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Peer Review File

Repurposing of a gill gene regulatory program for outer ear evolution

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
First of all, I think this is a pretty interesting EvoDevo study, and the technical level is sold according to my impression. However, I have a problem with incomplete course of ideas that are behind the major conclusions of this project.
The mammalian outer ear is the very late innovation in the tetrapod lineage. Obviously, this innovation has coopted and adopted some ancient mechanisms of patterning and generating cartilage in the outer parts of the ear. This is not a homology in a classical sense, but rather an innovation based on cooption of deep patterning circuits. Basically, the fish gill-specific enhancers were re-deployed to instruct the construction of the mammalian outer ear. Therefore, this is the story about how the loss of some structures led to release and cooption of the genetic regulatory material into new structures with similar properties (spatial and tissue type). However, when I read the text, I feel a totally different message. The authors conclude: “These findings suggest the mammalian outer ear arose from co-option of an ancestral gill program and show the power of cross-species enhancer testing to reveal cryptic homology across vast evolutionarily timescales.” This is not a homology. This is a re-deployment of circuits to build a novel structure. This is why I do not like the title. In my opinion it is wrong. What happened to this regulatory program during evolution between fishes and mammals? Why this program did not dissipate during long time of evolution prior to emergence of mammalian outer ear?
I feel that the story is incomplete without looking at the analyzed enhancers in amphibians – especially in salamander gills. Axolotls are now available via collaboration from many labs, and this part shall be explored to ensure that amphibian gills still develop in accord with discovered program of regulation. Also, the authors shall suggest the way of outer ear emergence and evolution in early mammalian groups. What about the outer ear in reptilians? They also have it though it appears very different and could evolve independently. Every crocodile possesses acute hearing abilities, characterized by an external (outer) ear composed of a brief canal sealed by a robust valvular flap that concludes at the eardrum. I would suggest to have a look at one of the reptilian models such as gecko. Could the outer ear be a synapomorphic trait that emerged immediately after water to land transition and emergence of amniotes? Or is it a result of homoplasy? Can we expand the comparative analysis of enhancers to make sure we understand this? I believe this conceptual line needs to be followed to have this story complete.

Referee #2

(Remarks to the Author)
This paper looks at the homology of the gills and mammalian outer ear.
I liked the story but was not convinced that the title was correct. The question is, did the mammalian ear co-op processes used in the gills during it formation, or did the gills turn into the ear. The title appears to suggest the latter but the former is more accurate (and stated in the abstract).

Are the pinna and gills homologous as stated here. The process appears to be homologous, i.e. the ability to make this type of cartilage might be homologous, but the actual structures are not homologous as they derive from distinct parts of the head/neck. The authors mention the transformation of the jaw to the middle ear in the intro. These structures are indeed homologous.

I think this paper, therefore, needs careful rewording so that it is not misleading. The main findings of the paper appears to be that elastic cartilage forms using conserved processes that are ancient, and that therian mammals reused this programme to make pinnas. This is very interesting, and fully backed up by the experiments. So there are elastic cartilage specific DARs that are found in gills and ears. This is subtly different from saying that there are gill DARs found in mammalian ears.

What about other elastic cartilages in mammals? Do these also use the same programme, or is the similarity only with the pinna? For example, the epiglottis or eustachian tubes. This should at least be discussed.

The analysis and mapping shown is well done, as it is difficult to do these comparisons across species.

Key experiments are driving LacZ in mice using a gill enhancer. I think it would be necessary to confirm whether the other elastic cartilages also stained for LacZ, not just showing that the hyaline cartilage doesn't. If they were positive, that would suggest a general elastic cartilage programme, if negative a more specific programme. Both results would be interesting. Only E15.5 is shown, and many of the elastic cartilages of the pinna etc form later, what happens to expression at other timepoints?
Regarding the lacZ histology, would be good to show LacZ overlapping with Sox9 or other cartilage marker as its difficult to say what population is actually labelled by the blue.

Why do the founder lines have such diverse patterns of expression? What does the digit expression represent as this appears quite consistent? Looks like there is also some expression in hair follicles near the ear?

The Dr-dlx5a/6a driven LacZ in the mouse in Figure 4 is not very convincing. Would be good to show what cells are actually labelled by sections and expression analysis.

The fact that Dlx5 is expressed in gills and ears doesn't really say much, as its also expressed in lots of other tissues in the head and body, including hyaline cartilages.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10363643/

Minor comments:

Intro: Cartilage is not preserved in fossils but is not a soft tissue, so this means to be reworded.

Pinnas are not found in all mammals, they evolved in therian and monotremes don't have them. A discussion point (not to actually try and do) could be whether monotremes have the same gill-specific/elastic cartilage specific DARs, as they do have a larynx, and also elastic cartilages in the outer ear canal.

The authors miss out the eustachian tube when talking about sites of elastic cartilage in the discussion.

In the discussion the authors mention a potential role for Fgf10 in gill and ears, but the two papers referred to are in Zf, so no ears.

In extended data figure 1, would be good to show the same stain across the animals given the point is to compare. It was difficult to see the gill elastic cartilage. The alcian blue in the gills was very faint.

