# Comprehensive series of Irx cluster mutants reveals diverse roles in facial cartilage development

D'Juan T Farmer, Punam Patel, Rachelle Choi, Chih-Yu Liu and J. Gage Crump
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**Original submission**

First decision letter

MS ID#: DEVELOP/2020/197244

MS TITLE: Comprehensive series of Irx cluster mutants reveals global requirements in cartilage individuation

AUTHORS: D'Juan T Farmer, Punam Patel, Rachelle Choi, Chih-Yu Liu, and J. Gage Crump

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The referees are split in their opinions on the suitability of your manuscript for publication in Development with reviewer 2 firmly of the opinion that despite the quality of the work, the level of novel mechanistic insights into cartilage formation is limited and not sufficient for publication in Development. This reviewer is very well qualified to make this assessment and so I do consider it to be important to add greater insight into how and why the phenotypes you describe arise. If you are able to address this concerns andother criticismsand suggestions, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

*Advance summary and potential significance to field*

In this manuscript, Farmer and colleagues study the function of 11 Irx zebrafish genes during embryonic and larval development. The authors use CRISPR based genome editing to generate fish lines with mutations in all 11 Irx genes. By crossing fish heterozygous for mutations in all 11 Irx genes, the authors generate fish with different Irx gene compound genotypes. The authors then test whether sets of offspring with specific morphological or skeletal phenotypes are enriched for different compound genotypes. The authors find that Irx mutants display regionally specific severe cartilage loss in the pharyngeal arches, neurocranium, and pectoral fin skeletons. In addition, a variety of facial cartilage fusions are found in different Irx mutant genotypes, similar to the hyoid joint loss previously described for Irx7 mutants. The authors show severe pharyngeal cartilage reductions are preceded by severe reductions in cranial neural crest. Interestingly, the cartilage fusion phenotypes are not associated with early pharyngeal pouch defects (as seen in other cartilage fusion mutants) but appear to result from inappropriate chondrification of perichondrial cells. Overall this study provides significant insight into the roles of Irx genes during skeletal development and should be of interest to readers of Development.

*Comments for the author*

The following questions/issues should be addressed before publication.

1. One strength of this manuscript is the comprehensive genetic analysis of 11 different Irx genes. However, the efficacy of the approaches tried here would be better documented by providing more data on genome editing efficacy. For example:

L94: “we injected sgRNAs targeting both irx3a and irx6a and identified linked alleles” How many fish were screened to find these doubly mutant alleles? What was the rate of single gene hits? Was the rate of double gene hits the product rule of the rate of each individual gene targeted by a guide?

L96: “injected a sgRNA for irx5b, identified a founder for an irx5b mutant allele” How many fish were screened to find this founder?

L99: same questions as above about “identifying founders”

2. Are alleles themselves recovered at a predicted 1:2:1 ratio? The data in Figs 1 C and E are elegant but don’t address whether any lethality occurs for any genotype before the stages assayed here. The results mention that the quintuple homozygous mutant was not observed, but it seems the authors might be able to comment on whether a 1:2:1 ratio was observed for each locus. Perhaps the authors can not answer this question if they only genotyped fish with phenotypes, and not the unaffected siblings. If this is the case, the authors should clarify around L109 that only affected fish were genotyped.

3. Fig. 1 : The “cluster generation strategy” column in Figure 1A is helpful information, however it doesn’t seem necessary to present in the figure. Perhaps explaining all of the info in this “cluster generation strategy” in the methods would provide the reader with this critical information without distracting from the visual panels in Figure 1. Or if Figure 1 is split into multiple figures (as suggested below), then maybe including all of this text in that figure would help.

4. In Figure 1, the panels should be more efficiently arranged to minimize wasted white space. As is, panel A and the graphs are hard to see without zooming way in, and the font throughout the figure is so small it is hard to read. One solution could be to break up this figure, with panel A being its own figure, and the phenotypes then presented in a separate figure.

5. Figure 1 should include specific sample numbers (both numbers of clutches, and total number of genotyped fish) in the figure or in the legend for each phenotype (body curvature, edema, and severe cartilage loss).

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6. Fig. 2 and Fig 3: Again, the authors should indicate in the figure or legend how many clutches and total fish with each phenotype were genotyped.

7. One hypothesis could be that Aa and Ba homo mutants are lethal due to a simple explanation that 2a and 5a (lacked by Ab and Bb) are required for viability. Not supporting this hypothesis are the data published in the Askary et al. 2015 paper showing 5a mutants lack phenotypes. Mentioning this result in this paper would help clarify the argument for redundancy amongst Irx genes.

8. The only appendage skeletal phenotypes presented in this study are severe cartilage loss, not cartilage fusions. Thus, the first sentence of the conclusion (L204-206) doesn't accurately summarize the data in that no data are presented showing that Irx proteins act as negative regulators of cartilage formation in the appendages. This conclusion should be corrected.

