SHORT COMMUNICATION

Restoration of *polr1c* in Early Embryogenesis Rescues the Type 3 Treacher Collins Syndrome Facial Malformation Phenotype in Zebrafish

Ernest Man Lok Kwong,^{*} Jeff Cheuk Hin Ho,^{*} Marco Chi Chung Lau,^{*} May-Su You,[†] Yun-Jin Jiang,[‡] and William Ka Fai Tse[‡]

From the Department of Biology,^{*} Hong Kong Baptist University, Kowloon Tong, Hong Kong; the Institute of Molecular and Genomic Medicine,[†] National Health Research Institutes, Miaoli, Taiwan; and the Faculty of Agriculture,[‡] Kyushu University, Fukuoka, Japan

Accepted for publication
October 5, 2017.

Address correspondence to
William Ka Fai Tse, Ph.D.,
Promotive Center for Interna-
tional Education and Research,
Faculty of Agriculture, Kyushu
University, Fukuoka 812-8581,
Japan. E-mail: kftse@agr.kyushu-u.ac.jp.

Treacher Collins syndrome (TCS) is a rare congenital birth disorder (1 in 50,000 live births) characterized by severe craniofacial defects. Recently, the authors' group unfolded the pathogenesis of *polr1c* Type 3 TCS by using the zebrafish model. Facial development depends on the neural crest cells, in which *polr1c* plays a role in regulating their expression. In this study, the authors aimed to identify the functional time window of *polr1c* in TCS by the use of photo-morpholino to restore the *polr1c* expression at different time points. Results suggested that the restoration of *polr1c* at 8 hours after fertilization could rescue the TCS facial malformation phenotype by correcting the neural crest cell expression, reducing the cell death, and normalizing the *p53* mRNA expression level in the rescued morphants. However, such recovery could not be reproduced if the *polr1c* is restored after 30 hours after fertilization. (*Am J Pathol* 2018, 188: 336–342; <https://doi.org/10.1016/j.ajpath.2017.10.004>)

Treacher Collins syndrome (TCS) [Online Mendelian Inheritance in Man (OMIM) no. 154500] is an autosomal-dominant mandibulofacial dysostosis that occurs in 1 out of 50,000 live births.¹ It is a rare congenital birth disorder characterized by severe craniofacial defects.² TCS affects the proper formation of facial structure, which leads to profound facial dysmorphism.³ Various facial phenotypes have been reported, including downward slant of palpebral fissure secondary to hypoplasia of the lateral orbit and cleft palate.⁴ Detailed human clinical description can be referred to clinical reviews.⁵

TCS can be classified into three types based on the origin of mutation: TCS1 has the indicated mutation in *TCOF1* [Type 1 TCS (TCS-1); OMIM no. 154500], whereas TCS2 has a mutation in *POLR1D* (TCS-2; OMIM no. 613717). TCS with a *POLR1C* mutation is classified as Type 3 TCS (TCS-3; OMIM no. 248390). Although most cases of TCS are attributed to mutations in *TCOF1*, mutations in *POLR1C* and *POLR1D* account for 9% of all non-*TCOF1*-related TCS cases.^{6,7} The disease mechanism is closely related to an increased expression of *p53*, leading to cell cycle arrest and apoptosis.^{8,9} Recently, our group has uncovered the

pathogenesis of the *POLR1C*-related TCS-3, and suggested the genetic suppression of *p53* may rescue the disease.¹⁰ In this report, the functional time window of the *polr1c* is identified, and more importantly, it is demonstrated that the restoration of *polr1c* in the early stage of development may partially rescue the TCS facial phenotype via restoring the neural crest cell (NCC) expression, reducing the cell death population, and guiding the *p53* pathway back to normal. Collectively, this is the first study to suggest the *polr1c* developmental functional time window in zebrafish, which provides new insight on reducing the treatment time under pharmacotherapy.

Supported by the Japan Society for the Promotion of Science Bilateral Open Partnership Joint Research Project AJ179064 (W.K.F.T.), National Health Research Institutes, Taiwan, grants MG-105-PP-12 and MG-106-PP-11, and the Ministry of Science and Technology, Taiwan, grants MOST 105-2311-B-400-001, MOST 106-2319-B-400-002, and MOST 106-2311-B-400-003-MY3 (Y.-J.J.).

E.M.L.K. and J.C.H.H. contributed equally to this work.