For clarity, the extended data figure 5 does not show the pinna. These are sections through the ear canal, so part of the outer ear. Its relevant as this region is not the pinna, which was the focus for the other parts of the paper. This region is also elastic cartilage.

Overall, this is a very good paper which sheds light on conserved ways to make elastic cartilage, which is very interesting.

Referee #3

(Remarks to the Author)
In the manuscript, the authors propose that the mammalian outer ear cartage evolved from gill filament cartilages, which may have been present in common ancestor of arthropods and vertebrates. To support of this, they use single-cell multi-omics to show that the elastic cartilage of the human outer ear and the gill filament cartilage of the zebrafish are transcriptionally very similar, and activate a similar Dlx-gene based gene regulatory network. These studies are then extended to horseshoe crabs, which are shown to possess a mesenchymal cell type that has some of the gene regulatory properties of the outer ear and gill filament cartilages.

Overall, the approach and data appear are sound. However, the narrow focus on human fetal ear and nose cartilages and zebrafish neural crest derivatives, limits the sampled cell types used. Thus, while zebrafish gill filament cartilage and human outer ear cartilages are transcriptionally more similar to each other than any other sampled cell type, the set of compared cell types (particularly skeletal cell types) is very small. This diminishes the homology argument somewhat.

The cross-species testing of multiple mouse enhancers in zebrafish (and one zebrafish enhancer in mouse) lends additional evidence that the gill filament and outer ear cartilage share similar gene regulatory networks. However, I would disagree with the author's interpretation that they are showing that the outer ear, as a structure, is homologous to gill filaments, as

structures (which is basically the title of the manuscript). Rather, I feel that the authors show that the elastic cartilage cells that compose both structures are likely homologous. The authors say as much when they acknowledge the lack of phylogenetic continuity of the outer ear structure, and speculate that other structures in heads of early tetrapods used a similar elastic cartilage differentiation program, preserving it to be coopted for outer ear evolution. The distinction between using a homologous GRN to produce a homologous cell type, and saying that two skeletal elements are homologous is critical and should be clearly stated in the title and throughout the manuscript.

The horseshoe crab data is a bit weaker than the zebrafish/mouse comparisons, though it is notable that a horseshoe crab enhancer that is (putatively) active in book lung mesenchyme cells can drive specific reporter in zebrafish gill filaments. This does suggests some evolutionary relationship between the two cell types. That being said, it is not totally clear what that relationship may be, and the proper controls of testing the horseshoe crab enhancer in a horseshoe crab itself is not performed.

Taken together, the authors present a reasonable argument that the elastic cartilage cells of the mammalian outer ear and the elastic cartilage cells of zebrafish gill filaments are homologous cell types. They also provide some evidence that this cell type may have been present in the last common ancestor of protostomes and deuterostomes. However, in my view, neither of these conclusions is particularly compelling. That a new structure (the outer ear) can be built from a pre-existing cell type (elastic cartilage cell) is very well-established concept, and would qualify as a foundational principle in the field of evolutionary developmental biology. This is yet another example of this. Also, the likely homology of protostome and deuterostome skeletal tissues has been established multiple recent publications.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
I am fully satisfied by the new efforts during this revision, and I am happy with the response.

Referee #2

(Remarks to the Author)
The authors have done a very good job of addressing my comments. I think the revised title is a huge improvement and the changes to the text are appropriate. The additional experiments have added important clarification on the links between elastic cartilage, pinna, and gills. Overall, this is an impressive body of work and will be of interest to many.

Referee #3

(Remarks to the Author)
The revised manuscript is improved. The writing is clear and methodical. The technical depth and phylogenetic breadth are both impressive.

However, I still strongly feel that the incorrect homology argument is being made. To me, the data support homology of a specific kind of *cell type* using single cell transcriptomic data and single cell DAR identification and reporter assays. I don't think it establishes homology of the outer ear and gills as *structures* consisting of multiple homologous cell types developing together over time via homologous developmental mechanisms. This is a critical distinction that I think the authors understand, but are glossing over a bit to increase perceived impact. The inability to convincingly homologize these structures reflects a limitation of the single cell -omics approach. There is an attempt to use comparisons between epiglottis elastic cartilage. But again, because the data is derived from single skeletal precursor cells, this falls short. Ultimately, more tissue-level developmental data is needed to go beyond a cell type homology. This would include spatial gene expression data across the multiple cell types that build the structure (not just the skeletal cells) and functional data showing that homlogous signaling pathways are mediating the development. This recent paper comparing cephalopod and vertebrate limbs is an example of structure homology argument: https://elifesciences.org/articles/43828. The authors' argument is akin to arguing that cephalopod and vertebrate limbs are homologous structures based on the fact that they share one type of specialized skeletal tissue. That isn't enough.

With all that being said, the evidence for cell type homology shown in the present manuscript is comprehensive and well done. I think re-written manuscript focused on a cell-level homology argument would be impactful. It may not be as sexy as "ears are really gills" but it would be impactful and (more importantly) closer to the truth.

