Zebrafish model for spondylo-megaepiphyseal-metaphyseal dysplasia reveals post-embryonic roles of Nkx3.2 in the skeleton

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Original submission

First decision letter

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MS TITLE: Zebrafish Model for Spondylo-Megaepiphyseal-Metaphyseal Dysplasia Reveals Post-Embryonic Nkx3.2 Requirements in Cartilage Growth

AUTHORS: Joanna Smeeton, Natasha Natarajan, Arati Naveen Kumar, Tetsuto Miyashita, Pranidhi Baddam, Peter Fabian, Daniel Graf, and 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, the referees express considerable interest in your work, but have some significant criticisms and recommend substantial revisions to your manuscript. If you are able to revise the manuscript along the lines suggested, 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 have limited 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.

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.

Reviewer 1

Advance summary and potential significance to field

In the manuscript, the authors report that rare surviving Nkx3.2 morphants do not show ectopic cartilage formation in the region of the missing jaw. On this basis, they conclude that Nkx3.2 has an additional postembryonic requirement restricting proliferation and dampening stress response in the joint adjacent cartilage. The latter is based on their findings from the single-cell analysis.

Comments for the author

In the manuscript by Smeeton and colleagues, the phenotypic consequences in the craniofacial and spine skeleton are described for Nkx3.2 mutations in Zebrafish. In addition, the authors provide single-cell RNAseq data performed on fli1a::GFP (labeling endothelial cells?! Giovannone et al. 2019) and sox10::DSRed double-positive cells from the micro-dissected craniofacial and pectoral fin skeletons of 21 dpf wildtype and Nkx3.2 mutant fish. Here they observed an upregulation of stress-induced pathways. Together with the observation that rare viable Nkx3.2 knock-down animals lack the ectopic cartilage in the jaw region and the scoliosis phenotype the authors come to the conclusion that Nkx3.2 has post-embryonic roles in dampening proliferation and buffering the stress response in joint-adjacent chondrocytes.

The manuscript is primarily descriptive and provides no functional data supporting the conclusions drawn on the basis of the single-cell RNAseq and rare viable Nkx3.2 knock-down animals.

Specific comments:

Introduction:
- The statement in the introduction that the pre-hypertrophic zone is identical with the columnar zone is incorrect. The proliferative zones of chondrocytes are subdivided into two zones comprising the zone of round proliferative chondrocytes and flattened stacked chondrocytes organized into columns which border and partially overlap with the prehypertrophic zone.
- According to the literature, nkx3.2 is expressed in proliferating and pre-hypertrophic chondrocytes of the growth plate (and not as stated in the text ‘only’ in the pre-hypertrophic zone).

Results:
General comment: please provide n-numbers of independent biological samples analyzed for all data shown.

- In the manuscript by Miyahita et al. 2019, the nkx3.2el802 mutation is described to have a 20 bp deletion, while here it is referred to as a 14 bp deletion. If the original description was incorrect then this should be clearly pointed out here.
- The deletion in the TALEN-induced nkx3.2el802 mutation should affect transcriptional levels, as (according to Fig. 1A) it appears to delete the region around the transcriptional start site. Yet, based on the in situ hybridizations shown in Figure 6, this does not seem to be the case. The authors should comment on this.
- FACS blots for cell sorting should be provided. In addition, it would be good if the authors could provide an image of a section of the double-labeled cells in the jaw region of 21 dpf heads. Were microdissected regions from different specimens or the different regions pooled for the analysis?
- Please provide the full names for abbreviated genes mentioned in the text.
- References for the features referred to in the paragraph entitled ‘Single-cell RNA sequencing ….’ need to be provided.
- The description of the ptgdsb.1 expression pattern is misleading as there appears to be a weaker signal in the articular region of the palatoquadrate element next to the joint in D as well.

Discussion:
Difficult to imagine how supernumerary bones (pseudoepiphysies) would be caused by cartilage overgrowth. Here an additional 'segmentation event' seems to have occurred.
Is defective ossification also observed in the fish spine, as it is seen in humans and mice?

The spine phenotype in human patients is described as kyphoscoliosis, the phenotype in fish resembles more kyphosis, in contrast to the sidewise bending observed in scoliosis (as such I find the title a bit misleading, as the data do not really provide any new insights into the human SMMD phenotype).
As the authors discuss the expression pattern of various markers in the bidirectional growth plate of zebrafish, it would be good to include a drawing of such a growth plate as part of the diagram in Fig. 6P and to state, for readers that may not be so familiar with zebrafish, that this exists in the Ch element. Yet, this bony element is not affected in the mutant.