Disclosures: None declared.

Materials and Methods

Fish Strains and Maintenance

The zebrafish strains used in this study were the AB wild-type and *Tg(fli1a:GFP)* lines that were obtained from the Zebrafish International Resource Center (Eugene, OR). They were raised and staged as previously described.¹¹ All experimental procedures on zebrafish embryos were approved by the Animal Experimental Committee, Hong Kong Baptist University.

MO Sequence Site Selection, Design, Injection, and Cleavage

The design of the morpholinos (MOs) was described previously,¹² and they were generated by Gene Tools, LLC (Philomath, OR). For the *polr1c* splicing Photo-morpholino (PMO), oligo targeting intron1 and exon2 (5'-TGGACCTGTAAAPATTAGATACAGT-3') was generated. The cleavage site is underlined. PMO was resuspended in distilled water and stored at -20°C. Two picomole of *polr1c* PMO was injected into 1- or 2-cell stage embryos. Embryos from four different pairs of fish were used for each PMO injection, and the injections were administered on multiple days. More than 200 embryos from four pairs of zebrafish were used for injection per day. For the PMO cleavage, a Gene Tools ultraviolet (UV) lightbox was used as per the manufacturer's protocol. Briefly, the control or PMO embryos (10 embryos each time) at selected developmental stages were put into the flat-bottomed tube provided, followed by 300 seconds of narrow-bandwidth 365-nm light. After exposure, embryos were placed back in the Petri dish with E3 medium.

RNA Extraction, Reverse Transcription, and Real-Time Quantitative PCR

Two and 5 days post-fertilization (dpf) larvae were collected. Ten larvae were pooled as 1 sample, and 12 samples with duplication were used for each experimental set.

Their total RNA was extracted by using TRIzol (Thermo Fisher Scientific, Waltham, MA). Purified RNA with an A260/A280 ratio of 1.8 to 2.0 was used. Briefly, 0.5 µg of total extracted RNA was reversely transcribed (ViLO; Thermo Fisher Scientific). Real-time PCR was conducted with the Applied Biosystems 7500 real-time PCR detection system using KAPA SYBR Green Supermix (Applied Biosystems, Foster City, CA). Beta-actin was used for normalization, and the relative expression ratio was calculated according to the described method.¹³ The sequences of the *p53* primer were forward: 5'-TTAAGT-GATGTGGTGCCTGCCT-3'; reverse: 5'-AGCTTCTTT-CCCTGTTTGGGCT-3'.

Alcian Blue Cartilage Staining and Facial Malformation Analysis

Five-dpf larvae were collected and fixed with 4% para-formaldehyde, and then followed by the general staining protocol as previously described.¹⁴ Quantification of the ethmoid plate morphology was used to represent the facial phenotype at 5 dpf. Samples were first randomly selected, and their whole-mount Alcian blue staining was confirmed by two individual researchers before dissecting the ethmoid plate for measurement. Length, width, length/width ratio, and relative area of the plate were measured. ImageJ software version 1.50 (NIH, Bethesda, MD) was used.

Whole-Mount *in Situ* Hybridization, Acridine Orange Staining, and Imaging

One-dpf embryos were collected and fixed with 4% para-formaldehyde. Single *in situ* hybridization of *dlx2a* was performed as previously described.¹⁵ For the Acridine orange staining, 2-dpf embryos were bathed in 10 µg/mL Acridine orange (Sigma-Aldrich, St. Louis, MO) in the dark for 1 hour, followed by replacing the Acridine orange solution with E3 medium before imaging. Images were captured by a Leica fluorescence microscope (Leica, Wetzlar, Germany) and quantified by ImageJ software as previously described.^{10,16}

Results

Restoration of *polr1c* at Early Developmental Stage May Rescue the Facial Phenotype

To unfold the critical developmental time window of the *polr1c*, the modified *polr1c* PMO with the reported sequence was used.¹⁰ The modification was made for the MO cleavage by the specific 365-nm UV wavelength, allowing the protein translation process. The use of PMO to "turn on" the gene has been reported in different models.^{17–19}