REVIEWER #1:

1.1 First of all, I think this is a pretty interesting EvoDevo study, and the technical level is solid according to my impression. However, I have a problem with incomplete course of ideas that are behind the major conclusions of this project. The mammalian outer ear is the very late innovation in the tetrapod lineage. Obviously, this innovation has coopted and adopted some ancient mechanisms of patterning and generating cartilage in the outer parts of the ear. This is not a homology in a classical sense, but rather an innovation based on cooption of deep patterning circuits. Basically, the fish gill-specific enhancers were re-deployed to instruct the construction of the mammalian outer ear. Therefore, this is the story about how the loss of some structures led to release and cooption of the genetic regulatory material into new structures with similar properties (spatial and tissue type). However, when I read the text, I feel a totally different message. The authors conclude: "These findings suggest the mammalian outer ear arose from co-option of an ancestral gill program and show the power of cross-species enhancer testing to reveal cryptic homology across vast evolutionarily timescales." This is not a homology. This is a re-deployment of circuits to build a novel structure. This is why I do not like the title. In my opinion it is wrong.

***Response*:** We agree with the reviewer that our paper is a story of evolutionary redeployment to create diverse head structures rather than homology by descent in the classic sense as the gills and outer ear arise from different positions in the embryonic head. We have extensively re-written the manuscript to avoid claims of homology (e.g. lines 361-384 of the Discussion) and provide a new title: **Redeployment of an ancestral gill program in outer ear evolution**.

1.2 What happened to this regulatory program during evolution between fishes and mammals? Why this program did not dissipate during long time of evolution prior to emergence of mammalian outer ear? I feel that the story is incomplete without looking at the analyzed enhancers in amphibians – especially in salamander gills. Axolotls are now available via collaboration from many labs, and this part shall be explored to ensure that amphibian gills still develop in accord with discovered program of regulation. Also, the authors shall suggest the way of outer ear emergence and evolution in early mammalian groups. What about the outer ear in reptilians? They also have it though it appears very different and could evolve independently. Every crocodile possesses acute hearing abilities, characterized by an external (outer) ear composed of a brief canal sealed by a robust valvular flap that concludes at the eardrum. I would suggest to have a look at one of the reptilian models such as gecko. Could the outer ear be a synapomorphic trait that emerged immediately after water to land transition and emergence of amniotes? Or is it a result of homoplasy? Can we expand the comparative analysis of enhancers to make sure we understand this? I believe this conceptual line needs to be followed to have this story complete.

***Response*:** We thank the reviewer for this suggestion as substantial new experiments in frogs and lizards have allowed us to provide plausible evolutionary continuity for the gill to outer ear transition. In reviewing the literature, we learned that tadpoles in several frog species have a gill filter system supported by an elastic-like cartilage, highly reminiscent of the elastic cartilage-containing gill filaments of fishes. We chose *Xenopus laevis* to conduct transgenic testing of zebrafish gill and human outer ear enhancers given its well-developed transgenesis protocols. To do so, we formed a new collaboration with Helen Willsey at UCSF, an expert in frog enhancer testing. In a new Fig. 5 and Extended Data Fig. 8a, we show that both a zebrafish gill developmental enhancer (*Dr_gata3-p1*) and a human outer ear developmental enhancer (*Hs_DLX5/6-p1*) drive expression in the developing tadpole gills, well before cartilage differentiation. These patterns are highly similar to the activities of these enhancers in zebrafish. We also show that a zebrafish gill elastic cartilage enhancer (*Dr_ucmaa-p1*) and a human outer ear elastic cartilage enhancer (*Hs_GAS8-p1*) drive expression in the gill filter cartilages of late-stage tadpoles. These findings show that the regulatory programs for both initial gill outgrowth and later elastic cartilage differentiation are conserved from fish gills and human outer ears to amphibian gills.

In order to understand how the gill regulatory program may have been conserved in reptiles, we formed a new collaboration with Thomas Lozito, an expert in lizard tail regeneration who maintains a colony of multiple different lizard species. While there are a few groups working on getting transgenesis to work in lizard species (e.g., Doug Menke made the first CRISPR anole in 2019), our discussions with multiple members of the lizard community made it clear that testing enhancers in lizards would not be feasible within the timeframe of this study. Instead, we performed histology of the ear in adult green anole lizards and discovered a permanent cartilage, the extracolumella, that had a cell-rich morphology similar to gill filament cartilage and stained strongly with Miller's elastin stain (Fig. 5f-h and Extended Data Fig. 8b,c). The extracolumella is a soft, flexible cartilage of mysterious developmental origins that is present in all reptiles. It helps to support the pharyngotympanic tube of the ear canal and connects the columella (stapes homolog) to the tympanic membrane. We discuss that starting in early mammals the stapes is now connected to the tympanic membrane via the incus and malleus, and this coincides with loss of the extracolumella elastic cartilage and new appearance of similar elastic cartilage in the outer ear canal and pinna. We also find that gill and outer ear enhancers sequence-conserved between fish and mammals are also conserved in the green anole genome. The extracolumella thus represents a plausible redeployment of the gill program in reptiles, though we are careful to discuss that this will require future experiments as transgenesis technology improves in reptiles.

