N and P indicate palatal shelf region,

627 while dashed lines indicate the palatal region used for quantification in R. O and Q are magnified images of
628 boxes in N and P, respectively. Red arrows in O and Q indicate representative EdU+ cells with less than 5
629 γ H2AX foci, while white arrowheads indicate representative cells that are positive for EdU and with >5 γ H2AX
630 foci. Scale bar: 50 μ m. (R) Quantification of EdU+ cells with >5 γ H2AX foci represented in N and P. Asterisk
631 indicates $P < 0.05$.

632 Figure 3-Source data 1 for 3H

633 Figure 3-Source data 2 for 3M

634 Figure 3-Source data 3 for 3R

635 **Figure 4. Altered *Trp53* expression is responsible for the developmental defects in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice**
636 (A-C) Cells collected from E13.5 palatal tissue are transfected with siRNA to knock down expression of *Trp53*.
637 Cell proliferation is evaluated using EdU labeling 3 days after transfection. A and B show proliferation of cells
638 assessed by EdU labeling. Difference in EdU+ cells between mock- and siRNA-transfected groups is quantified
639 in C. Scale bar: 100 μ m. Asterisk indicates $P < 0.05$. (D-F) Cells collected from E13.5 palatal tissue are
640 transfected with siRNA to knock down expression of *Trp53*. DNA damage is evaluated using γ H2AX 3 days
641 after transfection. D and E show γ H2AX+ cells. Difference in γ H2AX+ cells between mock- and siRNA-
642 transfected groups is quantified in F. Scale bar: 100 μ m. Asterisk indicates $P < 0.05$. (G-R) Histological analysis
643 of control and *Wnt1^{Cre};Kdm6b^{fl/fl}* mice treated with Nutlin-3. J, K, L, P, Q, and R are magnified images of
644 boxes in G, H, I, M, N, and O, respectively. Scale bar: 200 μ m. Mes: mesenchyme; PPM: palatine process of
645 maxilla; PB: palatine bone.

646 Figure 4-Source data 1 for 4C

647 Figure 4-Source data 2 for 4F

648 **Figure 5. Level of H3K27me3 is antagonistically regulated by *Kdm6b* and *Ezh2* during palatogenesis**
649 (A-E) Contribution of H3K27me3 in the palatal shelf is evaluated using immunostaining and western blot at
650 E13.5. Dotted lines in A and C indicate palatal shelf region. B and D are magnified images of boxes in A and C.
651 Asterisk in B indicates no accumulation of H3K27me3 observed in control mice. Arrowheads in D indicate

552 accumulation of H3K27me3 observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Scale bar: 50 μ m. (F-J) Contribution of *Ezh1*
 553 in the palatal shelf is evaluated using RNAscope *in situ* hybridization and western blot at E13.5. Dotted lines in
 554 F and H indicate palatal shelf region. G and I are magnified images of boxes in F and H. Arrowheads in G and I
 555 indicate representative *Ezh1*⁺ cells. Scale bar: 50 μ m. (K-O) Contribution of EZH2 in the palatal shelf is
 556 evaluated using immunostaining and western blot at E13.5. Dotted lines in K and M indicate palatal shelf region.
 557 L and N are magnified images of boxes in K and M. Arrowheads in L and N indicate representative EZH2⁺
 558 cells. Scale bar: 50 μ m. (P-V) Contribution of H3K27me3 in the palatal shelf of control mice, *Kdm6b* mutant
 559 mice and EZH2 haploinsufficient model is evaluated using immunostaining and western blot at E13.5. Dotted
 560 lines in P, R, and T indicate palatal shelf region. Q, S, and U are magnified images of boxes in P, R, and T,
 561 respectively. Asterisks in Q and U indicate no accumulation of H3K27me3 observed in control (Q) and
 562 *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* mice (U). White arrowheads in S indicate accumulation of H3K27me3 observed in
 563 *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Scale bar: 50 μ m.

564 Figure 5-source data 1 for 5E

565 Figure 5-source data 2 for 5J

566 Figure 5-source data 3 for 5O

567 Figure 5-source data 4 for 5V

568 **Figure 6. Haploinsufficiency of *Ezh2* rescues cleft palate in *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* mice**

569 (A-C) CT images at PN0.5. White arrowheads in A and C indicate palatine process of maxilla observed in
 570 control and *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* rescue model. Red arrows in A and C indicate palatine bone observed in
 571 control and *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* rescue model. White asterisk in B indicates missing palatine
 572 process of maxilla in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice and red asterisk indicates missing palatine bone in *Kdm6b* mutant
 573 mice. Scale bar: 0.4 mm. (D-F) Coronal views of CT images at PN0.5. Asterisk in E indicates cleft palate
 574 observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Scale bar: 0.3 mm. (G-I) Whole-mount oral view at PN0.5. Arrowhead in
 575 H shows complete cleft palate observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Dashed lines in G-I indicate location of
 576 sections in J-O. Scale bar: 2 mm. (J-O) Histological analysis of samples at PN0.5. Asterisk in K and N indicates

677 cleft palate in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. M-O are magnified images of boxes in J-L, respectively. Dotted lines in
678 M-O outline the bone structure. Scale bar: 200 μ m. Mes: mesenchyme; PPM: palatine process of maxilla.