Comments regarding figures and legends:
- Figure 1: A: please explain what the blue regions and the white boxed regions are (coding versus non-coding parts of the exons?)
C: what are the bright yellow cells to the right of the arrowhead in the mutant?
D: Position of ch bone should be pointed out in the wildtype as well.
E: Label for os and pts are very small and difficult to see. Arrows pointing to the position of the fusion of the two bones may be helpful.
- Figure 2: legend appears to be mixed up (e.g.: A,A',B,B'D,D',E, E' are microCT images and not A-D); arrows are mentioned in the legends but not present in the figure itself. What are the differences between images shown in C/C' and F/F' – magnification? If so then the size bars are missing. What is the frequency of this very abnormal spine curvature at the base of the fine shown in F/F' in the mutant? The alcian blue/alizarin red-stained images in Figure 4K look a lot less severe (which image is more representative?).
MicroCT images should be provided for the two mutant alleles and possibly also for the rare viable nkx3.2 morphants.
- Figure 3: The boxes around the regions in A and B are difficult to see. What does the darker stained region (dark bluish) in A' correspond to? Is this kind of a sesamoid-like element?
Quantification of the results shown in C-F would be more representative and meaningful.
What do the PCNA and BrdU stainings look like in the ceratohyal bone of the mutant?
- Figure 4: Alcian blue-stained sections may be better to represent the cartilage overgrowth in the jaw joint of the mutant. What happened to the palatoquadrate growth plate in the mutant?
- Figure 5: The legends next to the UMAP projection graph are too small to read. Also the labels of the x- and y-axes are too small. The same is true for the labels in C and D (not readable on the printed version). C) The organization is not self-explanatory; where are the top 5 enriched genes per cluster. Please explain color-coding; yellow corresponds to high levels of expression?
- Figure 6: What does the DIG-in situ hybridization for nkx3.2 in the mutant look like?
Fluorescent images in H-I' and N-O are of low resolution (pixels are visible at higher magnification). No convincing upregulation of ptgdsb.1 or sesn1 in the fluorescent images visible. Does the blue staining correspond to DAPI or HOECHST as a nuclear stain? If so, how do the authors explain that the nkx3.2 signal is not localized to the nucleus?

Reviewer 2

Advance summary and potential significance to field

Smeeton and colleagues investigate the roles of nkx3.2 during skeletal development in zebrafish. The authors report two genetic alleles of nkx3.2 which reveal new roles for nkx3.2 in regulating post-embryonic skeletal development. nkx3.2 mutants display jaw joint loss in larvae, phenocopying the previously reported morphant phenotype, as well as ectopic cartilage outgrowths around their jaw joints, scoliosis, and skull defects. In the jaw joint, ectopic proliferation was observed across the jaw joint region. Using single-cell RNA-sequencing, the authors discover stress pathways are upregulated in mutant chondrocytes, including components of the prostaglandin and mTORC1 pathways. These results are especially novel, and suggest many new questions on the role of these pathways during chondrocyte maturation. All three of the post-embryonic mutant skeletal phenotypes resemble phenotypes seen in human SMMD patients, suggesting this mutant could be a new model for this human disease. Overall this paper makes significant insights into skeletal development and will of great interest to readers of Development.

Comments for the author

Some questions should be addressed before publication.

1. Can the authors comment on why the morphant is lethal but the mutants are not? Are the genetic backgrounds similar? Could nkx3.2 mutations reported here elicit genetic compensation? At the very least it seems like the authors could comment on whether nkx3.1 is expressed in their scRNA-seq data and if so, if it is upregulated in the mutants.

2. The authors state that both alleles are likely "complete LOF alleles (L127-128)" but little data or justification of this prediction is provided. Could el802 initiate translation at a downstream ATG? Would the nature of the el802 allele predict that this mutation would not induce genetic compensation (if so, this could be argument against compensation occurring). The ua5011 allele seems like it would result in NMD so one might predict compensation.

3. The authors reference earlier papers suggesting that nkx3.2 upregulates sox9 and runx2, while repressing col10a1. The authors present data showing col10a1 is down not up in the mutant jaw joint, which is convincing. Can the authors comment on whether sox9 or runx2 are present and if so different by genotype in the scRNA-seq data?