With these findings we now present a model in Fig. 7 of how the gill program may have been retained and redeployed throughout vertebrate evolution to create fish respiratory gill filaments, amphibian gill filters, possibly the lizard ear-supporting extracolumella, and finally the mammalian outer ear.

REVIEWER #2:

2.1 This paper looks at the homology of the gills and mammalian outer ear. I liked the story but was not convinced that the title was correct. The question is, did the mammalian ear co-op processes used in the gills during it formation, or did the gills turn into the ear. The title appears to suggest the latter but the former is more accurate (and stated in the abstract).

***Response*:** Reviewer #1 had a similar comment and we agree that our paper is a story of evolutionary redeployment rather than strict homology. We have revised the title accordingly: **Redeployment of an ancestral gill program in outer ear evolution**.

2.2 Are the pinna and gills homologous as stated here. The process appears to be homologous, i.e. the ability to make this type of cartilage might be homologous, but the actual structures are not homologous as they derive from distinct parts of the head/neck. The authors mention the transformation of the jaw to the middle ear in the intro. These structures are indeed homologous.

***Response*:** We agree with the reviewer and have thoroughly revised the manuscript to reflect that outer ears arose through redeployment of a gill program in a distinct part of the head/neck (e.g. lines 361-384 of the Discussion).

2.3 I think this paper, therefore, needs careful rewording so that it is not misleading. The main findings of the paper appear to be that elastic cartilage forms using conserved processes that are ancient, and that therian mammals reused this programme to make pinnas. This is very interesting, and fully backed up by the experiments. So there are elastic cartilage specific DARs that are found in gills and ears. This is subtly different from saying that there are gill DARs found in mammalian ears.

***Response*:** In the original version, we did not make it clear that we see regulatory conservation between the gills and outer ear at both early developmental outgrowth stages, as well as later elastic cartilage differentiation stages. We have now more clearly separated elastic cartilage conservation in Fig. 3 and early developmental conservation in revised Fig. 4. In Fig. 3, we show that zebrafish gill elastic cartilage DARs can drive transgene expression in mouse outer ear elastic cartilage, and that reciprocally human outer ear elastic cartilage DARs can drive transgene expression in zebrafish gill filament cartilage. In contrast in Fig. 4, we identify DARs active in early developing gills well before cartilage differentiation occurs. Of these, 6 DARs are sequence-conserved in the human genome and selectively accessible in outer ears. We then show that both zebrafish and human versions of one of these near the Dlx5/6 locus drives expression in early forming fish gills, with the zebrafish version also driving expression in mesenchyme but not elastic cartilage of the developing outer ear (new Fig. 4h and Extended Data Fig. 7d,e). In addition, new data in Fig. 5 show that the human Dlx5/6 enhancer drives expression in early developing tadpole gills, again well before cartilage differentiation. We have revised the text to make it clear that both the initial outgrowth of gills and their later differentiation into an elastic cartilage subtype share regulatory conservation with mammalian outer ears.

> Lines 259-262: “These findings support conserved roles of DLX factors in the initial outgrowth of gills and outer ears, as well as the later differentiation of elastic cartilage, highlighting gene regulatory similarities of gills and outer ears beyond elastic cartilage.”
>
> Lines 278-280: “These findings support gene regulatory circuits for both initial gill outgrowth and later cartilage differentiation in amphibians being conserved with zebrafish gills and mammalian outer ears.”
>
> Lines 342-344: “Fish gills and the mammalian outer ear thus share a dedicated gene regulatory program for both their initial outgrowth and the later development of a specialized elastic cartilage subtype.”

2.4 What about other elastic cartilages in mammals? Do these also use the same programme, or is the similarity only with the pinna? For example, the epiglottis or eustachian tubes. This should at least be discussed.

***Response*:** In ongoing work before first submission, we had been working with two pediatric ENT surgeons to isolate epiglottis tissue from a second human fetus obtained through an NIH-approved biorepository at Mt Sinai Icahn School of Medicine (new co-authors Christian Hochstim, Alessandra Moscatello, Ya-Wen Chen; details of IRB approval provided). Given inter-human variability, we isolated both epiglottis and a second outer ear sample from this fetus and separately performed 10X single-nuclei multiomics which we now present in this revision (new Fig. 4a,b and revised Extended Data Figs. 5 and 6). First, we examined whether the elastic cartilage program is conserved between the outer ear and epiglottis. Based on RNA expression, epiglottis clusters with elastic cartilage from both outer ear samples and away from nose hyaline cartilage (Extended Data Fig. 5a-f). Of the 6 human outer ear elastic cartilage enhancers that we found to drive transgene expression in the fish gill filaments, 3 have similar accessibility, 1 has weaker accessibility, and 2 have no accessibility in epiglottis cartilage (Extended Data Fig. 5h). Overall, only ~17% of elastic cartilage enhancers are shared between outer ear and epiglottis (Extended Data Fig. 5g). In addition, we now show that the fish gill elastic cartilage enhancer *ucmaa_p1* drives outer ear cartilage but not epiglottis cartilage transgene expression in mouse (Fig. 3g,f and new Extended Data Fig. 4d,g). Thus, outer ear and epiglottis share some but not all of an elastic cartilage program with fish gills.

