Retinoic Acid Deficiency Underlies the Etiology of Midfacial Defects

Yanran Wu[1], Hiroshi Kurosaka[1], Qi Wang[1], Toshihiro Inubushi[1], Kohei Nakatsugawa[1], Masataka Kikuchi[2], Haruka Ohara[1], Takayuki Tsujimoto[1], Shota Natsuyama[1], Yuki Shida[1], Lisa L. Sandell[3], Paul Trainor[4,5] and Takashi Yamashiro[1]

1. Department of Orthodontics and Dentofacial Orthopedics, Graduate School of Dentistry, Osaka University
2. Department of Genome Informatics, Graduate School of Medicine, Osaka University
3. Department of Oral Immunology and Infectious Diseases, University of Louisville School of Dentistry
4. Stowers Institute for Medical Research
5. Department of Anatomy and Cell Biology, University of Kansas School of Medicine

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Author for correspondence

Hiroshi Kurosaka, DDS, PhD, MOrth

kurosaka@dent.osaka-u.ac.jp

Associate Professor

Department of Orthodontics and Dentofacial Orthopedics, Graduate School of Dentistry, Osaka University

Abstract

Embryonic craniofacial development depends on the coordinated outgrowth and fusion of multiple facial primordia, which are populated with cranial neural crest cells and covered by the facial ectoderm. Any disturbance in these developmental events, their progenitor tissues, or signaling pathways can result in craniofacial deformities such as orofacial clefts, which are among the most common birth defects in humans. In the present study, we showed that *Rdh10* loss-of-function leads to a substantial reduction in retinoic acid (RA) signaling in the developing frontonasal process during early embryogenesis, which results in a variety of craniofacial anomalies, including midfacial cleft and ectopic chondrogenic nodules. Elevated apoptosis and disturbed cell proliferation in post-migratory cranial neural crest cells and a substantial reduction in *Alx1* and *Alx3* transcription in the developing frontonasal process were associated with the midfacial cleft in *Rdh10*-deficient mice. More importantly, expanded *Shh* signaling in the ventral forebrain, as well as partial abrogation of midfacial defects in *Rdh10* mutants via inhibition of *Hh* signaling, indicates that misregulation of *Shh* signaling underlies the pathogenesis of reduced RA signaling-associated midfacial defects. Taken together, these data illustrate the spatiotemporal function of *Rdh10* and RA signaling during early embryogenesis and their importance in orchestrating molecular and cellular events essential for normal midfacial development.

Keywords

Median facial cleft, Tooth development, Mouse, Sonic hedgehog, Neural crest, Apoptosis

Introduction

Retinoic acid (RA), the active metabolic derivative of vitamin A, is a diffusible molecule that is critical for embryonic craniofacial development (Metzler and Sandell 2016). In fact, both excessive and deficient RA signaling can lead to congenital effects, including orofacial clefts (Sandell et al. 2007; Wang et al. 2019). Therefore, strict feedback and other mechanisms exist to regulate retinoic acid levels within a narrow physiological range (Shannon et al. 2017). Vitamin A is converted to RA via two sequential oxidation reactions. First, vitamin A (all-trans retinol) is oxidized to all-trans retinal by short-chain reductases/dehydrogenases such as RDH10. This is a reversible reaction in which DHRS3 converts the retinal back to retinol. In contrast, the second reaction, in which all-trans retinal is oxidized to retinoic acid by retinaldehyde dehydrogenases, such as RALDH1, 2, and 3, is irreversible. The demonstration that RDH10 is a critical determinant of RA synthesis revealed the oxidation of retinol to retinal as a critical nodal point in vitamin A metabolism and RA signaling during embryogenesis (Sandell et al. 2012; Sandell et al. 2007). Thus, it is interesting that the Rdh10trex/trex loss-of-function mouse, which was created by an ENU-induced point mutation, has been reported to exhibit a midfacial cleft at E12.5 (Sandell et al. 2007). However, due to the high incidence of early embryonic lethality, the detailed cellular and molecular mechanisms underlying the pathogenesis of midfacial clefts in this model remain unknown. Midfacial clefts, which are a subtype of orofacial cleft, occur much less frequently than cleft lip and cleft palate, and our understanding of their etiology and pathogenesis remains incomplete (Apesos and Anigian 1993; Mishra et al. 2015).