Minor comments:

9. The images in Figure 1B are quite washed out. Can the authors adjust levels digitally to make white areas more visible?

10. Fig 1: in the legend "f" is used to label "fin disk cartilage". This cartilage is more typically called the endoskeletal disc "ED" e.g. Heiner Grandel 1998, zfin.org, etc. It would be useful if the authors referred to this cartilage as the endoskeletal disc and cite Grandel's description of zebrafish pectoral fin anatomy.

11. L146: The authors should report the non-significant P-value for 0/2081 observed vs 1/1024. Can the authors indicate somehow (e.g. perhaps in a supplemental figure including the cluster generation strategy info) the physical distance between the Irx genes that are represented by double line breaks in the figure? If these distances are large, it seems like recombination would result in lower predicted ratios. If these distances are small, this concern is much less likely. Can the authors comment on whether or not evidence of recombination between linked Irx genes was observed in their genotyping?

12. L492: The authors should clarify that fish shown in Fig. S4 are wild-type.

Reviewer 2

*Advance summary and potential significance to field*

Farmer and colleagues build upon their previously published study of the role of zebrafish Irx5 and Irx7 in cartilage development by knocking out all the zebrafish irx genes. They produce compound mutants for each irx gene cluster by simultaneous injection of CRISPR/sgRNAs, combine cluster mutants through interbreeding and describe the mutant phenotypes. The results suggest distinct roles for individual IrxA or IrxB genes, or clusters, in limb cartilages and in subsets of craniofacial cartilages, and that cartilage fusions arise through ectopic chondrogenesis. These results are significant in that they reveal conserved roles for Irx genes in skeletogenesis in general as well as potentially region-specific roles in different skeletal elements.

*Comments for the author*

As the authors acknowledge, the study is limited by not including analysis of single mutants within each cluster. It seems that loss of irx7 function contributes to all of the phenotypes, so it would useful to know how each irx gene individually contributes to joint and cartilage defects. The authors also argue that localized irx expression accounts for their region-specific requirements, but do not provide any expression data for the irx genes in skeletal progenitors which could help confirm the specificity of mutant phenotypes. Successful generation of this large set of mutants is elegant and impressive, but the results are largely descriptive and confirm what has been previously described by this group and others without substantially adding to the understanding of Irx gene function or regulation.

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Major concerns:

1) The cartilage fusions described are dramatic and intriguing. However, it is already known that Irx genes function to inhibit cartilage formation and have specific requirements in skeletal and joint development. The argument that cartilage fusions observed in mutants is due to ectopic cartilage outgrowth from the perichondrium is based on very preliminary results (Fig. 4). How is perichondrium defined here and what is the evidence that it specifically contributes to outgrowths?

2) The authors argue that both redundancy and specificity in Irx function reflects their partially overlapping patterns of expression patterns, but do not provide any expression data in skeletal progenitors. While some have been described, many have not and this would seem a relatively easy set of experiments to do to support their arguments.

3) The quantification of results in Figs. 1-3 and S2 only indicate percentages of fish with particular genotypes (+/+, +/-, -/-) that have a given phenotype, but should include numbers of animals assayed with each of the different phenotypes.

4) Fig. 2C. Largely just from this figure the authors argue that in combinatorial irx mutants they observed “a near complete loss of sox10:dsRed+ cranial neural crest-derived cells (CNCCs) of the mandibular arch, a reduction in CNCCs of the hyoid (second) arch, but no changes in branchial CNCCs at 36 hpf.” However judging from this figure there is more going on, and without counting cells particularly for the second arch, the authors cannot conclude that there is a reduction in the number of CNCCs. Second arch morphology looks abnormal compared to the control, but a reduction in cell number is not clear. Arches 3-7 arches also appear different in mutant versus control. At 4 dpf, ceratobranchial cartilages also appear smaller than in the control.

Minor concerns:

Supp. Figure 3G: It is difficult to appreciate the M-Ch fusion from this ventral view. Related to major concern #2, where else in the forming skeleton is irx7:GFP expressed? There are numerous fusions due to irx7 dysfunction but expression is only shown for the Ch-Hs (expression at 5 dpf was previously described in Askary et al., 2015).

Reviewer 3

*Advance summary and potential significance to field*

The manuscript by Farmer et al. represents an impressive amount of work, carefully done and clearly presented, that supports new insights into the role of Irx proteins in negatively regulating cartilage growth.

*Comments for the author*

The study is a thorough and detailed examination of the genetic requirement for irx genes in zebrafish development. A mutant allele for each gene cluster was created by independently deleting a portion of the coding sequence of each gene. They thus avoided potential problems from deleting regulatory sequences that might have occurred with large deletions of entire clusters. The complex genetics (4 clusters and the single irx7 gene) necessitated an unusual approach; they intercrossed quintuple carriers, and examined >2000 progeny for phenotypes followed by genotyping. A variety of phenotypes were identified, but detailed characterization was focused on skeletal/craniofacial defects.