In contrast, analysis of early fish gill developmental enhancers that are sequence-conserved in humans revealed striking differences between the outer ear and epiglottis. We had originally reported 27

DARs active in early forming fish gills and sequence-conserved in the human genome, of which 9 had accessibility in the outer ear. Based on the second outer ear and new epiglottis datasets, we have been able to refine our analysis. 6 DARs are accessible in outer ear but not epiglottis and nose, including several linked to genes expressed in the developing mouse outer ear. These include Dlx5/6 and Dlx1/2 enhancers. We now show that mouse *Dlx5* is expressed in the outer ear but not epiglottis, and the zebrafish version of the conserved Dlx5/6 enhancer drives LacZ expression in the mouse outer ear but not epiglottis (new Extended Data Fig. 7c-e). These findings show that the DLX-mediated program is specific for the fish gills and outer ear but not the epiglottis. Interestingly, we find that 10 of the other 27 sequence-conserved enhancers are selectively accessible in the epiglottis but not the outer ear, with several of these linked to genes with epiglottis expression in mouse (new Extended Data Fig. 6b,d). While a detailed analysis of epiglottis development and regulation is beyond the scope of the current study, we briefly mention in Discussion the possibility that a distinct subset of the ancestral gill program was retained and redeployed in the epiglottis and possibly laryngeal tissues of intermediate species.

> Lines 356-359: “Moreover, accessibility and activity of Dlx1/2 and Dlx5/6 enhancers in the outer ear but not the epiglottis or jaw-forming arch indicate discrete roles of DLX factors in gill and outer ear development, distinct from roles in general elastic cartilage development, jaw patterning[1,2], and appendage outgrowth[3]. ”
>
> Lines 381-384: “Lastly, the selective accessibility of 10 sequence-conserved gill DARs in the epiglottis, combined with presence of elastic cartilage in the larynges of anoles, amphibia[4] and birds[5], hints at a separate redeployment of the gill program in the laryngeal regions of tetrapods.”

2.5 The analysis and mapping shown is well done, as it is difficult to do these comparisons across species.

***Response*:** We thank the reviewer for this kind comment.

2.6 Key experiments are driving LacZ in mice using a gill enhancer. I think it would be necessary to confirm whether the other elastic cartilages also stained for LacZ, not just showing that the hyaline cartilage doesn’t. If they were positive, that would suggest a general elastic cartilage programme, if negative a more specific programme. Both results would be interesting.

***Response*:** We now show that neither *Dr_ucmaa-p1:lacZ* or *Dr_dlx5a_p1-lacZ* are active in the epiglottis (new Extended Data Fig. 4d,g and Extended Data Fig. 7e). This supports a more specific program. We also show that *Dr_ucmaa-p1:lacZ* is active in elastic cartilage of the outer ear canal but not the pinna (new Extended Data Fig. 4e). We do not know if this is due to testing only a limited number of zebrafish gill enhancers in mice, or if the outer ear canal regulatory program is more similar to the fish gill program than that of the pinna.

2.7 Only E15.5 is shown, and many of the elastic cartilages of the pinna etc form later, what happens to expression at other timepoints?

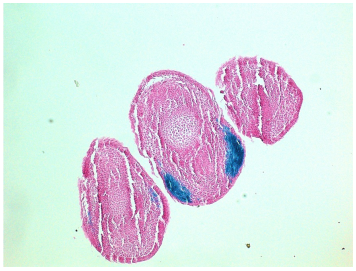
***Response*:** We have now also examined expression of *ucmaa_p1:LacZ* at P0 and continue to detect expression in the outer ear canal elastic cartilage but not the pinna or epiglottis (new Extended Data Fig. 4e-g).

2.8 Regarding the lacZ histology, would be good to show LacZ overlapping with Sox9 or other cartilage marker as its difficult to say what population is actually labelled by the blue.

***Response*:** In new Extended Data Fig. 4e,f, we perform anti-Sox9 protein staining on adjacent sections to demonstrate LacZ expression in elastic cartilage of the outer ear canal but not in adjacent hyaline cartilages.

2.9 Why do the founder lines have such diverse patterns of expression? What does the digit expression represent as this appears quite consistent? Looks like there is also some expression in hair follicles near the ear?

***Response*:** We do not fully understand what drives variability in founder line expression patterns, but we have focused on expression that was reproducible across the 4 founder lines: outer ear expression and digit expression. Nasal epithelial expression was only seen in Founders 1 and 2 and was quite weak and mosaic. Among Founder 2 animals only, some but not all embryos in each litter exhibited hair follicle and forebrain expression. We have now performed transverse sections through E15.5 *ucmaa-p1:lacZ* animals and identify expression in collateral ligaments of the digits (new Extended Data Fig. 4c). Below is a comparison of the LacZ digit sections to published expression of the ligament marker *Scx* (red) and cartilage marker *Sox9* (green)[6]. In separate work from our lab, we have found that *ucmaa* is transiently expressed in facial ligaments before it becomes confined to gill elastic cartilage, perhaps due to some overlap of elasticity programs in elastic cartilage and ligaments. We do not believe this extra digit ligament expression affects the major conclusions of our study given the many other enhancers we tested and additional lines of evidence linking gill and outer ear development.