679 **Figure 7. EdU+ and RUNX2+ cells are restored in *Wnt1^{Cre};Kdm6b^{fl/fl};Ezh2^{fl/+}* mice.**

680 (A-C) Immunostaining of EdU at E13.5 after 2 hours of EdU labeling. Dotted lines indicate palatal shelf region.
681 Dashed lines indicate the palatal region used for quantification in D. Scale bar: 50 μ m. (D) Quantification of
682 EdU+ cells represented in A-C. ANOVA is used for statistical analysis. Asterisk indicates $P < 0.05$. N.S: not
683 significant. (E-G) Immunostaining of RUNX2 at E13.5. Dotted lines indicate palatal shelf region. Dashed lines
684 indicate the palatal region used for quantification in H. Scale bar: 50 μ m. (H) Quantification of RUNX2+ cells
685 represented in E-G. ANOVA is used for statistical analysis. Asterisk indicates $P < 0.05$. N.S: not significant.

686 Figure 7-source data 1 for 7D

687 Figure 7-source data 2 for 7H

688 **Figure 8. *Kdm6b* regulates expression of *Trp53* through H3K27me3 and interacts with transcription**
689 **factor TFDP1 in activation of *Trp53***

690 (A) ChIP-qPCR shows H3K27me3 deposition at the promoter region of *Trp53* in palatal tissues of control,
691 *Kdm6b* mutant, and *Ezh2* haploinsufficient mice. ANOVA is used for statistical analysis. Asterisk indicates $P <$
692 0.05. N.S: not significant. (B-G) Expression of *Trp53* in palatal region at E13.5 using RNAscope *in situ*
693 hybridization. Dotted lines in B, C and D indicate palatal shelf. E, F, and G are magnified images of boxes in B,
694 C, and D, respectively. Asterisk in F indicates decreased expression of *Trp53* observed in *Wnt1^{Cre};Kdm6b^{fl/fl}*
695 mice. Scale bar: 50 μ m. (H) RT-qPCR analysis of *Trp53* expression in the palatal region of control, *Kdm6b*
696 mutant and *Ezh2* haploinsufficient mice. ANOVA is used for statistical analysis. Asterisk indicates $P < 0.05$.
697 N.S: not significant. (I) ChIP-qPCR shows KDM6B deposition at the promoter region of *Trp53* in the palatal
698 tissue of control mice. Asterisk indicates $P < 0.05$. (J-K) RT-qPCR analysis of *Trp53* expression in palatal
699 mesenchymal cells transfected with *Kdm6b*- or *Kdm6a*-overexpressing plasmids. Asterisk indicates $P < 0.05$.
700 N.S: not significant. (L) ATAC-seq analysis indicates that promoter region of *Trp53* is accessible for
701 transcription factor TFDP1. (M) ChIP-qPCR using palatal tissue shows that binding of TFDP1 to the promoter

of *Trp53* decreases in the *Kdm6b* mutant mice. Asterisk indicates $P < 0.05$. N.S: not significant. (N-O) Co-localization of TFDP1, *Kdm6b* and *Trp53* at E13.5 using immunostaining and RNAscope *in situ* hybridization. Dotted lines in N indicate palatal shelf. O is a magnified image of the box in N. Arrowheads in O indicate representative cells that are positive for TFDP1, *Kdm6b* and *Trp53*. Scale bar: 50 μm in N and 5 μm in O. (P) RT-qPCR quantification shows the expression of *Tfdp1* in samples collected at E13.5. N.S: not significant. (Q) RT-qPCR analysis of *Trp53* expression in palatal mesenchymal cells after *Tfdp1* siRNA transfection. Asterisk indicates $P < 0.05$. (R-S) RT-qPCR analysis of *Trp53* expression in palatal mesenchymal cells transfected with *Tfdp1* overexpressing plasmid. Asterisk in R indicates $P < 0.05$. N.S: not significant. (T) Co-IP experiment using protein extract from palatal tissues indicates that KDM6B and TFDP1 are present in the same complex. Anti-KDM6B antibody was used for immunoprecipitation. IgG served as negative control. IP: immunoprecipitation. IB: immunoblotting.

Figure 8-source data 1 for 8A

Figure 8-source data 2 for 8H

Figure 8-source data 3 for 8I

Figure 8-source data 4 for 8J

Figure 8-source data 5 for 8K

Figure 8-source data 6 for 8M

Figure 8-source data 7 for 8P

Figure 8-source data 8 for 8Q

Figure 8-source data 9 for 8R

Figure 8-source data 10 for 8S

Figure 8-source data 11 for 8T

Figure 9. Summary schematic drawing

Figure 1-figure supplement 1. Expression of KDM6 family

(A-L) Expression of KDM6 family genes in the palatal region at E13.5 using RNAscope *in situ* hybridization. D, E, F, J, K, and L are magnified images of boxes in A, B, C, G, H, and I respectively. Dotted lines in A, B, C, G, H, and I indicate region of palatal shelf. Dotted lines in D, E, F, J, K, and L indicate epithelium. Schematic drawing at bottom of the figure indicates the location of the presented section. Scale bar: 50 μ m. PS: palatal shelf; Mes: mesenchyme; Epi: epithelium. (M-N) Protein quantification of KDM6A and KDM6B in control and *Kdm6b* mutant palatal region at E13.5 using western blot. (O-P) Expression of *Kdm6b* in the first pharyngeal arch at E9.5 assessed using RNAscope *in situ* hybridization. Arrows in P indicate expression of *Kdm6b* at epithelium. PA1: first pharyngeal arch. Scale bar: 50 μ m.