4. Can the authors add segregation numbers to address lethality in the mutant alleles – i.e. were homozygous mutants recovered at the expected 25% frequency? Line 181 states "in marked contrast to the viability of most jaw-joint-less nkx3.2 mutants" which makes it sound like some mutants die. The discrepancy between the morphant and mutants is an important point, so presenting some kind of observed genotype ratios (or numbers for percent of mutants that die) would help clarify.

5. Can PCNA and/or BrdU results in Figure 3C-H somehow be quantified? Doing so, as well as some kind of statistical test if possible, would increase confidence in the claims of expanded cell proliferation across the jaw joint in the mutant.

6. The methods and results should both state more clearly which mutant allele was used for the scRNA-seq.

7. Do the fish mutants have spleens?

8. L29, wording makes it ambiguous whether rare mutations are GOF or LOF in humans, it might be clearer to add "Homozygosity for rare inactivating mutations … "

9. L421-422 How were "Fin and gill chondrocytes" identified and removed?

10. The font sizes in Figures 5 and 6 (especially in model in Figure 6) are so small they are very hard to read.

Reviewer 3

Advance summary and potential significance to field

The authors present a new zebrafish genetic model for SMMD. They attempt to distinguish between embryonic and post-embryonic roles of Nkx3.2, contrasting the previously known role in specifying the jaw joint in zebrafish, which they verify here with genetic mutants, with newly described cartilage overgrowth and spinal defects presented in the current study. These observations are nicely complemented by a single-cell RNAseq analysis of mutant cranial chondrocytes showing an apparent increase in mitotic (correlative evidence for hyperproliferation) and stress response genes.

Comments for the author

The paper falls short in providing strong enough evidence for many of its claims. It would be greatly improved by providing quantitative data to back up statements regarding cell numbers, as well as a more careful analysis of in situ hybridization results.

Major Concerns:

1) Lines 147-149, Fig 2. Details in the µCT images of the skeletal defects in nkx3.2 mutants are hard to see. Please provide higher-resolution insets particularly for the vertebral fusions. Please also indicate the number of animals that have the os-pts fusions and spinal defects described.

2) Lines 160-164, Fig 3C-D: Include quantitation for PCNA expression. At minimum the number of animals examined that display the general pattern of PCNA expression should be included.

3) Lines 165-166, Fig 3D: Provide evidence for continuous PCNA+ cells spanning the lower and upper jaw. For the joint region between Meckels and the adjacent cartilage there are few or no PCNA+ cells. Patterns of PCNA expression seem identical at the jaw joint and in Meckels between WT and mutant (Fig. 3C,D).

4) Lines 168-169, Figure 3E-F: Quantify the number of BrdU cells at the jaw joint in WT and presumptive jaw joint regions in mutants. If chondrocytes hyper-proliferate at the jaw joint in nkx3.2 mutants causing cartilage overgrowth, the number of BrdU+ cells in WT and mutant jaw joints should be striking. How much of the region displayed in panel F will give rise to what is observed in panel B'? I am wondering because it appears that there is just a single BrdU+ cell at the presumptive jaw joint in the nkx3.2 mutant (panel F) while most BrdU+ cells appear to be in regions that are largely Meckels and adjacent cartilages.

5) Line 171: Provide quantifications for the "increased numbers" of PCNA+ cells described in the text and definitive statements about the numbers of labelled cells in WT versus mutant.

6) Lines 197-199: The authors state that the absence of scoliosis in the 2 surviving nkx3.2 MO fish is evidence of a post-embryonic role for NKX3.2 in spine development by comparison to their nkx3.2 mutants. However, N=2 and perhaps given the high lethality in MO-injected fish (only 2/105 surviving) they may have developed scoliosis but died before 3 months of age for any number of reasons. Did the authors observe any spine defects in the other 103 MO-injected fish prior to their deaths (at late embryonic, larval, or other stages)? Additionally, because MO may be titrated-out by 2 dpf, it is still possible that nkx3.2 may play a role in ensuring proper spine development after 2 days, but still "embryonically." When do the authors first observe spine and/or tail defects in their genetic nkx3.2 mutants?

7) Figure 4, lines 616-617. Please provide higher magnification images of panels H and I, preferably at cellular resolution. In both panels, there appears to be alizarin staining between the Meckel's cartilage and the palatoquadrate, though this region is much reduced in the mutant compared to the MO-injected example. Also, while there is more alcian blue staining at this region in the mutant, there seems to be some light blue alcian staining in the region indicated by the arrowhead in panel I as well. Can the authors be sure that there is no delay in ossification of the palatoquadrate in mutants compared with knockdowns resulting in the observed phenotype?