[FIGURE REDACTED]

2.10 The Dr-dlx5a/6a driven LacZ in the mouse in Figure 4 is not very convincing. Would be good to show what cells are actually labelled by sections and expression analysis.

***Response*:** We acknowledge that the *Dr_dlx5a/6a-p1:LacZ* mouse shows weak expression. This is partly because we chose to use the zebrafish version of this enhancer, which drives weaker zebrafish gill expression than the human version (e.g., Fig. 4e,f). In addition to the original E13.5 stain, we have repeated LacZ staining at E15.5 and observe enhancer activity in the outer ear region as well as neural tube and brain. We sectioned through the outer ear canal of E15.5 mice and now show LacZ in the mesenchyme surrounding outer ear elastic cartilage but not the cartilage itself (new Fig. 4h and Extended Data Fig. 7e). This is consistent with this enhancer driving early gill mesenchyme expression rather than later elastic cartilage expression in zebrafish (Fig. 4e and Extended Data Fig. 7a). Following the advice of our imaging core director Seth Ruffins, we also used fluorescence lifetime imaging to detect endogenous X-gal fluorescence and confirmed activity associated with the outer ear (Extended Data Fig. 7d).

2.11 The fact that Dlx5 is expressed in gills and ears doesn't really say much, as its also expressed in lots of other tissues in the head and body, including hyaline cartilages.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10363643/

***Response*:** We agree that Dlx5 has many roles and sites of expression in the head and body, and we have extensively studied the role of Dlx5 and related DLX genes in the dorsoventral patterning of the jaw. However, at later stages we find that *dlx5a* in zebrafish becomes particularly enriched in the developing gill buds at 5 dpf and gill filaments at 60 dpf (new Fig. 4d). In our single-cell RNAseq datasets and recent spatial transcriptomics analysis of zebrafish, we consistently identify *dlx5a* as one of the most specific markers of gills and gill filaments, and to a certain extent several other members of the DLX family. In mouse, we find that *Dlx5* expression strongly marks the developing outer ear at E13.5 but not the epiglottis or hyaline cartilages in general (Fig. 4c and new Extended Data Fig. 7c). The paper referenced by the reviewer refers to a role of Dlx5 in chondrocyte hypertrophy in osteoarthritis, but we do not observe *Dlx5* to be generally expressed in hyaline cartilages at the embryonic stages we examined.

As we acknowledge that *Dlx5* has many expression domains, we also focused on a sequence-conserved Dlx5/6 enhancer with more restricted activity in the gills and outer ear (Fig. 4). This enhancer drives expression in gill and outer ear mesenchyme associated with elastic cartilage but not the cartilage itself (Extended Data Fig. 7a,b and new E15.5 sections in Fig. 4h). It is also not accessible or active in the epiglottis (Extended Data Fig. 7e), pointing to a more specific role of Dlx5 in outer ear versus general elastic cartilage development. In addition, we identify a nearly identical DLX binding motif enriched in both gill and outer ear DARs. In new data, we show that activity of the zebrafish *Dr_gata3-p1:GFP* enhancer line, one of the earliest markers of gill-forming mesenchyme that we have identified in zebrafish, depends on the presence of multiple DLX motifs (Fig. 4i, Extended Data Fig. 7i). Importantly, destroying DLX sites only affects activity of *Dr_gata3-p1:GFP* in the forming gills and not other minor expression domains such as the jaw joint. These multiple lines of evidence argue for a key conserved role of Dlx5 and potentially other DLX transcription factors in gill and outer ear development, distinct from its more general roles in facial patterning.

Minor comments:
2.12 Intro: Cartilage is not preserved in fossils but is not a soft tissue, so this means to be reworded.

***Response*:** We have re-worded this to state that non-mineralized tissues rarely survive in the fossil record.

> Lines 53-54: "However, uncovering the origins of non-mineralized structures, which rarely survive in the fossil record, is more challenging."

2.13 Pinnas are not found in all mammals, they evolved in therian and monotremes don't have them. A discussion point (not to actually try and do) could be whether monotremes have the same gill-specific/elastic cartilage specific DARs, as they do have a larynx, and also elastic cartilages in the outer ear canal.

***Response*:** In the introduction, we state pinna in relation to the outer ear of therian mammals. We also provide new data in Fig. 5f-h that lizards have elastic cartilage associated with their pharyngotympanic tube and tympanic membrane. The sequence-conserved gill DARs accessible in the human outer ear are also sequence-conserved in amphibia, reptiles, and monotremes. With the extensive new data added, we felt the manuscript was already on the longer side and chose not to include a discussion of monotreme mammals, but we would be happy to include this if the reviewer felt strongly it would improve the manuscript.

2.14 The authors miss out the eustachian tube when talking about sites of elastic cartilage in the discussion.

***Response*:** We now add this in the Introduction.

> Lines 56-58: "The pinna and external auditory canal of the outer ear, as well as the eustachian tube, are supported by specialized elastic cartilage, distinct from the hyaline cartilage found throughout most of the body."