Figure 1-figure supplement 1-source data 1 for 1M

Figure 1-figure supplement 1-source data 2 for 1N

Figure 1-figure supplement 2. Loss of *Kdm6b* in epithelium and CNC-derived cells

(A-C and E-G) CT images and histological analysis of *Krt14^{Cre};Kdm6b^{fl/fl}* mice at PN0.5. Scale bars in A and E: 0.4 mm; Scale bars in B and F: 0.6 mm; Scale bars in C and G: 100 μ m. (D and H) Expression of *Kdm6b* assessed using RNAscope *in situ* hybridization at E13.5. *Kdm6b* is efficiently knocked out from epithelium in *Krt14^{Cre};Kdm6b^{fl/fl}* mice. Dotted lines in D and H indicate epithelium. Scale bar: 100 μ m. Epi: epithelium; DE: dental epithelium. (I-N) No obvious phenotype was observed in skull bones or mandible in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice at PN0.5. Yellow triangles in J and M indicate location of palatine process of maxilla and red triangle indicates location of palatine bone. Arrowhead in N indicates flattened skull observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Scale bar in I and L: 0.6 mm; Scale bar in J and M: 1 mm.

Figure 1-figure supplement 3. Loss of *Kdm6b* in CNC-derived cells results in soft palate muscle defects

(A-P) Histological analysis of soft palate muscles at PN0.5. Boxes in A, B, C, D, I, J, K, and L are shown magnified in E, F, G, H, M, N, O, and P, respectively. Dotted lines outline each muscle. Asterisks in I, J, K, and L indicate cleft palate observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. PP: pterygoid plate; PLG: palatoglossus; TVP: tensor veli palatini; LVP: levator veli palatini; PLP: palatopharyngeus. Scale bar: 100 μ m. Asterisks in I-L indicate cleft in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. (Q-X) Immunostaining of MHC at PN0.5. Dotted lines outline each

751 muscle. PLG: palatoglossus; TVP: tensor veli palatini; LVP: levator veli palatini; PLP: palatopharyngeus. Scale
752 bar: 50 μ m.

753 **Figure 2-figure supplement 1. *Kdm6b* is not required for CNCCs to populate pharyngeal arches but is**
754 **critical for survival of palatal mesenchymal cells**

755 (A-B) Whole-mount images of tdTomato reporter mice at E10.5. Arrowheads indicate CNCCs that have
756 successfully migrated to the pharyngeal arch at E10.5. No differences were observed between control and
757 *Wnt1^{Cre};Kdm6b^{fl/fl}* mutant mice. Insets show immunostaining of tdTomato at E10.5. Dotted lines in the insets
758 indicate first pharyngeal arch. PA1: first pharyngeal arch. Scale bars: 1 mm. (C-D) Histological analysis of
759 samples at E14.5. Asterisk in D indicates cleft palate observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. PS: palatal shelf.
760 Scale bar: 100 μ m. (E-F) Senescence β -Galactosidase staining using cell culture from E13.5 palatal
761 mesenchymal cells. Arrowheads in E and F indicate representative β -galactosidase+ cells. Scale bar: 100 μ m.
762 (G) Quantification of senescence-associated β -galactosidase activity represented in E and F. Asterisk indicates P
763 < 0.05 . (H-K) Co-localization of EdU and Lamin B1 at E13.5 after 48 hours of EdU labeling. Dotted lines
764 indicate palatal shelf region. Dashed lines indicate the palatal region used for quantification in L. J and K are
765 magnified images of boxes in H and I, respectively. Arrows in J indicate representative cells that are positive for
766 both Lamin B1 and EdU, while arrowheads in K indicate representative cells that are EdU positive and Lamin
767 B1 negative. Scale bar: 50 μ m in H and I, 25 μ m in J and K. (L) Quantification of cells that are EdU positive
768 and Lamin B1 negative represented in H and I. Asterisk indicates $P < 0.05$.

769 Figure 2-figure supplement 1-source data 1 for 1G

770 Figure 2-figure supplement 1-source data 1 for 1L

771

772 **Figure 4-figure supplement 1. *Trp53* plays a critical role in regulating palatogenesis**

773 (A-C) RT-qPCR analysis of *Trp53*, *Runx2* and *Sp7* expression in cells isolated from palatal region of control
774 mice 3 days after transfection with *Trp53* siRNA. Asterisk indicates $P < 0.05$. (D-E) Histological analysis of
775 samples treated with 10% DMSO in corn oil at E10.5, E12.5 and E14.5. Asterisk in E indicates cleft palate

776 observed in *Wnt1^{Cre};Kdm6b^{fl/fl}* mouse. Scale bar: 200 μ m. (F) P53 protein in the palatal region quantified using
 777 western blot. (G) Quantification of western blot represented in F. Asterisk indicates $P < 0.05$. N.S: not
 778 significant.

779 Figure 4-figure supplement 1-source data 1 for 1A

780 Figure 4-figure supplement 1-source data 2 for 1B

781 Figure 4-figure supplement 1-source data 3 for 1C

782 Figure 4-figure supplement 1-source data 4 for 1F

783 Figure 4-figure supplement 1-source data 5 for 1G

784

Figure 8-figure supplement 1. KDM6B and transcription factors are involved in regulating *Trp53*

786 (A-B) RT-qPCR analysis of *Kdm6a* and *Kdm6b* expression in palatal mesenchymal cells isolated from
 787 *Wnt1Cre;Kdm6b^{fl/fl}* mice after transfection with *Kdm6a*- or *Kdm6b*- overexpressing plasmids. Asterisks indicate
 788 $P < 0.05$. (C) ATAC-seq analysis suggests that the promoter of *Trp53* is accessible to transcription factors E2F4
 789 and E2F6. (D-E) Expression of *Wrap53* in palatal region at E13.5 using RNAscope *in situ* hybridization. Dotted
 790 lines in D and E indicate palatal shelf. Scale bar: 50 μ m. (F) Co-expression of *Kdm6b* and *Tfdp1* in the palate
 791 region at E13.5 using published scRNA-seq analysis (GEO: GSE155928). (G-H) Immunostaining of TFDP1 in
 792 the palatal region of control and *Kdm6b* mutant mice. Arrows indicate representative TFDP1+ cells. Scale bar:
 793 50 μ m. (I) RT-qPCR analysis of *Tfdp1* expression in cells isolated from palatal region of control mice 3 days
 794 after transfection with *Tfdp1* siRNA. Asterisk indicates $P < 0.05$. (J-K) RT-qPCR analysis of *Tfdp1* expression
 795 in palatal mesenchymal cells isolated from control and *Kdm6b* mutant mice after transfection with *Tfdp1*-
 796 overexpressing plasmid. J represents the result using cells isolated from control mice and K represents the result
 797 using cells isolated from *Wnt1^{Cre};Kdm6b^{fl/fl}* mice. Asterisks indicate $P < 0.05$.