Therefore, we conditionally deleted *Rdh10* using tamoxifen-inducible ubiquitous Cre in mice, which was used in a previous study on salivary glands, nasal airways, and secondary

palates. Inactivating *Rdh10* activity prior to E8.5 elicited a variety of midfacial defects, including a midfacial cleft (Friedl et al. 2019; Kurosaka et al. 2017; Metzler et al. 2018). These phenotypes were associated with a substantial reduction in RA signaling and, consequently, apoptosis of post-migratory cranial neural crest cells, especially in the developing frontonasal processes. *Shh* activity was expanded in the ventral forebrain, leading to an increase in facial width and midfacial clefts. The interaction between *Shh* and RA signaling has been proposed to play critical roles during craniofacial development. In particular, exaggerated *Shh* signaling from the developing brain has been shown to split the frontonasal ectodermal zone (FEZ), a signaling center of embryonic craniofacial development, in chicken embryos, which results in a lack of growth at the facial midline (Hu and Marcucio 2009). Furthermore, it is well known that disturbed *Shh* signaling in both humans and mice results in morphological defects in the midfacial area, with elevated *Shh* signaling related to a wider face, occasionally with a midfacial cleft (Brugmann et al. 2010). Consistent with these observations, inhibition of *Shh* signaling partially abrogated midfacial defects in *Rdh10* mutants.

Materials and Methods

Mice, Tamoxifen Administration, and Cyclopamine Injection

Rdh10$^{flox/flox}$ and *Rdh10*$^{\beta geo/+}$ (Sandell et al. 2012), *Cre-ERT2* (Ventura et al. 2007), and *RARE-lacZ* (Rossant et al. 1991) mice were sustained and employed as previously described (Kurosaka et al. 2017; Sandell et al. 2012; Sandell et al. 2007). To eliminate *RDH10* from developing embryos, *Rdh10*$^{flox/flox}$ female mice were crossbred with *Cre-*

ERT2:Rdh10$^{flox/flox}$ male mice, followed by administration of tamoxifen, as previously reported (Kurosaka et al. 2017). Cyclopamine (LKT, #c9710) was administered to pregnant dams at a dose of 20 mg/kg of body weight via intraperitoneal injection at E11.0, after tamoxifen treatment at E7.0 (Firulli et al. 2014).

Whole-mount *In Situ* Hybridization

Whole-mount *in situ* hybridization was performed as previously described (Kurosaka et al. 2014). A minimum of three embryos per probe of each genotype were examined.

Quantitative PCR (qPCR)

Total RNA was extracted from the six dissected heads (three samples from each genotype) of E9.5 *Rdh10*$^{flox/flox}$ control and *CreERT2: Rdh10*$^{flox/flox}$ mutants using RNeasy (Qiagen) according to the manufacturer's protocol. cDNA samples were then synthesized from the six individual heads using a TaKaRa RNA PCR kit (Takara). The sets of synthetic primers used for the amplification in the six samples were as follows: mouse *GAPDH* sense 5‐GTCCCGTAGACAAAATGGTG‐3 and antisense 5‐CAATGAAGGGGTCGTTGATG‐3; mouse *Tfap2a*, sense 5‐CCCGATCCACTCCTTACCTC‐3 and antisense 5‐GCATTGCTGTTGGACTTGGA‐3; mouse *Sox9*, sense 5‐AGGAAGTCGGTGAAGAACGG‐3′, and antisense 5-GGACCCTGAGATTGCCCAGA‐3′.