2.15 In the discussion the authors mention a potential role for Fgf10 in gill and ears, but the two papers referred to are in Zf, so no ears.

***Response*:** We apologize for this error and now include the proper Fgf10 mouse outer ear reference (Zhang et al, 2020).

2.16 In extended data figure 1, would be good to show the same stain across the animals given the point is to compare. It was difficult to see the gill elastic cartilage. The alcian blue in the gills was very faint.

***Response*:** The point of Extended Data Fig. 1f is to show that the very first Alcian Blue reactivity of elastic cartilage in the zebrafish gill filaments becomes apparent around 14 dpf, relatively late in development. However, we agree with the reviewer that it would be good to show comparable images of Alcian Blue cartilage staining in the mature gills across all the species. We have now included this as Extended Data Fig. 1d.

2.17 For clarity, the extended data figure 5 does not show the pinna. These are sections through the ear canal, so part of the outer ear. Its relevant as this region is not the pinna, which was the focus for the other parts of the paper. This region is also elastic cartilage.

***Response*:** We obtained these images from GenePaint, which we reference as a public repository of mouse expression patterns. We now clarify in the legend to what is now Extended Data Fig. 6 that the sections are through the ear canal and not the pinna.

REVIEWER #3:

3.1 Overall, the approach and data appear are sound. However, the narrow focus on human fetal ear and nose cartilages and zebrafish neural crest derivatives, limits the sampled cell types used. Thus, while zebrafish gill filament cartilage and human outer ear cartilages are transcriptionally more similar to each other than any other sampled cell type, the set of compared cell types (particularly skeletal cell types) is very small. This diminishes the homology argument somewhat.

***Response*:** We now better describe that cell type homology of elastic cartilage is also supported by extensive enhancer data and not just transcriptional similarity. In order to expand the number of sampled cell types (see also response to **2.4**), we have now also performed single-cell multiomics of human fetal epiglottis, the other major site of elastic cartilage besides the outer ear. This analysis shows that 4/6 elastic cartilage enhancers with cross-species activity in zebrafish have accessibility in both the outer ear and

epiglottis, and that overall ~17% of elastic cartilage enhancers are shared between the outer ear and epiglottis. In contrast, gill developmental enhancers are selectively conserved in either the outer ear or epiglottis but not both. In particular, we identified 27 putative early gill enhancers that are sequence conserved in the human genome. Of these, 6 are selectively accessible in the outer ear but not the epiglottis and nose, and we show that one of these near the Dlx5/6 locus drives transgene expression in the mouse outer ear but not epiglottis. These findings strengthen the argument for regulatory similarities between the gills and outer ear, versus related structures such as the epiglottis that also contain elastic cartilage, although as described below we now discuss that this reflects redeployment of the gill program in the outer ear rather than homology by descent in the class sense.

3.2 The cross-species testing of multiple mouse enhancers in zebrafish (and one zebrafish enhancer in mouse) lends additional evidence that the gill filament and outer ear cartilage share similar gene regulatory networks. However, I would disagree with the author's interpretation that they are showing that the outer ear, as a structure, is homologous to gill filaments, as structures (which is basically the title of the manuscript). Rather, I feel that the authors show that the elastic cartilage cells that compose both structures are likely homologous. The authors say as much when they acknowledge the lack of phylogenetic continuity of the outer ear structure, and speculate that other structures in heads of early tetrapods used a similar elastic cartilage differentiation program, preserving it to be coopted for outer ear evolution. The distinction between using a homologous GRN to produce a homologous cell type, and saying that two skeletal elements are homologous is critical and should be clearly stated in the title and throughout the manuscript.

***Response*:** Both of the other reviewers had similar comments about our previous use of the term homology in relation to the gills and outer ear. We agree that the term redeployment better reflects our findings, and we have extensively revised the text to reflect this and provide a more accurate title: **Redeployment of an ancestral gill program in outer ear evolution**.

3.3 The horseshoe crab data is a bit weaker than the zebrafish/mouse comparisons, though it is notable that a horseshoe crab enhancer that is (putatively) active in book lung mesenchyme cells can drive specific reporter in zebrafish gill filaments. This does suggests some evolutionary relationship between the two cell types. That being said, it is not totally clear what that relationship may be, and the proper controls of testing the horseshoe crab enhancer in a horseshoe crab itself is not performed.

***Response*:** Unfortunately, horseshoe crabs have a very long generation time and to our knowledge no group has developed methods to test enhancers in horseshoe crabs. As the reviewer notes, the ability of the horseshoe crab *dll* enhancer accessible in book gill mesenchyme to drive gill-specific transgene expression in zebrafish is striking. In addition, horseshoe crab book gill DARs were enriched for a nearly identical DLX binding motif as DARs in fish gills and human outer ears. While the types of experiments that can be performed in horseshoe crabs is limited, we feel this supports an ancestral DLX-mediated program being important for gill development in both invertebrates and vertebrates.