One main conclusion, that Irx proteins have broadly conserved functions across evolution, is well supported by the observed skeletal defects. More specifically they define two types of cartilage defects in irx mutants. They observe deficits in cranial crest populations early, that correlate with diminished or missing cartilage elements later in development. Second, they find some mutants with fusions between cartilages, resulting in obliterated joints. They draw a parallel between these latter phenotypes and the fused toes mouse mutant (Irx3/5 mutations). Interestingly, the mouse phenotype has been attributed to loss of programmed cell death between digits, but here they

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show instead by sequential live imaging that there is ectopic cartilage formation at joints, resulting in overgrowth or fusion.

The imaging was of the highest quality, the figures were well organized, and they presented the large amount of (sometimes complicated) data very clearly. The writing was also very clear – I found only one mistake (redundant word on line 54: “are generally function”).

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**First revision**

Author response to reviewers' comments

Reviewer #1

**1.1** One strength of this manuscript is the comprehensive genetic analysis of 11 different Irx genes. However, the efficacy of the approaches tried here would be better documented by providing more data on genome editing efficacy. For example:

L94: “we injected sgRNAs targeting both irx3a and irx6a and identified linked alleles” How many fish were screened to find these doubly mutant alleles? What was the rate of single gene hits? Was the rate of double gene hits the product rule of the rate of each individual gene targeted by a guide?

L96: “injected a sgRNA for irx5b, identified a founder for an irx5b mutant allele” How many fish were screened to find this founder?

L99: same questions as above about “identifying founders”

***Response:*** We thank the reviewer for this request. To our knowledge, this is the first report in zebrafish of using CRISPR to independently target multiple linked genes in a single generation. In Results Lines 99-115 and Methods Lines 348-376, we now provide extensive details of the precise number of injected fish screened and the rates of single, double, and triple gene mutations required to identify the alleles reported in this manuscript. We also add a sentence at the end of the Discussion highlighting this technical strength of our study.

> Lines 327-329: “We find that co-injection of Cas9 and guide RNAs targeting multiple genes can result in independent mutations in up to three linked genes in a single injection, thus demonstrating feasibility of gene editing of complex gene loci in zebrafish.”

**1.2** Are alleles themselves recovered at a predicted 1:2:1 ratio? The data in Figs 1 C and E are elegant but don’t address whether any lethality occurs for any genotype before the stages assayed here. Perhaps the authors cannot answer this question if they only genotyped fish with phenotypes, and not the unaffected siblings. If this is the case, the authors should clarify around L109 that only affected fish were genotyped.

***Response:*** We clarify in the text that only animals that did not inflate their swim bladders at 4 dpf were analyzed for skeletal defects.

> Lines 124-126: “In total, we collected 2081 embryos from 13 clutches. Animals that failed to inflate their swim bladders by 4 dpf were collected, visually inspected for abnormalities, and processed for staining of cartilage (Alcian blue) and bone (Alizarin red).”

We also note that for phenotypes in which alleles did not contribute, we identify ratios that are not significantly different from the anticipated 1:2:1 ratio, arguing against early lethality of specific genotypes before the stages analyzed.

**1.3** Fig. 1 : The “cluster generation strategy” column in Figure 1A is helpful information, however it doesn’t seem necessary to present in the figure. Perhaps explaining all of the info in this “cluster generation strategy” in the methods would provide the reader with this critical information without distracting from the visual panels in Figure 1. Or if Figure 1 is split into

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multiple figures (as suggested below), then maybe including all of this text in that figure would help.

***Response:*** We have removed the cluster generation strategy section from the figure, as it is now extensively discussed in the new Results and Methods sections (see response to **1.1**).

**1.4** In Figure 1, the panels should be more efficiently arranged to minimize wasted white space. As is, panel A and the graphs are hard to see without zooming way in, and the font throughout the figure is so small it is hard to read. One solution could be to break up this figure, with panel A being its own figure, and the phenotypes then presented in a separate figure.

***Response:*** We have separated Figure 1 into two figures and reorganized the panels to minimize white space and increase legibility.

**1.5** Figure 1 should include specific sample numbers (both numbers of clutches, and total number of genotyped fish) in the figure or in the legend for each phenotype (body curvature, edema, and severe cartilage loss). Fig. 2 and Fig 3: Again, the authors should indicate in the figure or legend how many clutches and total fish with each phenotype were genotyped.

***Response:*** We have included the n value for each phenotype within each figure and have documented the number of clutches collected from quintuple heterozygous crosses for this study (Line 124).

**1.6** One hypothesis could be that Aa and Ba homo mutants are lethal due to a simple explanation that 2a and 5a (lacked by Aa and Ba) are required for viability. Not supporting this hypothesis are the data published in the Askary et al. 2015 paper showing 5a mutants lack phenotypes. Mentioning this result in this paper would help clarify the argument for redundancy amongst Irx genes.