Results

Loss of *Rdh10* and RA Signaling in the Developing Frontonasal Process Leads to Midfacial Defects in Mice

To investigate the spatiotemporal requirement for *Rdh10* in midfacial morphogenesis, we initially investigated the expression pattern of *Rdh10* during embryonic craniofacial development. Staining for *β-galactosidase* activity in mice carrying the $Rdh10^{\beta geo}$ allele and its sectioned images revealed that *Rdh10* is expressed in the ventral groove of the forebrain at E9.5 (Fig. 1A, A'). From E10.5 to E12.5, expression was detected in the caudal region of the nasal pit epithelium and mesenchyme (Fig. 1B-D and B'-D').

Rdh10 is a critical determinant of RA synthesis and signaling. Therefore, to determine the spatiotemporal effects of *Rdh10* loss-of-function on RA signaling during frontonasal development, we bred RARE-lacZ into the background of *Rdh10flox/flox* mice and performed β-galactosidase staining. Following tamoxifen treatment at E7.0, a substantial reduction in *RARE-lacZ* expression was observed in the frontonasal process of E9.5 (Fig. 1E, E', H and H') and E10.5 (Fig. 1F and I) *CreERT2: Rdh10flox/flox* embryos. At the same time, whole-mount in situ hybridization using an excised Exon 2 specific Rdh10 riboprobe showed diminished expression in the frontonasal process of *CreERT2: Rdh10flox/flox* embryos compared to controls (Fig. 1G and J).

We then conditionally deleted a floxed allele of *Rdh10* (*CreERT2:* $Rdh10^{flox/flox}$) via tamoxifen administration at either E7.0 or E7.5. After treatment at E7.0, 36 of 38 (92.6%) *CreERT2:* $Rdh10^{flox/flox}$ mice exhibited midfacial clefts by E11.5, while only 11 of 51 (21.5%) E7.5-treated mice presented with a midfacial cleft. Nuclear fluorescent staining of control and

CreERT2: Rdh10$^{flox/flox}$ embryos treated with tamoxifen at E7.0 and examined at E10.5. This revealed larger distances between nasal pits, without major loss of tissue in the mutants as compared to controls (Fig. 1K, N, and Q). Consistently, by E11.5, the medial nasal processes failed to fuse in the facial midline in *CreERT2: Rdh10*$^{flox/flox}$ mice relative to controls (Fig. 1L and O). By E12.5, the midfacial cleft became more pronounced and ectopic nodules formed in the medial nasal region of *CreERT2: Rdh10*$^{flox/flox}$ mice relative to controls (Fig. 1M and P).

Rdh10-Mediated RA Signaling Is Required for the Survival of Post-Migratory Cranial Neural Crest Cells by Maintaining Expression of *Alx* Family Genes

The frontonasal process is populated by cranial neural crest cells, and perturbation of their development can result in craniofacial defects, such as the midfacial cleft. In our previous study, we did not assess the development of cranial neural crest cells in *Rdh10* mutants. Furthermore, the anatomical position that was investigated in the previous study was more posterior than the focused area in the present study (Kurosaka et al. 2021; Kurosaka et al. 2017). To ascertain whether the midfacial cleft in *CreERT2: Rdh10*$^{flox/flox}$ mouse embryos was due to a disruption in neural crest cell development, we initially performed *in situ* hybridization with *Tfap2a* and *Sox9*, both of which are key transcriptional regulators of neural crest cell development and are expressed during formation and migration. The expression patterns of both genes in the frontonasal process and first pharyngeal arch of E9.5 *CreERT2: Rdh10*$^{flox/flox}$ mutant embryos were indistinguishable from those of the controls (Fig. 2A-H). The results of quantitative PCR of these two genes did not show significant differences