3.4 Taken together, the authors present a reasonable argument that the elastic cartilage cells of the mammalian outer ear and the elastic cartilage cells of zebrafish gill filaments are homologous cell types. They also provide some evidence that this cell type may have been present in the last common ancestor of protostomes and deuterostomes. However, in my view, neither of these conclusions is particularly compelling. That a new structure (the outer ear) can be built from a pre-existing cell type (elastic cartilage cell) is very well-established concept, and would qualify as a foundational principle in the field of

evolutionary developmental biology. This is yet another example of this. Also, the likely homology of protostome and deuterostome skeletal tissues has been established multiple recent publications.

***Response*:** We feel our study is a major conceptual advance as it uses cross-species enhancer discovery and testing to uncover how an ancestral developmental program for gills was redeployed to make multiple different types of elastic cartilage-containing structures across vertebrate evolution. The regulatory conservation of both initial gill outgrowth and then later development of a unique elastic cartilage subtype goes beyond simple co-option of a cell type in a different part of the body. Instead, it shows how a developmental program, that in this case uses neural crest-derived cells to form elastic cartilage-containing outgrowths, can be spatially shifted and modified during evolution to form structures with new functions. Also in this revision, substantial new data in amphibians and reptiles (Fig. 5 and Extended Data Fig. 8) now provide a plausible evolutionary model for how the gill program was retained and then redeployed in different ways across tetrapods, thus increasing the broad appeal of this study to evolutionary and developmental biologists.

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Reviewer 3

I still strongly feel that the incorrect homology argument is being made. To me, the data support homology of a specific kind of *cell type* using single cell transcriptomic data and single cell DAR identification and reporter assays. I don't think it establishes homology of the outer ear and gills as *structures* consisting of multiple homologous cell types developing together over time via homologous developmental mechanisms. This is a critical distinction that I think the authors understand but are glossing over a bit to increase perceived impact. The inability to convincingly homologize these structures reflects a limitation of the single cell -omics approach. There is an attempt to use comparisons between epiglottis elastic cartilage. But again, because the data is derived from single skeletal precursor cells, this falls short. Ultimately, more tissue-level developmental data is needed to go beyond a cell type homology… With all that being said, the evidence for cell type homology shown in the present manuscript is comprehensive and well done. I think a re-written manuscript focused on a cell-level homology argument would be impactful. It may not be as sexy as "ears are really gills" but it would be impactful and (more importantly) closer to the truth.

Response: We agree with the reviewer that ears are not simply gills in a different position, and that we have only established homology of elastic cartilage and not other cell types in these two structures. However, we do provide multiple lines of evidence that the initial outgrowth of gills and ears also shares a common gene regulatory circuitry, separate from the later development of the shared elastic cartilage subtype. For example, we identified 6 enhancers for early gill development in zebrafish that are sequence conserved in the human genome and accessible in the human fetal outer ear. For one of these (Dlx5/6), we show that the human version drives expression in early forming gills of zebrafish but not elastic cartilage, and that the zebrafish version drives expression in the early forming outer ear of mouse but not in elastic cartilage. We also show conserved expression of *Dlx5* and *Gata3* in early forming gills and ears, and cite prior work showing a similar role of the Fgf10 signaling pathway in formation of both structures. We now clarify these two examples of homology in the revised Summary:

> Lines 37-39, “Here we show that the outer ear shares gene regulatory programs with the gills of fishes and amphibians for both its initial outgrowth and later development of elastic cartilage.”

To address the concerns of the reviewer and make the cell-level homology more accurate, we have re-written the manuscript in the following ways:

1. Rather than stating that the entire gill program was reutilized during evolution of the outer ear, we now more clearly state that certain “elements” of the gill program were reutilized. Some examples include:

> Lines 45-47 of Summary, “We propose that elements of an invertebrate gill program were reutilized in vertebrates to generate first gills and then the outer ear.”

Lines 64-66, “This reveals how elements of an ancestral gill program may have been iteratively repurposed during evolution to create morphologically diverse structures in the head.”

Lines 318-320, “Our findings suggest that elements of an ancestral gill developmental program were reutilized multiple times through the course of vertebrate evolution to generate diverse gill and ear structures (**Fig. 5g**).”

2. We have modified text to state that elastic cartilage and not cell types in general are homologous between gills and the outer ear.

Lines 132-134, “These findings indicate conservation of gene expression and enhancer architectures between zebrafish gill and human outer ear elastic cartilage.”

Lines 157-159, “Selective activity of human outer ear enhancers in zebrafish gill cartilage and other gill cell types, versus nose enhancers in zebrafish hyaline cartilage, supports homology of elastic cartilage between the mammalian outer ear and fish gills.”

3. We now more clearly state in the last paragraph of the Discussion that ears are not simply gills and contain distinct cell types other than elastic cartilage that are not homologous.

Lines 367-371: “For example, the respiratory zebrafish gills contain CNCC-derived pillar cells that facilitate gas exchange, while *Xenopus* gill filters and outer ears lack respiratory function and hence pillar cells. As many features of outer ears are not shared with gills, it will be interesting to examine how new gene regulatory programs were integrated into pre-existing gill programs to facilitate the evolutionary emergence of the outer ear.”