798 Figure 8-figure supplement 1-source data 1 for 1A

799 Figure 8-figure supplement 1-source data 2 for 1B

800 Figure 8-figure supplement 1-source data 3 for 1I

801 Figure 8-figure supplement 1-source data 4 for 1J

802 Figure 8-figure supplement 6-source data 5 for 1K

803

804 **Supplementary Files**

805 **Supplementary File 1**

806 Antibodies used for *in vivo* immunostaining.

807 **Supplementary File 2**

808 Probes used for *in situ* RNAscope.

809 **Supplementary File 3**

810 Primers used for ChIP-qPCR and RT-qPCR.

811 **Supplementary File 4**

812 Antibodies used for western blot and CoIP.

813 **Supplementary File 5**

814 siRNA used in cell culture experiments.

815 **Supplementary File 6**

816 Plasmids used in cell culture experiments.

817 **Supplementary File 7**

818 QC report for ATAC-seq.

819 **Source Data Files**

820

821 Figure 2-Source data 1 for 2C

822 Figure 2-Source data 2 for 2H

823 Figure 2-Source data 3 for 2Q

824 Figure 2-Source data 4 for 2R

825 Figure 2-source data 5 for 2W

826 Figure 3-Source data 1 for 3H

827 Figure 3-Source data 2 for 3M
828 Figure 3-Source data 3 for 3R
829 Figure 4-Source data 1 for 4C
830 Figure 4-Source data 2 for 4F
831 Figure 5-source data 1 for 5E
832 Figure 5-source data 2 for 5J
833 Figure 5-source data 3 for 5O
834 Figure 5-source data 4 for 5V
835 Figure 7-source data 1 for 7D
836 Figure 7-source data 2 for 7H
837 Figure 8-source data 1 for 8A
838 Figure 8-source data 2 for 8H
839 Figure 8-source data 3 for 8I
840 Figure 8-source data 4 for 8J
841 Figure 8-source data 5 for 8K
842 Figure 8-source data 6 for 8M
843 Figure 8-source data 7 for 8P
844 Figure 8-source data 8 for 8Q
845 Figure 8-source data 9 for 8R
846 Figure 8-source data 10 for 8S
847 Figure 8-source data 11 for 8T
848 Figure 1-figure supplement 1-source data 1 for 1M
849 Figure 1-figure supplement 1-source data 2 for 1N
850 Figure 2-figure supplement 1-source data 1 for 1G
851 Figure 2-figure supplement 1-source data 1 for 1L

852 Figure 4-figure supplement 1-source data 1 for 1A
853 Figure 4-figure supplement 1-source data 2 for 1B
854 Figure 4-figure supplement 1-source data 3 for 1C
855 Figure 4-figure supplement 1-source data 4 for 1F
856 Figure 4-figure supplement 1-source data 5 for 1G
857
858 Figure 8-figure supplement 1-source data 1 for 1A
859 Figure 8-figure supplement 1-source data 2 for 1B
860 Figure 8-figure supplement 1-source data 3 for 1I
861 Figure 8-figure supplement 1-source data 4 for 1J
862 Figure 8-figure supplement 1-source data 5 for 1K
863
864
865
866
867