between E9.5 heads of control and *CreERT2: Rdh10*$^{flox/flox}$ embryos (Appendix fig 1). These results indicate that the formation and migration of cranial neural crest cells were not affected by reduced RA signaling. To assess the behavior and viability of post-migratory cranial neural crest cells, we performed TUNEL staining and phospho-Histone-H3 (PHH3) immunostaining to detect apoptosis and cell proliferation, respectively, in the frontonasal process of E10.5 embryos. We observed a significant increase in the percentage of apoptotic cells in the neural crest cell-derived frontonasal mesenchyme of E10.5 *CreERT2: Rdh10*$^{flox/flox}$ embryos relative to controls (Fig. 2I, K, and M). In contrast, the percentage of PHH3-positive cells in the frontonasal mesenchyme was not significantly different between the control and *CreERT2: Rdh10*$^{flox/flox}$ embryos (data not shown). However, when we further regionalized the medial nasal process (MNP) into the medial and lateral regions, PHH3-positive cells showed a significant reduction in the medial MNP in E10.5 *CreERT2: Rdh10*$^{flox/flox}$ embryos compared to control embryos (Figure 2J, L, and N). These data indicate that proper RA signaling is required for the survival and proliferation of post-migratory cranial neural crest cells, and that elevated apoptosis and disruption of the profile of dividing cells in the frontonasal mesenchyme may contribute to the cellular basis of the midfacial cleft. Furthermore, we detected a shorter vertical length for medial MNPs in E10.5 *CreERT2: Rdh10*$^{flox/flox}$ embryos than in control embryos (Fig. 2O, P, and Q).

To determine the molecular mechanisms underlying the craniofacial defects in *CreERT2: Rdh10*$^{flox/flox}$ mice, we re-evaluated the results of our previously generated RNAseq dataset, which was collected from the E11.5 maxillary complex (including medial nasal, lateral nasal, and maxillary processes) in our previous publication (Kurosaka et al. 2017). We identified several genes known to be critical to midfacial development with significant differences in

expression levels; specifically, the *Alx* family and *Pax9* of transcription factors, whose primary domain of activity is confined to the frontonasal mesenchyme during early craniofacial morphogenesis. The expression of *Alx1* and *Alx3* reduced the area of expression around the frontonasal processes in E10.5 *CreERT2: Rdh10*$^{flox/flox}$ embryos compared to that in the controls (Fig. 2R-U). This is important because mutations in *Alx1* and *Alx3* are known to be associated with elevated apoptosis in the frontonasal processes and midfacial clefts (Beverdam et al. 2001).

Furthermore, we assessed other signaling molecules and found that *Pax9* and *Pitx2,* which play crucial roles in early craniofacial development, have smaller areas of expression in the developing frontonasal area in *CreERT2: Rdh10*$^{flox/flox}$ embryos (Fig. 3A, B, E, and F). On the other hand, the expression of *Fgf8* and *Bmp4* showed subtle differences in *CreERT2: Rdh10*$^{flox/flox}$ embryos (Fig.3C, D, G, and H).

Perturbed Expression of *Shh* Contributes to Midfacial Defects Associated with Reduced RA Signaling

Shh signaling also plays a critical role in midfacial development (Abzhanov and Tabin 2004; Brugmann et al. 2010), and in a recently published study, we revealed that excess RA downregulates *Shh* signaling during craniofacial development (Wang et al. 2019). For these reasons, we compared the differences in *Shh* signaling between control and *CreERT2: Rdh10*$^{flox/flox}$ embryos, which have reduced RA signaling. As a result, we detected persistent expression of *Shh* in the ventral forebrain at E11.5 *CreERT2: Rdh10*$^{flox/flox}$ embryos without evidence of persistent buccopharyngeal membrane (Fig. 4B, F, J and N). It showed

comparable expression at E10.5 (Fig. 4A, E, I, and M). Moreover, the receptor *Ptch1* and activator *Gli1*, which provide a readout of *Shh* signaling, exhibited ectopic expression in the facial midline in E11.5 *CreERT2: Rdh10*$^{flox/flox}$ mice (Fig. 4C, D, G, and H). Examination of histological sections also revealed elevated expression of *Ptch1* and *Gli1* in the cranial mesenchyme around the facial midline (Fig. 4K, L, O, and P). These data suggest that persistent expansion of *Shh* expression in the ventral forebrain caused by reduced RA signaling underlies the etiology of the midfacial cleft.