***Response:*** We now note in the Discussion that *irx5a* single mutants lack the larval lethality observed in the IrxBa cluster mutants.

> Lines 295-299: "Although we lacked the resources to analyze contributions of individual members of each Irx cluster, we note that the larval lethality and pouch defects observed in IrxBa$^{-/-}$ mutants were not seen when only *irx5a* was mutated (Askary et al., 2015), indicating roles for the two other members of the IrxBa cluster (*irx3a* and *irx6a*) in these processes."

**1.7** The only appendage skeletal phenotypes presented in this study are severe cartilage loss, not cartilage fusions. Thus, the first sentence of the conclusion (L204-206) doesn't accurately summarize the data in that no data are presented showing that Irx proteins act as negative regulators of cartilage formation in the appendages. This conclusion should be corrected.

***Response:*** We have modified the first sentence of the Discussion to not imply that we observe cartilage fusions in the zebrafish fin.

> Line 272: "Here we uncover diverse roles for Irx proteins in the development of the facial and fin skeletons of zebrafish."

**1.8** The images in Figure 1B are quite washed out. Can the authors adjust levels digitally to make white areas more visible?

***Response:*** We have adjusted the images as requested.

**1.9** Fig 1: in the legend "f" is used to label "fin disk cartilage". This cartilage is more typically called the endoskeletal disc "ED" e.g. Heiner Grandel 1998, zfin.org, etc. It would be useful if the authors referred to this cartilage as the endoskeletal disc and cite Grandel's description of zebrafish pectoral fin anatomy.

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***Response:*** We now label this cartilage as the “endoskeletal disc” in Figure 2.

**1.10** L146: The authors should report the non-significant P-value for 0/2081 observed vs 1/1024.

***Response:*** We now report “p = 0.07, one-tailed student’s T test” on Line 165.

**1.11** Can the authors indicate somehow (e.g. perhaps in a supplemental figure including the cluster generation strategy info) the physical distance between the Irx genes that are represented by double line breaks in the figure? If these distances are large, it seems like recombination would result in lower predicted ratios. If these distances are small, this concern is much less likely. Can the authors comment on whether or not evidence of recombination between linked Irx genes was observed in their genotyping?

***Response:*** We have added the physical distances in Figure 1. In the Methods, we now describe that each individual mutant allele within each cluster of heterozygous zebrafish was screened periodically to ensure that recombination had not occurred.

> Lines 378-380: “Quintuple heterozygous zebrafish were genotyped every other generation for every mutation within each cluster to ensure recombination had not taken place between wildtype loci. Across multiple generations, we did not detect any signs of recombination.”

**1.12** L492: The authors should clarify that fish shown in Fig. S4 are wild-type.

***Response:*** We have added “Wild type” to Fig. S5 (formerly Fig. S4).

<u>Reviewer #2</u>

**2.1** The study is limited by not including analysis of single mutants within each cluster. It seems that loss of irx7 function contributes to all of the phenotypes, so it would be useful to know how each irx gene individually contributes to joint and cartilage defects.

***Response:*** While we agree that further dissecting the roles of individual members of each Irx cluster would be interesting, to do so properly would be well beyond the financial, personnel, and space resources of this study. In general, we found that many of the phenotypes were seen only upon combinatorial loss of multiple Irx clusters, and to produce all possible genotypes of the four Irx clusters and *irx7* we screened 2081 embryos. Unfortunately, primarily due to space limitations of our zebrafish facility, we did not recover and maintain Irx cluster alleles harboring mutations in subsets of cluster genes. The one exception is *irx5a*, which we had previously studied, and we now describe in the results differences between the *irx5a* mutant and the full IrxBa cluster mutant.

> Lines 295-299: “Although we lacked the resources to analyze contributions of individual members of each Irx cluster, we note that the larval lethality and pouch defects observed in IrxBa$^{-/-}$ mutants were not seen when only *irx5a* was mutated (Askary et al., 2015), indicating roles for the two other members of the IrxBa cluster (*irx3a* and *irx6a*) in these processes.”

**2.2** The authors also argue that localized irx expression accounts for their region-specific requirements, but do not provide any expression data for the irx genes in skeletal progenitors, which could help confirm the specificity of mutant phenotypes.

***Response:*** In new data, we find that the IrxAb cluster and *irx7* play a role in early cranial neural crest specification (new Fig. 4), and IrxBa in endodermal pouch formation (new Fig. 5). In Fig. 4E, we use in situ hybridization to show that the requirement for IrxAb and *irx7* in neural crest formation correlates with expression of the IrxAb gene *irx1b* and *irx7* in the early *sox10*+ neural crest domain. In Fig. 5D, new in situ hybridization data show that *irx3a* is expressed in both posterior arch *dlx2a*+ neural crest cells and in the *dlx2a*-negative pouch-forming region, consistent with a role of this IrxBa gene in endodermal pouch formation.