References

- Arya AK, El-Fert A, Devling T, Eccles RM, Aslam MA, Rubbi CP, Vlatkovic N, Fenwick J, Lloyd BH, Sibson DR et al. 2010. Nutlin-3, the small-molecule inhibitor of MDM2, promotes senescence and radiosensitises laryngeal carcinoma cells harbouring wild-type p53. *Br J Cancer* **103**: 186-195. <https://doi.org/10.1038/sj.bjc.6605739>
- Bannister AJ, Kouzarides T. 2011. Regulation of chromatin by histone modifications. *Cell Res* **21**: 381-395. <https://doi.org/10.1038/cr.2011.22>
- Bernadotte A, Mikhelson VM, Spivak IM. 2016. Markers of cellular senescence. Telomere shortening as a marker of cellular senescence. *Aging-Us* **8**: 3-11. <https://doi.org/DOI.10.18632/aging.100871>
- Blagoev KB. 2009. Cell proliferation in the presence of telomerase. *PLoS One* **4**: e4622. <https://doi.org/10.1371/journal.pone.0004622>
- Bowen ME, Attardi LD. 2019. The role of p53 in developmental syndromes. *J Mol Cell Biol* **11**: 200-211. <https://doi.org/10.1093/jmcb/mjy087>
- Bowen ME, McClendon J, Long HK, Sorayya A, Van Nostrand JL, Wysocka J, Attardi LD. 2019. The Spatiotemporal Pattern and Intensity of p53 Activation Dictates Phenotypic Diversity in p53-Driven Developmental Syndromes. *Dev Cell* **50**: 212-228 e216. <https://doi.org/10.1016/j.devcel.2019.05.015>
- Bruneau BG, Koseki H, Strome S, Torres-Padilla ME. 2019. Chromatin and epigenetics in development: a Special Issue. *Development* **146**. <https://doi.org/10.1242/dev.185025>
- Buenrostro JD, Wu B, Chang HY, Greenleaf WJ. 2015. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. *Curr Protoc Mol Biol* **109**: 21 29 21-21 29 29. <https://doi.org/10.1002/0471142727.mb2129s109>
- Bush JO, Jiang R. 2012. Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development. *Development* **139**: 231-243. <https://doi.org/10.1242/dev.067082>
- Cardiff RD, Miller CH, Munn RJ. 2014. Manual hematoxylin and eosin staining of mouse tissue sections. *Cold Spring Harb Protoc* **2014**: 655-658. <https://doi.org/10.1101/pdb.prot073411>
- Chai Y, Maxson RE, Jr. 2006. Recent advances in craniofacial morphogenesis. *Dev Dyn* **235**: 2353-2375. <https://doi.org/10.1002/dvdy.20833>
- Chandler RL, Magnuson T. 2016. The SWI/SNF BAF-A complex is essential for neural crest development. *Dev Biol* **411**: 15-24. <https://doi.org/10.1016/j.ydbio.2016.01.015>
- Chandrasekharan D, Ramanathan A. 2014. Identification of a novel heterozygous truncation mutation in exon 1 of ARHGAP29 in an Indian subject with nonsyndromic cleft lip with cleft palate. *Eur J Dent* **8**: 528-532. <https://doi.org/10.4103/1305-7456.143637>
- Chen S, Jing JJ, Yuan Y, Feng JF, Han X, Wen Q, Ho TV, Lee C, Chai Y. 2020. Runx2+Niche Cells Maintain Incisor Mesenchymal Tissue Homeostasis through IGF Signaling. *Cell Reports* **32**. <https://doi.org/ARTN.108007>
- Cobourne MT, Xavier GM, Depew M, Hagan L, Sealby J, Webster Z, Sharpe PT. 2009. Sonic hedgehog signalling inhibits palatogenesis and arrests tooth development in a mouse model of the nevoid basal cell carcinoma syndrome. *Dev Biol* **331**: 38-49. <https://doi.org/10.1016/j.ydbio.2009.04.021>
- Cordero DR, Brugmann S, Chu Y, Bajpai R, Jame M, Helms JA. 2011. Cranial neural crest cells on the move: their roles in craniofacial development. *Am J Med Genet A* **155A**: 270-279. <https://doi.org/10.1002/ajmg.a.33702>
- den Broeder MJ, Ballangby J, Kamminga LM, Alestrom P, Legler J, Lindeman LC, Kamstra JH. 2020. Inhibition of methyltransferase activity of enhancer of zeste 2 leads to enhanced lipid

accumulation and altered chromatin status in zebrafish. *Epigenetics Chromatin* **13**: 5.
<https://doi.org/10.1186/s13072-020-0329-y>

Dimitrova E, Turberfield AH, Klose RJ. 2015. Histone demethylases in chromatin biology and beyond. *EMBO Rep* **16**: 1620-1639. <https://doi.org/10.15252/embr.201541113>

Dixon MJ, Marazita ML, Beaty TH, Murray JC. 2011. Cleft lip and palate: understanding genetic and environmental influences. *Nat Rev Genet* **12**: 167-178. <https://doi.org/10.1038/nrg2933>

Flavahan WA, Gaskell E, Bernstein BE. 2017. Epigenetic plasticity and the hallmarks of cancer. *Science* **357**. <https://doi.org/10.1126/science.aal2380>

Gokbuget D, Billewicz R. 2019. Epigenetic control of transcriptional regulation in pluripotency and early differentiation. *Development* **146**. <https://doi.org/10.1242/dev.164772>

Grosshans BL, Grottsch H, Mukhopadhyay D, Fernandez IM, Pfannstiel J, Idrissi FZ, Lechner J, Riezman H, Geli MI. 2006. TEDS site phosphorylation of the yeast myosins I is required for ligand-induced but not for constitutive endocytosis of the G protein-coupled receptor Ste2p. *Journal of Biological Chemistry* **281**: 11104-11114. <https://doi.org/10.1074/jbc.M508933200>

Gurrieron C, Uriostegui M, Zurita M. 2017. Heterochromatin Reduction Correlates with the Increase of the KDM4B and KDM6A Demethylases and the Expression of Pericentromeric DNA during the Acquisition of a Transformed Phenotype. *J Cancer* **8**: 2866-2875. <https://doi.org/10.7150/jca.19477>

Han X, Feng J, Guo T, Loh YE, Yuan Y, Ho TV, Cho CK, Li J, Jing J, Janeckova E et al. 2021. Runx2-Twist1 interaction coordinates cranial neural crest guidance of soft palate myogenesis. *Elife* **10**. <https://doi.org/10.7554/eLife.62387>

Hanna CW, Demond H, Kelsey G. 2018. Epigenetic regulation in development: is the mouse a good model for the human? *Hum Reprod Update* **24**: 556-576. <https://doi.org/10.1093/humupd/dmy021>

He F, Chen Y. 2012. Wnt signaling in lip and palate development. *Front Oral Biol* **16**: 81-90. <https://doi.org/10.1159/000337619>

Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, Cheng JX, Murre C, Singh H, Glass CK. 2010. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell* **38**: 576-589. <https://doi.org/10.1016/j.molcel.2010.05.004>

Henckel A, Toth S, Arnaud P. 2007. Early mouse embryo development: could epigenetics influence cell fate determination? *Bioessays* **29**: 520-524. <https://doi.org/10.1002/bies.20591>