Inhibition of Excessive *Hh* Signaling Partially Restores Midfacial Phenotype in *CreERT2: Rdh10*$^{flox/flox}$ Mice

To test our hypothesis that elevated and ectopic *Shh* signaling results from reduced RA signaling and contributes to midfacial clefting in *CreERT2: Rdh10*$^{flox/flox}$ embryos, we posited that suppressing *Shh* signaling should rescue the cleft phenotype. Therefore, we injected cyclopamine, an inhibitor of Smoothened, which is a component of the *Hh* signaling pathway, into pregnant dams at E11.0. The treated embryos were harvested at E13.0. We have also confirmed upper incisor defects in cyclopamine-treated wild-type embryos, which recapitulates the phenotype of reduced *Shh* signaling (Appendix Fig. 2) (Dassule et al. 2000).

Compared to *CreERT2: Rdh10*$^{flox/flox}$ mutants, which exhibit a midfacial cleft (92.6%, 36 out of 38 embryos) when treated with tamoxifen at E7.0, only 56% (9 out of 16) *CreERT2: Rdh10*$^{flox/flox}$ mutant embryos exhibited midfacial cleft when treated with cyclopamine (Fig. 5A-E). Not only was the incidence of midfacial cleft diminished, the average distance of the nostrils in *CreERT2: Rdh10*$^{flox/flox}$ mutant embryos treated with cyclopamine was reduced,

indicating that the severity and penetrance of the midfacial cleft were reduced by the administration of cyclopamine (Fig. 5F and G). However, no embryos showed complete restoration of ectopic nodules in our analysis (0/4). These results indicate that there are uninvestigated molecules that cause ectopic nodules under the control of RA signaling, which will be investigated in the future.

Discussion

Rdh10 is expressed in a spatiotemporally regulated manner during craniofacial development starting at E8.0, and is a critical regulator of vitamin A oxidation, RA synthesis, and signaling. (Metzler and Sandell 2016; Sandell et al. 2012; Sandell et al. 2007; Shannon et al. 2017). Therefore, we compared the expression of *Rdh10* and the activity of RA signaling (*RARE-LacZ* reporter transgene) between control and *CreERT2: Rdh10*$^{flox/flox}$ embryos treated with tamoxifen at E7.0. In wild-type embryos, we detected the craniofacial expression of *Rdh10* from E9.5–E12.5. Based on the timing of embryonic facial development, we hypothesized that *Rdh10* at an earlier stage is critical for midfacial development, whereas later stage expression could impact organ and/or tissue development. Remarkably, the degree of disruption in *Shh* signaling was more severe in the group of mice that were administered tamoxifen at E7.0 than at E7.5, which also correlated with the severity of the midfacial cleft (Appendix Fig. 3). We observed a considerable reduction in *Rdh10* expression and *RARE-LacZ* activity in the developing frontonasal process in association with the midfacial cleft, which recapitulates the phenotype observed in *Rdh10* germline null mutants (Sandell et al. 2012; Sandell et al. 2007). Furthermore, we determined that the E7.0-7.5 time period is

critical for tamoxifen administration of *CreERT2: Rdh10*$^{flox/flox}$ embryos to produce the midfacial cleft phenotype. Previous reports revealed that tamoxifen administered at E8.5, completely eliminated *Rdh10* RNA by E10.5, which diminished RA signaling activity to 30% in that of control embryos by E11.5 (Metzler et al. 2018). Taken together with the substantial reduction in *RARE-LacZ* activity in E9.5-10.5 and wider nostrils in E10.5 *CreERT2: Rdh10*$^{flox/flox}$ E7.0 tamoxifen-treated embryos, the critical timing for *Rdh10* expression and RA signaling for midfacial morphogenesis is around E9.5-E10.5. These results are distinguishable from the result showing that removing *Raldh2*, another critical enzyme for synthesizing retinoic acid, causes severe frontonasal truncation at E9.5, in mice (Niederreither et al. 1999). Thus, we propose a model in which the face of *CreERT2: Rdh10*$^{flox/flox}$ embryos is normal up until E10.0, and the lack of inhibition of *Shh* signaling after E10.5 causes the midfacial cleft.