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**2.3** Successful generation of this large set of mutants is elegant and impressive, but the results are largely descriptive and confirm what has been previously described by this group and others without substantially adding to the understanding of Irx gene function or regulation.

***Response:*** We agree that in the initial submission our data for skeletal phenotypes was largely descriptive. We have therefore performed extensive additional mechanistic analysis, resulting in 2.5 new main figures and 1 additional supplemental figure of data. We summarize the new mechanistic insights below.

1. Previous studies using morpholino knockdown in frog and analysis of the large *Fused toes* deletion in mouse had implicated the IrxB cluster in neural crest migration. By examining neural crest development at four stages in zebrafish (Fig. 4), we find that IrxA genes are required for the timely specification of cranial neural crest cells, as opposed to simply their migration. Later recovery of neural crest formation manifests as a selective loss of the anterior neural crest streams and preferential defects in the anterior jaw skeleton. We also perform new assays in Fig. 4D showing that reductions of first arch neural crest cells at 24 hpf are not due to changes in proliferation and apoptosis at this stage, consistent with the earlier defects in generation of *sox10*+/*dlx2a*+ neural crest cells. This early role for IrxA genes and *irx7* in cranial neural crest specification is further supported by the expression of *irx1b* and *irx7* in the early neural crest domain (Fig. 4E, see **2.2**).

2. We also further investigated the preferential loss of the posterior gill cartilages in IrxB mutants. Rather than affecting neural crest cells directly, we find that IrxB mutants have a profound loss of endodermal pouches, whose requirements for neural crest survival and differentiation have been well documented. In Fig. 5A-C, we now show that *her5*:GFP+ endoderm is present early in IrxB mutants but fails to undergo pouch morphogenesis, which corresponds to lack of segmentation of *sox10*:dsRed+ neural crest cells into discrete posterior arches. This role for IrxB genes in endoderm morphogenesis, which had not previously been reported in any species, is further supported by the expression of *irx3a* in the pouch-forming region (Fig. 5D, see **2.2**).

3. In our previous study (Askary et al., 2015), we had shown that Irx5a and Irx7 function together to promote the development of a single fish-specific joint, the hyoid. Whether Irx genes had more general roles in joint formation, or in the separation of adjacent cartilages, remained unknown. In the mouse *Fused toes* mutant, it had been proposed that fusion of adjacent toe bones was due to lack of programmed cell death in the intervening mesenchyme. In Fig. 6, we show that combinatorial Irx mutants have fusions at a number of joints, as well as extensive fusions between neighboring cartilages. In new data in Fig. 7B, we now use a perichondrium-enriched transgenic line – *trps1*:GFP – to show that fusions between adjacent Meckel's and ceratohyal cartilages likely result from inappropriate chondrogenesis in the perichondrium. These findings expand our understanding of Irx function, with these factors serving to not only limit cartilage maturation within joints, but also cartilage formation within the perichondrium to maintain cartilage separation.

4. We also note that in Fig. 2 we report a specific role of IrxB genes in formation of the scapula homolog of the zebrafish pectoral fin. As mouse IrxB mutants have a similarly specific loss of the scapula (the most proximal element of the forelimb), our finding points to deep conservation of proximal-distal patterning of the fin and limb by IrxB proteins.

**2.4** The argument that cartilage fusions observed in mutants is due to ectopic cartilage outgrowth from the perichondrium is based on very preliminary results (Fig. 4). How is perichondrium defined here and what is the evidence that it specifically contributes to outgrowths?

***Response:*** In new Fig. 7B, we use a perichondrium-enriched *trps1*:GFP transgene in combination with the cartilage *sox10*:dsRed transgene to further define the source of ectopic outgrowths in Irx mutants. Compared to the flattened *trps1*:GFP-only cells in the wild-type perichondrium of the ceratohyal, we observed flattened *trps1*:GFP+;*sox10*:dsRed+ cells within the mutant

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perichondrium, in a position consistent with the fusions observed in our serial imaging analysis (Fig. 7A). This new finding provides better support for a perichondrium origin of ectopic outgrowths, though we are careful to note that we have only examined one specific type of fusion between the ceratohyal and Meckel's cartilages. We therefore clarify in the Discussion that the origin of other fusions and joint loss requires further investigation.

> Lines 317-323: "In addition to loss of the hyoid joint and fusion of Meckel's and ceratohyal cartilages, we observed numerous other joint losses and cartilage fusions in Irx mutant combinations. It remains unclear which particular Irx members are expressed in these other zones of cartilage fusions. Moreover, it is possible that some of these other fusions are indirect consequences of earlier defects, such as reduced neural crest-derived cells. Indeed, reductions of mandibular arch neural crest-derived cells in zebrafish *h3f3a* mutants was also found to correlate with loss of the jaw joint in some cases (Cox et al., 2012)."