Hobbs CA, Chowdhury S, Cleves MA, Erickson S, MacLeod SL, Shaw GM, Shete S, Witte JS, Tycko B. 2014. Genetic epidemiology and nonsyndromic structural birth defects: from candidate genes to epigenetics. *JAMA Pediatr* **168**: 371-377. <https://doi.org/10.1001/jamapediatrics.2013.4858>

Hu N, Strobl-Mazzulla P, Sauka-Spengler T, Bronner ME. 2012. DNA methyltransferase3A as a molecular switch mediating the neural tube-to-neural crest fate transition. *Genes Dev* **26**: 2380-2385. <https://doi.org/10.1101/gad.198747.112>

Hu N, Strobl-Mazzulla PH, Bronner ME. 2014. Epigenetic regulation in neural crest development. *Dev Biol* **396**: 159-168. <https://doi.org/10.1016/j.ydbio.2014.09.034>

Jambhekar A, Dhali A, Shi Y. 2019. Roles and regulation of histone methylation in animal development. *Nat Rev Mol Cell Biol* **20**: 625-641. <https://doi.org/10.1038/s41580-019-0151-1>

Jiang W, Wang J, Zhang Y. 2013. Histone H3K27me3 demethylases KDM6A and KDM6B modulate definitive endoderm differentiation from human ESCs by regulating WNT signaling pathway. *Cell Res* **23**: 122-130. <https://doi.org/10.1038/cr.2012.119>

Jones NC, Lynn ML, Gaudenz K, Sakai D, Aoto K, Rey JP, Glynn EF, Ellington L, Du C, Dixon J et al. 2008. Prevention of the neurocristopathy Treacher Collins syndrome through inhibition of p53 function. *Nature Medicine* **14**: 125-133. <https://doi.org/10.1038/nm1725>

- Kang S, Chovatiya G, Tumber T. 2019. Epigenetic control in skin development, homeostasis and injury repair. *Exp Dermatol* **28**: 453-463. <https://doi.org/10.1111/exd.13872>
- Kim H, Kim D, Choi SA, Kim CR, Oh SK, Pyo KE, Kim J, Lee SH, Yoon JB, Zhang Y et al. 2018. KDM3A histone demethylase functions as an essential factor for activation of JAK2-STAT3 signaling pathway. *Proc Natl Acad Sci U S A* **115**: 11766-11771. <https://doi.org/10.1073/pnas.1805662115>
- Kim KC, Friso S, Choi SW. 2009. DNA methylation, an epigenetic mechanism connecting folate to healthy embryonic development and aging. *J Nutr Biochem* **20**: 917-926. <https://doi.org/10.1016/j.jnutbio.2009.06.008>
- Kim WT, Kim H, Katanaev VL, Joon Lee S, Ishitani T, Cha B, Han JK, Jho EH. 2012. Dual functions of DP1 promote biphasic Wnt-on and Wnt-off states during anteroposterior neural patterning. *EMBO J* **31**: 3384-3397. <https://doi.org/10.1038/emboj.2012.181>
- Klemm SL, Shipony Z, Greenleaf WJ. 2019. Chromatin accessibility and the regulatory epigenome. *Nat Rev Genet* **20**: 207-220. <https://doi.org/10.1038/s41576-018-0089-8>
- Kohn MJ, Bronson RT, Harlow E, Dyson NJ, Yamasaki L. 2003. Dp1 is required for extra-embryonic development. *Development* **130**: 1295-1305. <https://doi.org/10.1242/dev.00355>
- Lee YH, Saint-Jeannet JP. 2011. Sox9 function in craniofacial development and disease. *Genesis* **49**: 200-208. <https://doi.org/10.1002/dvg.20717>
- Leoyklang P, Suphadeetiporn K, Siriwan P, Desudchit T, Chaowanapanja P, Gahl WA, Shotelersuk V. 2007. Heterozygous nonsense mutation SATB2 associated with cleft palate, osteoporosis, and cognitive defects. *Hum Mutat* **28**: 732-738. <https://doi.org/10.1002/humu.20515>
- Lessard JA, Crabtree GR. 2010. Chromatin regulatory mechanisms in pluripotency. *Annu Rev Cell Dev Biol* **26**: 503-532. <https://doi.org/10.1146/annurev-cellbio-051809-102012>
- Levi G, Mantero S, Barbieri O, Cantatore D, Paleari L, Beverdam A, Genova F, Robert B, Merlo GR. 2006. Msx1 and Dlx5 act independently in development of craniofacial skeleton, but converge on the regulation of Bmp signaling in palate formation. *Mech Dev* **123**: 3-16. <https://doi.org/10.1016/j.mod.2005.10.007>
- Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv:13033997v2 [q-bioGN]*.
- Li Y, Stockton ME, Bhuiyan I, Eisinger BE, Gao Y, Miller JL, Bhattacharyya A, Zhao XY. 2016. MDM2 inhibition rescues neurogenic and cognitive deficits in a mouse model of fragile X syndrome. *Sci Transl Med* **8**. <https://doi.org/ARTN.336ra61>
- Lindgren AM, Hoyos T, Talkowski ME, Hanscom C, Blumenthal I, Chiang C, Ernst C, Pereira S, Ordulu Z, Clericuzio C et al. 2013. Haploinsufficiency of KDM6A is associated with severe psychomotor retardation, global growth restriction, seizures and cleft palate. *Hum Genet* **132**: 537-552. <https://doi.org/10.1007/s00439-013-1263-x>
- Lu S, Yang Y, Du Y, Cao LL, Li M, Shen C, Hou T, Zhao Y, Wang H, Deng D et al. 2015. The transcription factor c-Fos coordinates with histone lysine-specific demethylase 2A to activate the expression of cyclooxygenase-2. *Oncotarget* **6**: 34704-34717. <https://doi.org/10.18632/oncotarget.5474>
- Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR et al. 2010. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci* **13**: 133-140. <https://doi.org/10.1038/nn.2467>
- Mahmoudi S, Henriksson S, Corcoran M, Mendez-Vidal C, Wiman KG, Farnebo M. 2009. Wrap53, a Natural p53 Antisense Transcript Required for p53 Induction upon DNA Damage. *Molecular Cell* **33**: 462-471. <https://doi.org/10.1016/j.molcel.2009.01.028>
- Manna S, Kim JK, Bauge C, Cam M, Zhao Y, Shetty J, Vacchio MS, Castro E, Tran B, Tessarollo L et al. 2015. Histone H3 Lysine 27 demethylases Jmjd3 and Utx are required for T-cell differentiation. *Nat Commun* **6**: 8152. <https://doi.org/10.1038/ncomms9152>