To identify the functional time window of *polr1c*, different time points [8, 16, 24, 30, and 48 hours post-fertilization (hpf)] were screened for *polr1c* restoration. The data are reported in [Supplemental Figure S1](#). The cleavage and the Alcian blue staining were performed at different time points. Results showed that the ethmoid plate structure was malformed in morphants that were exposed to UV light at 16, 24, 30, and 48 hpf ([Figure 1](#) and [Supplemental Figure S1](#)). Morphants exposed to UV light at 16 and 24 hpf exhibited a wide range of ethmoid plate structures (in terms of length, width, and relative area), which indicated the restoration of *polr1c* at such time points was unable to rescue the facial structure at a high successful rate. On the other hand, the morphants exposed to UV light at 8 and 30 hpf may result in a more consolidate phenotype. Two representative time points were chosen for further investigation. The cleavage was performed at 8 and 30 hpf. The modified PMO mimicked the facial

Figure 1 Restoration of *polr1c* via PMO in the early developmental stage may rescue TCS facial phenotypes at 5 dpf. **A–D:** *polr1c* P-morphants exhibit a rounded head, smaller eyes, and jaw agenesis at 5 dpf. **Arrows** indicates the malformation of the lower jaw in morphants (**B** and **D**). The phenotype can be rescued by *polr1c* restoration at 8 hours (**C**), but not at 30 hours (**D**). **E–H:** Alcian blue staining clearly demonstrates the loss of facial cartilage in P-morphants (**F**) and P-morphants that were exposed to 365-nm UV light at 30 hours (**H**). On the other hand, the P-morphants that were exposed to UV light at 8 hours show a rescued facial phenotype (**G**). **I–L:** Flat mount of the neurocranium shows the rudimentary ethmoid plate structure (**red dashed boxes**) in P-morphants (**J**) and P-morphants that were exposed to UV light at 30 hours (**L**). **M–P:** Enlarged view of the ethmoid plate. The plate is outlined in red. The P-morphants (**L**) and P-morphants that were exposed to the UV light at 30 hours (**P**) have a shorter and smaller ethmoid plate. **Dashed lines** indicate the length of the plate; **solid lines**, the width (*w*). The malformation phenotypes shown are the representative figures of severe cases. Anterior is to the left in all views. **E–P:** Images were recolored grey for better contrast. **Q–T:** Quantified data of the ethmoid plate structure. The length (**Q**), width (**R**), and the relative area (**T**) of the ethmoid plate are greatly reduced in the P-morphants and P-morphants that were exposed to the UV light at 30 hours. However, there is no significant difference in the P-morphants that were exposed to the UV light at 8 hours. In addition, there is no significant change in length-to-width ratio among all of the samples (**S**). *n* = 35 (**Q–T**). **P* < 0.05, ***P* < 0.01. Scale bars: 500 μm (**A–H**); 200 μm (**I–L**). Original magnification, ×100 (**M–P**). Ctrl, control; ep, ethmoid plate; no, notochord; pc, parachordal; tr, trabecula.

Figure 3 The restoration of *polr1c* at 8 hpf can normalize the *p53* pathway in the P-morphants. **A** and **B**: Real-time quantitative PCR shows that the *polr1c* P-morphants and P-morphants that were exposed to UV light at 30 hpf have a higher mRNA expression level of *p53* expression at 2 dpf (**A**) and 5 dpf (**B**); whereas the P-morphants exposed to 365-nm UV light at 8 hpf have a similar *p53* expression level as controls. **C**: Summary of the study. In the wild-type case (**top**), *polr1c* is expressed throughout the development, and normal facial structure is formed. In the morphant case (**bottom**), *polr1c* is not expressed during the development, and thus results in facial malformation, which is due to the misexpression of NCCs, increase of cell death, and activation of *p53* pathway. In the case of P-morphants exposed to UV light, the facial malformation phenotype can be recovered if *polr1c* is restored at 8 hpf; however, the anomalies cannot be solved if *polr1c* restoration is done after 30 hpf. The restoration of *polr1c* at an earlier developmental stage can rescue the facial phenotype via correcting the NCC expression and *p53* pathway. Data are expressed as means $\pm$ SEM. $n = 12$ (**A** and **B**). * $P < 0.05$. Ctrl, control.