The severity of defects, including the midfacial cleft observed in *CreERT2: Rdh10*$^{flox/flox}$ embryos treated with tamoxifen at E7.0, was reduced when tamoxifen was administered at E7.5. Consistent with the temporal importance of *Rdh10* and RA signaling observed here, previous studies revealed that eliminating *Rdh10* at later developmental stages resulted in substantially milder craniofacial phenotypes. More specifically, administration of tamoxifen at E7.5 would result in choanal atresia. At E8.5, 36% of the embryos exhibited secondary cleft palate in *CreERT2: Rdh10*$^{flox/flox}$ and *CreERT2: Rdh10*$^{delta/flox}$ embryos, respectively (Friedl et al. 2019; Kurosaka et al. 2017). From previous RNAseq analysis together with our *in situ* hybridization analyses, we identified a significant reduction in *Alx1* and *Alx3* expression in the developing frontonasal process of RA signaling-deficient embryos. *Alx* homeobox transcription factors, such as *Alx1, Alx3, and Alx4*, have been associated with

distinct types of human frontonasal dysplasia that exhibit midfacial cleft (El-Ruby et al. 2018; Twigg et al. 2009; Uz et al. 2010). Moreover, the midfacial cleft is exhibited together with elevated apoptosis in the frontonasal mesenchyme of *Alx3/4* compound mutant mice. Although the mechanistic connection between RA signaling and *Alx* gene expression during craniofacial development is largely unknown, these results suggest that midfacial cleft and elevated apoptosis due to RA deficiency are at least partially mediated by reduced expression of *Alx* family genes.

Shh signaling regulates facial width and midfacial patterning during craniofacial development (Brugmann et al. 2010; Muenke and Beachy 2000). Loss-of-function and gain-of-function of *Shh* signaling in the ventral forebrain or frontonasal process result in holoprosencephaly and hypertelorism, respectively, both of which can manifest with other midfacial defects, including midfacial cleft and upper incisor defects (Brugmann et al. 2010; Firulli et al. 2014; Nanni et al. 2001). Evidence for potential interactions between RA and *Shh* signaling during early embryonic craniofacial development has been reported previously (Helms et al. 1994; Schneider et al. 2001; Wang et al. 2019). Additionally, the locus of *the Shh* gene is reported to contain functional RA-response elements in some species, which indicates possible direct interaction (Rhinn and Dolle 2012). However, the role of *Shh* signaling in the etiology of midfacial cleft in retinoid-deficient mice has not been investigated (Friedl et al. 2019; Kurosaka et al. 2017; Metzler et al. 2018). In the present study, genetic readouts of *Shh* signaling through *Ptch1* and *Gli1* expression revealed an expansion in the midfacial mesenchyme of E11.5 *CreERT2: Rdh10*$^{flox/flox}$ embryos treated with tamoxifen. Furthermore, the degree of *Shh* expression in the ventral forebrain corresponded with the incidence of midfacial clefting (Appendix Fig. 3). Altogether, these

findings suggest that retinoid signaling regulates *Shh* expression in the developing craniofacial epithelium, which then impacts the neural crest cell-derived craniofacial mesenchyme. Consistent with this idea, our previous results revealed that mice with a neural crest cell-specific deletion of *Rdh10* did not exhibit gross craniofacial phenotypes (Kurosaka et al. 2017). This implies that the effects of RA signaling on the frontonasal mesenchyme occur in a paracrine manner through the epithelium.

Furthermore, we detected smaller expression areas of *Pax9* and *Pitx2* genes that are important for craniofacial development in the developing medial nasal process of *CreERT2: Rdh10*$^{flox/flox}$ embryos treated with tamoxifen. These genes have been shown to interact with each other at the molecular level and regulate multiple organ development, including midfacial development (Liu et al. 2003; Liu et al. 2005; Peters et al. 1998). The disturbed expression pattern of *Fgf8,* which resembles our previous publication (Kurosaka et al. 2017), has also been shown to result in defects in midfacial integration. This underlies the etiology of midfacial cleft in *CreERT2: Rdh10*$^{flox/flox}$ embryos (Griffin et al. 2013). These results indicate that retinoid signaling is critical for maintaining the expression of these genes in the developing medial nasal process, and its reduction results in the midfacial cleft. It is also possible that the reduced area of the *Alx* gene expression in the mutant could impact the expression of *Shh*, since several reports showed crosstalk of these molecules in embryonic limb development (Qu et al. 1997; Takahashi et al. 1998).