**2.5** The quantification of results in Figs. 1-3 and S2 only indicate percentages of fish with particular genotypes (+/+, +/-, -/-) that have a given phenotype, but should include numbers of animals assayed with each of the different phenotypes.

***Response:*** We now report n values for every scored phenotype in each figure.

**2.6** Fig. 2C. Largely just from this figure the authors argue that in combinatorial irx mutants they observed "a near complete loss of sox10:dsRed+ cranial neural crest-derived cells (CNCCs) of the mandibular arch, a reduction in CNCCs of the hyoid (second) arch, but no changes in branchial CNCCs at 36 hpf." However, judging from this figure there is more going on, and without counting cells, particularly for the second arch, the authors cannot conclude that there is a reduction in the number of CNCCs. Second arch morphology looks abnormal compared to the control, but a reduction in cell number is not clear. Arches 3-7 arches also appear different in mutant versus control. At 4 dpf, ceratobranchial cartilages also appear smaller than in the control.

***Response:*** As mentioned earlier, we have now greatly expanded our analysis of the early neural crest defects in Fig. 4. In situ analyses of the early neural crest marker *sox10* at 11 hpf and the neural crest ectomesenchyme marker *dlx2a* at 16.5 hpf reveal an early defect in neural crest specification in *IrxAb*; *irx7* mutants, with partial recovery leading to selective loss of first arch neural crest cells (Fig. 4A,B; quantification in new Fig. S3 confirms first arch specificity of defects). We then used *sox10*:dsRed to visualize neural crest cells at 16.5 hpf and 24 hpf, and further examined proliferation (pHH3) and apoptosis (Caspase-3) of *sox10*:dsRed+ cells at 24 hpf in wild types and mutants (Fig. 4C,D). Quantification in Fig. 4F-H reveal a preferential loss of first arch neural crest cells in *IrxAb*; *irx7* but not *IrxBa* mutants, consistent with expression of the IrxAb gene *irx1b* and *irx7* in the neural crest-forming domain at 11 hpf (Fig. 4E). These results reveal a new role for IrxAb and Irx7 in the timely specification of cranial neural crest, with defects in the process manifesting as a selective loss of the anterior facial skeleton.

**2.7.** Supp. Figure 3G: It is difficult to appreciate the M-Ch fusion from this ventral view.

***Response:*** We replaced this image with a lateral view that now clearly shows the M-Ch fusion.

**2.8.** Related to major concern #2, where else in the forming skeleton is irx7:GFP expressed? There are numerous fusions due to irx7 dysfunction but expression is only shown for the Ch-Hs (expression at 5 dpf was previously described in Askary et al., 2015).

***Response:*** In addition to the Ch-Hs junction where the hyoid joint affected in *irx7* mutants develops, we show in Fig. S5 that *irx7*:GFP is also expressed in the periochondrium where we observe the ectopic *trps1*:GFP+;*sox10*:dsRed+ outgrowths in new Fig. 7B. This is consistent with roles for Irx7 in preventing both hyoid joint fusions and Meckel's-ceratohyal fusions. At the same time, we agree with the lack of obvious expression of irx7:GFP at other joints and hence clarify in the Discussion that the origin of other fusions requires further investigation (see **2.4**). Of note, the Meckel's-Meckel's joint fusion does not depend on *irx7* loss (Fig. 6M).

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Reviewer #3

This reviewer stated that our study "represents an impressive amount of work, carefully done and clearly presented, that supports new insights into the role of Irx proteins in negatively regulating cartilage growth", and they did not request any changes.

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Second decision letter

MS ID#: DEVELOP/2020/197244

MS TITLE: Comprehensive series of Irx cluster mutants reveals diverse roles in facial cartilage development

AUTHORS: D'Juan T Farmer, Punam Patel, Rachelle Choi, Chih-Yu Liu, and J. Gage Crump

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, two of the referees are happy with your revisions while the third has several concerns that he/she considers to be essential to be addressed prior to publication. Please attend to these comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

*Advance summary and potential significance to field*

In this manuscript, Farmer and colleagues study the function of 11 Irx zebrafish genes during embryonic and larval development. The authors use CRISPR based genome editing to generate fish lines with mutations in all 11 Irx genes. By crossing fish heterozygous for mutations in all 11 Irx genes, the authors generate fish with different Irx gene compound genotypes. The authors then test whether sets of offspring with specific morphological or skeletal phenotypes are enriched for different compound genotypes. The authors find that Irx mutants display regionally specific severe cartilage loss in the pharyngeal arches, neurocranium, and pectoral fin skeletons. In addition, a variety of facial cartilage fusions are found in different Irx mutant genotypes, similar to the hyoid joint loss previously described for Irx7 mutants. The authors show severe pharyngeal cartilage reductions are preceded by severe reductions in cranial neural crest. Interestingly, the cartilage fusion phenotypes are not associated with early pharyngeal pouch defects (as seen in other cartilage fusion mutants) but appear to result from inappropriate chondrification of perichondrial cells. Overall this study provides significant insight into the roles of Irx genes during skeletal development and should be of interest to readers of Development.