malformation of the *polr1c* mutant at 5 dpf, which the photo-morphants (P-morphants) exhibited a rounded head, smaller eyes, and jaw agenesis at 5 dpf (Figure 1, A–D). The facial phenotypes were further confirmed by Alcian blue staining (Figure 1, E–H). The P-morphant displayed a shorter and smaller ethmoid plate structure (Figure 1, I–L). The result can be clearly seen in the enlarged ethmoid plate (Figure 1, M–P). Different degrees of cranioskeletal anomalies were found in the morphants, and the result was quantified by various parameters of the ethmoid plate. Briefly, the horizontal length, longitudinal width, and the relative area of the ethmoid plate were measured. Results clearly showed that the length, width, and relative area in P-morphants and the 30-hours UV light–exposed P-morphants were significantly smaller than those in the controls. However, the 8-hours UV light–exposed morphants exhibited a similar structure as controls. In addition, the length to width ratio of the ethmoid plates was similar among controls and P-morphants (Figure 1, Q–T).

Restoration of *polr1c* Plays Regulatory Role in NCC Expression, Cell Death Mechanism, and *p53* Pathway

The expression pattern of the NCCs was monitored by the use of transgenic fish and *in situ* hybridization. The *Tg(fli1a:GFP)* line was used to monitor the pharyngeal arches development. *fli1a* is a marker of neural crest–derived ectomesenchyme,²⁰ whose expression was severely reduced in P-morphants when compared to controls and 8-hpf P-morphants (Figure 2, A–D and M). A similar result was found in the other early cranial NCC marker *dlx2a*. Its expression at pharyngeal arches was diminished at 1 dpf in the P-morphants (Figure 2, E–H and N). These results clearly demonstrated that the restoration of *polr1c* in early embryogenesis (8 hpf) may bring back the normal

NCC specification at 1 dpf, which is required for normal facial development.

In addition, extensive cell death in early embryogenesis was found in the TCS morphant and mutant.¹⁰ As expected, the P-morphants and P-morphants exposed to UV light at 30 hpf showed extensive cell death when compared to the controls at 2 dpf. However, the P-morphants that were exposed to the UV light at 8 hpf displayed reduced cell death phenotype (Figure 2, I–L and O). Moreover, the activation of *p53* expression in TCS was suppressed in the P-morphants exposed to the UV light at 8 hpf (Figure 3, A and B). The activation of the *p53* pathway leads to cell cycle arrest and apoptosis,²¹ together with the altered NCC expression, resulted in the facial malformation in the TCS.²² Taken together, these results suggested that *polr1c* contributes to facial development during early embryogenesis in a critical time period in which *polr1c* contributes to regulation of NCC specification and function of the *p53* pathway (Figure 3C).

Discussion

Zebrafish have been recruited as a new human disease model,^{23,24} in which they share similar developmental pathways and signaling pathways as humans. In terms of craniofacial development, there are a number of regulatory developmental mechanisms shared between fish and mammals.^{25,26}

Facial development requires proper cranial NCC specification and migration. The facial malformation in TCS was found to be closely related to NCC expression.^{8,27–30} Correct migration of NCC is critical for palatogenesis.⁹ One of the major causes of the facial malformation phenotype in Type 3 TCS was due to the improper NCC localization.^{10,22,31} It is crucial for correct NCC migration during the early developmental stage. Our data showed that the

reactivation of *polr1c* at early embryogenesis can partially rescue the facial phenotype, which was due to the recovery of NCC expression. The study by Noack Watt and colleagues³¹ suggested that the excessive cell death in *polr1c* mutants diminished the pool of pre-migratory NCCs, which then led to reduced migrating NCCs, and resulted in facial malformation. Our result here showed that the recovery of the facial structure may only be achieved by the early restoration of *polr1c* expression, which further supported the notion of the regulatory roles of *polr1c* in the early stage of facial formation. However, it should be noted that the recent study used solely the ethmoid plate as the parameter for the facial malformation. Furthermore, the rescued morphants could not totally recover the facial structure, such as the rostral edge of the ethmoid plate. Such phenotype can be classified as “cleft palate.”^{32,33} As with other rescue experiments, 100% recovery was not found in zebrafish or mouse models.^{9,10,31,34} Nevertheless, our study showed the restoration of *polr1c* at early developmental stage may improve the ethmoid plate structure significantly. It should be noted that various facial phenotypes can be found in TCS patients. In the zebrafish model, the underdevelopment of the lower jaw and trabecula of the neurocranium in the morphants from the whole-mount Alcian blue staining could be seen. Various signaling pathways are tightly regulated to form the facial structure, and our group is now undergoing different experiments to understand the regulatory roles of *polr1c* in the facial formation.