- Marino S, Vooijs M, van Der Gulden H, Jonkers J, Berns A. 2000. Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. *Genes Dev* **14**: 994-1004.
- Miermont A, Antolovic V, Lenn T, Nichols JME, Millward LJ, Chubb JR. 2019. The fate of cells undergoing spontaneous DNA damage during development. *Development* **146**. <https://doi.org/UNSPdev174268>
- Mijit M, Caracciolo V, Melillo A, Amicarelli F, Giordano A. 2020. Role of p53 in the Regulation of Cellular Senescence. *Biomolecules* **10**. <https://doi.org/10.3390/biom10030420>
- Molina-Serrano D, Kyriakou D, Kirmizis A. 2019. Histone Modifications as an Intersection Between Diet and Longevity. *Front Genet* **10**: 192. <https://doi.org/10.3389/fgene.2019.00192>
- Moller M, Schotanus K, Soyer JL, Haueisen J, Happ K, Stralucke M, Happel P, Smith KM, Connolly LR, Freitag M et al. 2019. Destabilization of chromosome structure by histone H3 lysine 27 methylation. *PLoS Genet* **15**: e1008093. <https://doi.org/10.1371/journal.pgen.1008093>
- Nakamura Y, Yamamoto K, He XJ, Otsuki B, Kim Y, Murao H, Soeda T, Tsumaki N, Deng JM, Zhang ZP et al. 2011. Wwp2 is essential for palatogenesis mediated by the interaction between Sox9 and mediator subunit 25. *Nature Communications* **2**. <https://doi.org/ARTN251>
- Nelms BL, Labosky PA. 2010. in *Transcriptional Control of Neural Crest Development*, San Rafael (CA).
- Noden DM. 1983. The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. *Dev Biol* **96**: 144-165. [https://doi.org/10.1016/0012-1606\(83\)90318-4](https://doi.org/10.1016/0012-1606(83)90318-4)
- . 1991. Cell movements and control of patterned tissue assembly during craniofacial development. *J Craniofac Genet Dev Biol* **11**: 192-213.
- Parada C, Chai Y. 2012. Roles of BMP signaling pathway in lip and palate development. *Front Oral Biol* **16**: 60-70. <https://doi.org/10.1159/000337617>
- Pediconi N, Salerno D, Lupacchini L, Angrisani A, Peruzzi G, De Smaele E, Levrero M, Belloni L. 2019. EZH2, JMJD3, and UTX epigenetically regulate hepatic plasticity inducing retro-differentiation and proliferation of liver cells. *Cell Death Dis* **10**: 518. <https://doi.org/10.1038/s41419-019-1755-2>
- Reynolds K, Kumari P, Sepulveda Rincon L, Gu R, Ji Y, Kumar S, Zhou CJ. 2019. Wnt signaling in orofacial clefts: crosstalk, pathogenesis and models. *Dis Model Mech* **12**. <https://doi.org/10.1242/dmm.037051>
- Rigueur D, Lyons KM. 2014. Whole-mount skeletal staining. *Methods Mol Biol* **1130**: 113-121. https://doi.org/10.1007/978-1-62703-989-5_9
- Rinon A, Molchadsky A, Nathan E, Yovel G, Rotter V, Sarig R, Tzahor E. 2011. p53 coordinates cranial neural crest cell growth and epithelial-mesenchymal transition/delamination processes. *Development* **138**: 1827-1838. <https://doi.org/10.1242/dev.053645>
- Roessler E, Velez JI, Zhou N, Muenke M. 2012. Utilizing prospective sequence analysis of SHH, ZIC2, SIX3 and TGIF in holoprosencephaly probands to describe the parameters limiting the observed frequency of mutant gene x gene interactions. *Mol Genet Metab* **105**: 658-664. <https://doi.org/10.1016/j.ymgme.2012.01.005>
- Ruijtenberg S, van den Heuvel S. 2016. Coordinating cell proliferation and differentiation: Antagonism between cell cycle regulators and cell type-specific gene expression. *Cell Cycle* **15**: 196-212. <https://doi.org/10.1080/15384101.2015.1120925>
- Satokata I, Maas R. 1994. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet* **6**: 348-356. <https://doi.org/10.1038/ng0494-348>
- Schwarz D, Varum S, Zemke M, Scholer A, Baggiolini A, Draganova K, Koseki H, Schubeler D, Sommer L. 2014. Ezh2 is required for neural crest-derived cartilage and bone formation. *Development* **141**: 867-877. <https://doi.org/10.1242/dev.094342>

Seelan RS, Mukhopadhyay P, Pisano MM, Greene RM. 2012. Developmental epigenetics of the murine secondary palate. *ILAR J* **53**: 240-252. <https://doi.org/10.1093/ilar.53.3-4.240>