*Comments for the author*

The authors have carefully and thoroughly responded to each of the many suggestions/questions from the reviewers. The revised manuscript is now significantly improved, especially with respect to the clarity of content in the first three figures.

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Reviewer 2

*Advance summary and potential significance to field*

Farmer and colleagues build upon their previously published study of the role of zebrafish Irx5 and Irx7 in cartilage development by knocking out all the zebrafish irx genes. They produce compound mutants for each irx gene cluster by simultaneous injection of CRISPR/sgRNAs, combine cluster mutants through interbreeding and describe the mutant phenotypes.
The results suggest: 1) distinct roles for individual IrxA or IrxB genes, or clusters, in limb cartilages and in subsets of craniofacial cartilages, 2) that the craniofacial defects in Irx mutants result from early requirements in neural crest specification and pharyngeal pouch formation, and 3) that cartilage fusions arise through ectopic chondrogenesis. These results are significant in that they reveal conserved roles for Irx genes in skeletogenesis in general as well as potentially region-specific roles in different skeletal elements.

*Comments for the author*

The manuscript is improved over the previous submission and the authors have addressed many of my concerns. The addition of new figures quantifying the effects of Irx mutants on cranial neural crest cells and pharyngeal pouches strengthens the arguments for cell type-specific roles for Irx genes and provides some mechanistic insights (defects in neural crest specification and pouch formation) to explain these defects. However, I still have one major and several more minor concerns to be addressed.

Major:

Figure 7 - I am not convinced from the data presented in Fig. 7B that perichondrial outgrowths cause the cartilage fusions in Irx mutants. First, it is unclear how the cartilages depicted in Fig. 7B relate to the fusions shown in Fig. 7A. Fig. 7B appears to show ventral views of the ceratohyal palatoquadrate, and symplectic cartilages (labels are needed), while the authors state that “ectopic double positive cells were seen precisely in the region where the ceratohyal was seen to fuse with Meckel’s cartilage.” Neither Meckel’s cartilage nor a clear fusion can be seen in Fig. 7B. Of the 3/7 mutants that showed sox10:dsRed; trps1:GFP double-positive, perichondrial-like cells, were any located at sites of ceratohyal-Meckel’s fusions? Second, even if the examples shown in Fig. 7B are relevant the sox10(+) chondrocytes indicated by the arrowhead seem more likely to form the fusions than the sox10;trps1 double-positive, perichondrial-like cells and simply penetrate the perichondrium. sox10;trps1 double-positive cells appear restricted to the perichondrium while sox10(+) cells within the outgrowth have chondrocyte-like morphologies and similar levels of trps1:GFP as other chondrocytes in the ceratohyal.

Minor:

Figure 5: There appear to be rudiments of both first and second pharyngeal pouches based on her5:GFP labeling, in contrast to the description in the text (lines 222-224). Lines 103-104, 111: Please clarify numbers of animals. As written, it is unclear if only 23 animals were injected and assayed or, more likely, only 23 of many injected animals were screened.

Fig. S4G. The arrowhead should be moved closer to the Meckel’s-ceratohyal fusion.

Concerns about developmental delay. The authors state (lines 279-281) that “loss of anterior irxab-/-; irx7-/- jaw cartilages in mutants is due to a delay in cranial neural crest formation, resulting in a preferential loss of the anterior-most skeletogenic neural crest-derived cells.” This seems to contradict their previous interpretation that these phenotypes reflect defects in “specification” of CNCCs (lines 208-209).

Reviewer 3

*Advance summary and potential significance to field*

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The study provides a comprehensive view of the role of irx genes in cartilage specification and craniofacial development. The large number of irx genes, and their redundant functions, required separately targeting each gene in all five loci, and intercrossing quintuple carriers of mutations in all 11 genes. They uncover two requirements for irx genes in formation of the cartilage skeleton, an early requirement in cranial neural crest differentiation, and a later negative regulation of cartilage differentiation. Their findings provide support for conserved roles for Irx genes across vertebrates, but also new insights into the mechanisms underlying those roles.

*Comments for the author*

The authors have throughly addressed significant comments from the previous reviews, and the paper does not require further revisions.