Studies have found that the *p53* suppression can rescue the TCS phenotype in mouse and zebrafish models.^{9,10,31} However, the long-term suppression of *p53* may result in a higher risk of tumors during their life time.^{30,35,36} Thus, alternative therapeutic treatment, such as the use of anti-oxidants, has been suggested recently.³⁴ Because possible off-target effects may be generated via the treatment, limiting the treatment by designed time period would reduce the possible side effects. We here suggest that *polr1c* may not be critical for facial development after 30 hpf, and thus propose the possibility of withdrawing the chemotherapies after that time point. Although such withdrawal is not easy in *in utero* mammalian models, the identification of the *polr1c* functional time window in this study should provide some insights on potential treatment scheme.

Conclusion

The restoration of *polr1c* at early embryogenesis can partially rescue the TCS facial malformation phenotype via correcting the NCC specification and normalizing the *p53* mRNA expression level.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpathol.2017.10.004>.

References

1. The Treacher Collins Syndrome Collaborative Group, Dixon J, Edwards SJ, Gladwin AJ, Dixon MJ, Loftus SK, Bonner CA, Koprivnikar K, Wasmuth JJ: Positional cloning of a gene involved in the pathogenesis of Treacher Collins syndrome. *Nat Genet* 1996, 12: 130–136
2. Collins ET: Case with symmetrical congenital notches in the outer part of each lower lid and defective development of the malar bones. *Trans Ophthalmol Soc U K* 1900, 20:190
3. Su PH, Chen JY, Chen SJ, Yu JS: Treacher Collins syndrome with a de Novo 5-bp deletion in the TCOF1 gene. *J Formos Med Assoc* 2006, 105:518–521
4. Kadakia S, Helman SN, Badhey AK, Saman M, Ducic Y: Treacher Collins Syndrome: the genetics of a craniofacial disease. *Int J Pediatr Otorhinolaryngol* 2014, 78:893–898
5. Plomp RG, van Lieshout MJ, Joosten KF, Wolvius EB, van der Schroeff MP, Versnel SL, Poulblon RM, Mathijssen IM: Treacher Collins syndrome: a systematic review of evidence-based treatment and recommendations. *Plast Reconstr Surg* 2016, 137:191–204
6. Dauwerse JG, Dixon J, Seland S, Ruivenkamp CA, van Haeringen A, Hoefsloot LH, Peters DJ, Boers AC, Daumer-Haas C, Maiwald R, Zweier C, Kerr B, Cobo AM, Toral JF, Hoozeboom AJ, Lohmann DR, Hehr U, Dixon MJ, Breuning MH, Wiczorek D: Mutations in genes encoding subunits of RNA polymerases I and III cause Treacher Collins syndrome. *Nat Genet* 2011, 43:20–22
7. Vincent M, Genevieve D, Ostertag A, Marlin S, Lacombe D, Martin-Coignard D, et al: Treacher Collins syndrome: a clinical and molecular study based on a large series of patients. *Genet Med* 2016, 18:49–56
8. Dixon J, Jones NC, Sandell LL, Jayasinghe SM, Crane J, Rey JP, Dixon MJ, Trainor PA: Tcof1/Treacle is required for neural crest cell formation and proliferation deficiencies that cause craniofacial abnormalities. *Proc Natl Acad Sci U S A* 2006, 103:13403–13408
9. Jones NC, Lynn ML, Gaudenz K, Sakai D, Aoto K, Rey JP, Glynn EF, Ellington L, Du C, Dixon J, Dixon MJ, Trainor PA: Prevention of the neurocrisopathy Treacher Collins syndrome through inhibition of p53 function. *Nat Med* 2008, 14:125–133
10. Lau MC, Kwong EM, Lai KP, Li JW, Ho JC, Chan TF, Wong CK, Jiang YJ, Tse WK: Pathogenesis of POLR1C-dependent Type 3 Treacher Collins syndrome revealed by a zebrafish model. *Biochim Biophys Acta* 2016, 1862:1147–1158
11. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF: Stages of embryonic development of the zebrafish. *Dev Dyn* 1995, 203:253–310
12. Tse WKF, Eisenhaber B, Ho SHK, Ng Q, Eisenhaber F, Jiang YJ: Genome-wide loss-of-function analysis of deubiquitylating enzymes for zebrafish development. *BMC Genomics* 2009, 10:637
13. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001, 29:e45
14. Solomon KS, Kudoh T, Dawid IB, Fritz A: Zebrafish foxi1 mediates otic placode formation and jaw development. *Development* 2003, 130: 929–940
15. Tse WK, Jiang YJ: Functional screen of zebrafish deubiquitylating enzymes by morpholino knockdown and in situ hybridization. *Methods Mol Biol* 2012, 815:321–331
16. Fong HC, Ho JC, Cheung AH, Lai KP, Tse WK: Developmental toxicity of the common UV filter, benophenone-2, in zebrafish embryos. *Chemosphere* 2016, 164:413–420
17. Tallafuss A, Gibson D, Morcos P, Li Y, Seredick S, Eisen J, Washbourne P: Turning gene function ON and OFF using sense and antisense photo-morpholinos in zebrafish. *Development* 2012, 139: 1691–1699
18. Saxena A, Peng BN, Bronner ME: Sox10-dependent neural crest origin of olfactory microvillous neurons in zebrafish. *Elife* 2013, 2:e00336
19. Deng W, Farnham MM, Goldys EM, Mohammed S, Pilowsky PM: Gene interference with morpholinos in a gold nanoparticle-based