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Author Contributions

Y. W. contributed to design, data acquisition, analysis, and interpretation, and drafted and revised the manuscript; H. K. contributed to analysis and interpretation and critically drafted and revised the manuscript; Q. W, T.I, M. K, H. O, K. N, and T. Y. contributed to the analysis and interpretation. T. T., S. N., and Y. S contributed to the data acquisition. L.L.S. contributed to the analysis and revised the manuscript. P.A.T. provided tools and reagents, interpreted the data, and revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

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Figure legends

Figure 1. Patterns of *Rdh10* expression, RA signaling activity, and midfacial phenotypes in *CreERT2:Rdh10*$^{flox/flox}$ mutant mice. **(A-D, A'-D')** *Rdh10* expression in *Rdh10*$^{\beta geo}$ embryos at E9.5, E10.5, E11.5 and E12.5. Frontal views of *Rdh10 β-galactosidase* staining pattern in *Rdh10*$^{\beta geo}$ mice(A-D). Frontal sections (A'-D') of *Rdh10*$^{\beta geo}$ embryos shown in A-D. The red arrowheads in A-D and A'-D' indicate *Rdh10* expression in the frontonasal and medial and lateral nasal processes. Scale bar in A, 200 μm. **(E-J)** Comparison of *RARE-lacZ* and *Rdh10* expression between control and *CreERT2:Rdh10*$^{flox/flox}$ embryos. Lateral views (E, H) and Frontal views (E', H') of *RARE-lacZ* expression in E9.5 control (E, E') and *CreERT2: Rdh10*$^{flox/flox}$ embryos (H, H'). Frontal views of *RARE-lacZ* expression in E10.5 control (F) and *CreERT2: Rdh10*$^{flox/flox}$ embryos (I). Frontal views of *Rdh10* mRNA expression in E10.5 control (G) and *CreERT2: Rdh10*$^{flox/flox}$ embryos (J). The red arrowheads in E-J indicate RA signaling activity and *Rdh10* expression in the frontonasal and medial and lateral nasal processes. **(K-P)** Frontal views of whole-mount nuclear fluorescent imaging of control and *CreERT2: Rdh10*$^{flox/flox}$ embryos administered with tamoxifen at E7.0. E10.5(K, N), E11.5(L, O), E12.5(M, P) whole-mount nuclear fluorescent imaging of control (K-M) and *CreERT2: Rdh10*$^{flox/flox}$ embryos (N-P). The brackets in K, N indicate the distances between the nostrils. The yellow arrowheads in P indicate ectopic nodules on the face. Asterisks in O and P indicate midfacial clefts. **(Q)** Statistical analysis of the distances between the nostrils of E10.5 embryos. Asterisk, $P<0.05$. fb, forebrain; fn, frontonasal process; lnp, lateral nasal process; mnp, medial nasal process; mx, maxillary process; mn, mandible process.