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**Second revision**

Author response to reviewers' comments

Reviewer #2

**2.1** Major Concern: Figure 7 - I am not convinced from the data presented in Fig. 7B that perichondrial outgrowths cause the cartilage fusions in Irx mutants. First, it is unclear how the cartilages depicted in Fig. 7B relate to the fusions shown in Fig. 7A. Fig. 7B appears to show ventral views of the ceratohyal, palatoquadrate, and symplectic cartilages (labels are needed), while the authors state that "ectopic double positive cells were seen precisely in the region where the ceratohyal was seen to fuse with Meckel's cartilage." Neither Meckel's cartilage nor a clear fusion can be seen in Fig. 7B. Of the 3/7 mutants that showed sox10:dsRed; trps1:GFP double-positive, perichondrial-like cells, were any located at sites of ceratohyal-Meckel's fusions? Second, even if the examples shown in Fig. 7B are relevant the sox10(+) chondrocytes indicated by the arrowhead seem more likely to form the fusions than the sox10;trps1 double-positive, perichondrial-like cells and simply penetrate the perichondrium. sox10;trps1 double-positive cells appear restricted to the perichondrium while sox10(+) cells within the outgrowth have chondrocyte-like morphologies and similar levels of trps1:GFP as other chondrocytes in the ceratohyal.

***Response:*** We agree that in the absence of proper lineage tracing, we cannot definitively conclude whether mutant fusions result from aberrant perichondral chondrogenesis or expansion of pre-existing chondrocytes (or both). We have addressed this limitation in two ways. First, we have modified the text to be more conservative in our interpretations. For example:

> Lines 270-274: "Our findings suggest that, in addition to preventing inappropriate cartilage maturation in the hyoid joint (Askary et al., 2015), Irx7 and IrxB family members also function to prevent inappropriate chondrogenesis in the perichondrium, although we cannot rule out that proliferative expansion of chondrocytes alternatively or also contributes to mutant fusions (Fig. 7C)."

> Lines 313-315. "…in vivo imaging in zebrafish revealed that fusion between the lower jaw Meckel's and the ceratohyal cartilages, which form from distinct pharyngeal arches, occurs through ectopic chondrogenesis ~~of the ceratohyal perichondrium~~."

Second, we now show two additional examples of fusions in Fig. 7B. Whereas the severe fusion example is less informative, the mild fusion shows several *trps1*:GFP$^{high}$; *sox10*:dsRed+ cells at the fusion site, consistent with a perichondral origin of the fusion, at least in this example. We also clarify that the original example shown is a case where an ectopic cartilage outgrowth was seen from the ceratohyal that failed to fuse with Meckel's. In both cases, we observe a mixture of *trps1*:GFP$^{high}$; *sox10*:dsRed+ and *trps1*:GFP$^{low}$; *sox10*:dsRed+ cells in the mutant perichondrium –

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in contrast to the wild-type perichondrium composed of *trps1*:GFP$^{high}$; *sox10*:dsRed- cells. We describe these new images in more detail on Lines 261-270. We have also added zoomed out views of the cartilages in Fig. 7B to show the position of Meckel's cartilage in mutants, have added labels to the cartilages, and have added arrows and arrowheads to distinguish *trps1*:GFP$^{high}$; *sox10*:dsRed+ and *trps1*:GFP$^{low}$; *sox10*:dsRed+ cells in the mutant perichondrium.

**2.2** Figure 5: There appear to be rudiments of both first and second pharyngeal pouches based on her5:GFP labeling, in contrast to the description in the text (lines 222-224).

***Response:*** We thank the reviewer for this observation and agree that there is also a rudimentary (though much reduced) second pouch in mutants. We modified Fig. 5B,C to now show both rudimentary first and second pouches in mutants, and have modified the text accordingly.

> Lines 220-222: "These phenotypes became even more apparent by 36 hpf, with six pouches in wild types and only rudimentary first and second pouches in mutants, ..."

**2.3** Lines 103-104, 111: Please clarify numbers of animals. As written, it is unclear if only 23 animals were injected and assayed or, more likely, only 23 of many injected animals were screened.

***Response:*** We now clarify this on Line 101: "Of the 23 injected animals that were screened, …" and similarly on Lines 109 and 111.

2.4 Fig. S4G. The arrowhead should be moved closer to the Meckel's-ceratohyal fusion.

***Response:*** Moved as requested.

2.5 Concerns about developmental delay. The authors state (lines 279-281) that "loss of anterior irxab-/-; irx7-/- jaw cartilages in mutants is due to a delay in cranial neural crest formation, resulting in a preferential loss of the anterior- most skeletogenic neural crest-derived cells." This seems to contradict their previous interpretation that these phenotypes reflect defects in "specification" of CNCCs (lines 208-209).

***Response:*** We agree that this line in the Discussion was confusing and have therefore modified it to more clearly describe the results shown in Fig. 4.

> Lines 285-287: "We find that loss of jaw cartilages in IrxAb$^{-/-}$; *irx7*$^{-/-}$ mutants is due to preferential defects in the specification of the anterior-most skeletogenic neural crest-derived cells."

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Third decision letter

MS ID#: DEVELOP/2020/197244

MS TITLE: Comprehensive series of Irx cluster mutants reveals diverse roles in facial cartilage development

AUTHORS: D'Juan T Farmer, Punam Patel, Rachelle Choi, Chih-Yu Liu, and J. Gage Crump
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

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