Sen R, Lencer E, Geiger EA, Jones KL, Shaikh TH, Artinger KB. 2020. The role of KMT2D and KDM6A in cardiac development: A cross-species analysis in humans, mice, and zebrafish. *bioRxiv*: 2020.2004.2003.024646. <https://doi.org/10.1101/2020.04.03.024646>

Shahbazi MN, Jedrusik A, Vuoristo S, Recher G, Hupalowska A, Bolton V, Fogarty NNM, Campbell A, Devito L, Ilic D et al. 2016. Self-organization of the human embryo in the absence of maternal tissues. *Nat Cell Biol* **18**: 700-708. <https://doi.org/10.1038/ncb3347>

Shen X, Liu Y, Hsu YJ, Fujiwara Y, Kim J, Mao X, Yuan GC, Orkin SH. 2008. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. *Mol Cell* **32**: 491-502. <https://doi.org/10.1016/j.molcel.2008.10.016>

Shpargel KB, Starmer J, Wang C, Ge K, Magnuson T. 2017. UTX-guided neural crest function underlies craniofacial features of Kabuki syndrome. *Proc Natl Acad Sci U S A* **114**: E9046-E9055. <https://doi.org/10.1073/pnas.1705011114>

Smith ZD, Meissner A. 2013. DNA methylation: roles in mammalian development. *Nat Rev Genet* **14**: 204-220. <https://doi.org/10.1038/nrg3354>

Soares LM, He PC, Chun Y, Suh H, Kim T, Buratowski S. 2017. Determinants of Histone H3K4 Methylation Patterns. *Mol Cell* **68**: 773-785 e776. <https://doi.org/10.1016/j.molcel.2017.10.013>

Soldatov R, Kaucka M, Kastriti ME, Petersen J, Chontorotzea T, Englmaier L, Akkuratova N, Yang YS, Haring M, Dyachuk V et al. 2019. Spatiotemporal structure of cell fate decisions in murine neural crest. *Science* **364**: 971-+. <https://doi.org/ARTN eaas9536>

Sugii H, Grimaldi A, Li J, Parada C, Vu-Ho T, Feng J, Jing J, Yuan Y, Guo Y, Maeda H et al. 2017. The Dlx5-FGF10 signaling cascade controls cranial neural crest and myoblast interaction during oropharyngeal patterning and development. *Development* **144**: 4037-4045. <https://doi.org/10.1242/dev.155176>

Tateossian H, Morse S, Simon MM, Dean CH, Brown SD. 2015. Interactions between the otitis media gene, Fbxo11, and p53 in the mouse embryonic lung. *Dis Model Mech* **8**: 1531-1542. <https://doi.org/10.1242/dmm.022426>

Trainor P, Krumlauf R. 2000. Plasticity in mouse neural crest cells reveals a new patterning role for cranial mesoderm. *Nat Cell Biol* **2**: 96-102. <https://doi.org/10.1038/35000051>

Wiles ET, Selker EU. 2017. H3K27 methylation: a promiscuous repressive chromatin mark. *Curr Opin Genet Dev* **43**: 31-37. <https://doi.org/10.1016/j.gde.2016.11.001>

Williams AB, Schumacher B. 2016. p53 in the DNA-Damage-Repair Process. *Cold Spring Harb Perspect Med* **6**. <https://doi.org/10.1101/cshperspect.a026070>

Wilson S, Filipp FV. 2018. A network of epigenomic and transcriptional cooperation encompassing an epigenomic master regulator in cancer. *NPJ Syst Biol Appl* **4**: 24. <https://doi.org/10.1038/s41540-018-0061-4>

Wysocka J, Swigut T, Xiao H, Milne TA, Kwon SY, Landry J, Kauer M, Tackett AJ, Chait BT, Badenhorst P et al. 2006. A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature* **442**: 86-90. <https://doi.org/10.1038/nature04815>

Xu J, Liu H, Lan Y, Aronow BJ, Kalinichenko VV, Jiang R. 2016. A Shh-Foxf-Fgf18-Shh Molecular Circuit Regulating Palate Development. *PLoS Genet* **12**: e1005769. <https://doi.org/10.1371/journal.pgen.1005769>

Young JI, Slifer S, Hecht JT, Blanton SH. 2021. DNA Methylation Variation Is Identified in Monozygotic Twins Discordant for Non-syndromic Cleft Lip and Palate. *Front Cell Dev Biol* **9**. <https://doi.org/ARTN 656865>

1101 Yu L, Gu S, Alappat S, Song Y, Yan M, Zhang X, Zhang G, Jiang Y, Zhang Z, Zhang Y et al. 2005. Shox2-
 1102 deficient mice exhibit a rare type of incomplete clefting of the secondary palate. *Development*
 1103 **132**: 4397-4406. <https://doi.org/10.1242/dev.02013>
 1104 Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W
 1105 et al. 2008. Model-based analysis of ChIP-Seq (MACS). *Genome Biol* **9**: R137.
 1106 <https://doi.org/10.1186/gb-2008-9-9-r137>
 1107 Zhao H, Oka K, Bringas P, Kaartinen V, Chai Y. 2008. TGF-beta type I receptor Alk5 regulates tooth
 1108 initiation and mandible patterning in a type II receptor-independent manner. *Dev Biol* **320**: 19-
 1109 29. <https://doi.org/10.1016/j.ydbio.2008.03.045>
 1110 Zoghbi HY, Beaudet AL. 2016. Epigenetics and Human Disease. *Cold Spring Harb Perspect Biol* **8**: a019497.
 1111 <https://doi.org/10.1101/cshperspect.a019497>
 1112

PN0.5

Figure 1

EdU — 2 HRs — Sampling

EdU — 48 HRs — Sampling

Figure 4

Figure 5

PN0.5

Figure 7