- delivery platform in rat PC12 cells. *J Biomed Nanotechnol* 2015, 11: 2111–2123
20. Das A, Crump JG: Bmps and id2a act upstream of Twist1 to restrict ectomesenchyme potential of the cranial neural crest. *PLoS Genet* 2012, 8:e1002710
 21. Rubbi CP, Milner J: Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. *EMBO J* 2003, 22:6068–6077
 22. Tse WK: Treacher Collins syndrome: new insights from animal models. *Int J Biochem Cell Biol* 2016, 81:44–47
 23. Zon LI, Peterson RT: In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* 2005, 4:35–44
 24. Seth A, Stemple DL, Barroso I: The emerging use of zebrafish to model metabolic disease. *Dis Model Mech* 2013, 6:1080–1088
 25. Swartz ME, Sheehan-Rooney K, Dixon MJ, Eberhart JK: Examination of a palatogenic gene program in zebrafish. *Dev Dyn* 2011, 240: 2204–2220
 26. Yelick PC, Schilling TF: Molecular dissection of craniofacial development using zebrafish. *Crit Rev Oral Biol Med* 2002, 13:308–322
 27. Poswillo D: The pathogenesis of the Treacher Collins syndrome (mandibulofacial dysostosis). *Br J Oral Surg* 1975, 13:1–26
 28. Wiley MJ, Cauwenbergs P, Taylor IM: Effects of retinoic acid on the development of the facial skeleton in hamsters: early changes involving cranial neural crest cells. *Acta Anat (Basel)* 1983, 116: 180–192
 29. Herring SW, Rowlatt UF, Pruzansky S: Anatomical abnormalities in mandibulofacial dysostosis. *Am J Med Genet* 1979, 3:225–229
 30. McKeown SJ, Bronner-Fraser M: Saving face: rescuing a craniofacial birth defect. *Nat Med* 2008, 14:115–116
 31. Noack Watt KE, Achilleos A, Neben CL, Merrill AE, Trainor PA: The roles of RNA polymerase I and III subunits Polr1c and Polr1d in craniofacial development and in zebrafish models of Treacher Collins syndrome. *PLoS Genet* 2016, 12:e1006187
 32. Kamel G, Hoyos T, Rochard L, Dougherty M, Kong Y, Tse W, Shubinets V, Grimaldi M, Liao EC: Requirement for frzb and fzd7a in cranial neural crest convergence and extension mechanisms during zebrafish palate and jaw morphogenesis. *Dev Biol* 2013, 381:423–433
 33. Eberhart JK, He X, Swartz ME, Yan YL, Song H, Boling TC, Kunerth AK, Walker MB, Kimmel CB, Postlethwait JH: MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. *Nat Genet* 2008, 40:290–298
 34. Sakai D, Dixon J, Achilleos A, Dixon M, Trainor PA: Prevention of Treacher Collins syndrome craniofacial anomalies in mouse models via maternal antioxidant supplementation. *Nat Commun* 2016, 7:10328
 35. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A: Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992, 356:215–221
 36. Levine AJ: p53, the cellular gatekeeper for growth and division. *Cell* 1997, 88:323–331