Figure 2. Analysis of cranial neural crest cell behavior and assessment of gene expression profiles in developing facial processes. **(A-H)** Migration of cranial neural crest cells in control and *CreERT2: Rdh10*$^{flox/flox}$ embryos. Lateral views (A, E, C, G) and frontal views (B, F, D, H) of *Tfap2a* and *Sox9* expression in E9.5 control (A-D) and *CreERT2: Rdh10*$^{flox/flox}$ embryos (E-H). Scale bar in A, 200 μm**. (I-N)** Apoptosis and proliferation of cranial neural crest cells in control and *CreERT2: Rdh10*$^{flox/flox}$ embryos. Frontal sections of E10.5 heads from control (I-J) and *CreERT2: Rdh10*$^{flox/flox}$ mice (K-L). TUNEL staining is shown in green (I, K), and phospho-Histone-H3 (PHH3) is in red (J, L). Scale bar in I, 100 μm. (M-N) Statistical analysis of the percentages of TUNEL-positive cells (TUNEL+) (M) within the mesenchyme of the frontonasal process and PHH3-positive cells (PHH3+) (N) within the mesenchyme of the medial part of the medial nasal process(medial-MNP) which is artificially divided by the white lines (J, L) according to the comparable amount of DAPI stained cells on each side. Asterisk, P<0.05. **(O-Q)** Morphometric analysis of the medial nasal process at E11.5. (O-P) Frontal sections of E11.5 heads from control and *CreERT2: Rdh10*$^{flox/flox}$ mice. The length of the white lines indicate the depth of the medial nasal process between the ventral and dorsal sides of the embryos. (Q) Statistical analysis of the depth of the medial nasal process. Asterisk, P<0.05. **(R-U)** Whole-mount *in situ* hybridization of *Alx1* (R, T), *Alx3* (S, U) in control (R-S) and *CreERT2:Rdh10*$^{flox/flox}$ (T-U) frontonasal processes. The yellow arrowheads in R-U indicate the reduction of *Alx1* and *Alx3* expression levels. Tamoxifen was administered at E7.0 (A-U). fn, frontonasal process; lnp, lateral nasal process; mnp, medial nasal process; np, nasal pit.

Figure 3. Molecular assessment during midfacial development. **(A-H)** Whole-mount *in situ* hybridization of *Pax9* (A, E), *Pitx2* (B, F), *Pitx2* (C, G) and *Bmp4* (D, H) in control (A-D) and *CreERT2: Rdh10*$^{flox/flox}$(E-H) mice. lnp, lateral nasal process; mnp, medial nasal process.

Figure 4. Disrupted *Shh* signaling in *CreERT2:Rdh10*$^{flox/flox}$ embryos. **(A-H)** Whole-mount *in situ* hybridization of *Shh*, *Ptch1* and *Gli1* in control (A-D) and *CreERT2:Rdh10*$^{flox/flox}$ (E-H) embryos. Ventral views of *Shh* (A, B, E and F), *Ptch1* (C and G) and *Gli1* (D and H) expression in E10.5 and E11.5 embryos. The white arrows in A, B, E and F indicate *Shh* expression in the ventral forebrain of *CreERT2:Rdh10*$^{flox/flox}$ embryos. The black arrows in C, D, G and H indicate ectopic *Ptch1* and *Gli1* expression in the midline of the face in *CreERT2:Rdh10*$^{flox/flox}$ embryos. **(I-P)** Frontal sections of the heads from control (I-L) and *CreERT2:Rdh10*$^{flox/flox}$ (M-P) embryos shown in A-H. The positions to cut the sections are indicated by the white lines in A-H. The red arrows in J-L and N-P indicate enhancement of *Shh* (J and N), *Ptch1* (K and O) and *Gli1* (L and P) expression. fb, forebrain; lnp, lateral nasal process; mnp, medial nasal process.

Figure 5. Modification of midfacial cleft phenotype via inhibition of *Hh* signaling. **(A-D)** Frontal views of whole-mount nuclear fluorescent imaging of cyclopamine-treated *CreERT2: Rdh10*$^{flox/flox}$ embryos at E13.0. The white line in A and B indicate the distance between nostrils. Scale bar in A, 200 μm. **(E)** Phenotypic variation of *CreERT2: Rdh10*$^{flox/flox}$ embryos with or without cyclopamine treatment. **(F)** The incidence of midfacial

cleft in cyclopamine-treated *CreERT2: Rdh10*$^{flox/flox}$ embryos showed a significant reduction compared to the nontreated group. Asterisk, $P<0.05$; N=16. **(G)** The distance between nostrils in cyclopamine-treated *CreERT2: Rdh10*$^{flox/flox}$ embryos showed a significant reduction compared to the nontreated group. Asterisk, $P<0.05$.