8) Lines 238-239: The authors state that "In mutants, col10a1a was not expressed in the missing jaw joint region, consistent with lack of col10a1a reads in the mutant chondrocyte cluster in the single-cell data (Figure 6C)". However, it is impossible to correlate position (jaw joint region) with mutant chondrocyte cluster 2 from the single-cell data. Cells from this cluster could be derived from any col10a1a-low chondrocytes from multiple cartilages in the head.

9) Line 242: The authors state that ptgdsb.1 expression is excluded from the jaw joint in WT, but in Figure 6F', there clearly are ptgdsb.1(+) cells at the jaw joint. Also, the authors state that ptgdsb.1 is expressed at "...lower levels in Meckels cartilage of the lower jaw..." but this region (Figure 6D) appears to overlap with the expression domain of nkx3.2 (Figure 6A), which marks the jaw joint. What delineates the "jaw joint" from the "lower levels in Meckels cartilage" here? There appear to be some cells with both ptgdsb.1 and nkx3.2 spots in panel F.

10) Line 243: The authors state that sesn1 is excluded from the jaw joint in WT but it looks like there is some sesn1 expression in cells that have nkx3.2+ spots (Figure H, H'). Please confirm or deny using the color picker tool of an image editing program or some other image analysis method.

The authors should clarify how they classify cells as being in the “jaw joint region” or not, as well as how they define the presence or absence of expression in this and the case above. Is there a way to quantify the level of expression in jaw joint cells? If so, it would probably be more accurate to say that ptgdsb.1 and sesn1 expression is increased at the jaw joint in mutant versus WT.

11) Lines 278-280: Similarly, clarify what is meant by “...generally excluded from the jaw joint, although there does appear to be weak co-expression of ptgdsb.1 and nkx3.2 on the lower jaw side...” As discussed above, where there appears to be co-expression when the authors claim there is none, they claim a “general” exclusion but then follow-up with an invalidating statement that there is some co-expression. Please provide further analysis and clarification in the results portion of the text to reflect these discrepancies.

12) Lines 291-292: The authors frame the difference in expression levels of nkx3.2 in the jaw versus the growth plate as possibly “reflect[ing] slower growth of the subarticular zone relative to the expansive growth of the endochondral cartilage template” but provide no references for this distinction. Their data (Figure 3C,D) also do not support this statement as no quantitation of number of proliferative cells at the jaw joint versus ceratohyal growth plate in WT was performed. There appear to be similar numbers of PCNA(+) cells in the Meckels cartilage part of the jaw joint and the ceratohyal in WT.

Minor concerns:

The resolution of the µCT images in Figure 2, in particular panels A’, D, E’, E E’ is quite low. It is hard to appreciate what is being displayed here.

The authors should update the Miyashita et al. 2019 BioRx citations tp reflect the accepted manuscript status at the Journal of Experimental Biology.

Figure 1: The authors should consider labeling some major cartilage elements and bones in panels B-E in addition to areas of interest such as the jaw joint and presumptive jaw joint region referred to later in the text (Lines 156-157).

Lines 596-597: The figure legend describes arrows to indicate spinal abnormalities, but there are no arrows in any of the panels (A-D). Similarity the legend describes arrows for panels (E-F), but there are none.

Figure 2: How consistent are the tail defect morphologies in nkx3.2 mutants? While the images in Figure 2F, F’ closely resemble their counterparts in E, E’,
the E and F image sets were acquired from different fish (E is ua5011 and F is el802). Were the F examples chosen because they were most similar to E or does this morphology reflect the vast majority of tail defects in nkx3.2 mutants?

Figure 2 legend: Please specify that panels with the prime symbol indicate dorsal views.
Consider changing “60 dpf” in Figure 1 to “2 mpf” to be consistent with the labelling in Figure 2 or change Figure 2 labels to “30 dpf” and “60 dpf.”

Figure 3: The authors should label some of the major bones/skeletal elements in all panels. This would be particularly helpful when visually comparing panels A and B considering the altered morphologies of most jaw bones in nkx3.2 mutants.
They should also consider adding labels for “lower jaw,” “upper jaw,” and/or Meckel’s cartilage, palatoquadrate to panels C and E as the authors refer to all in the text and/or legend but these are difficult to follow for a reader not familiar with zebrafish anatomy.

Figure 3 legend: The authors should indicate in the legend that panels A-F are ventral views. They should also describe in the legend the nuclear stain in panels C-F and include a colored label for it as for “PCNA” and “BrDU” in panels C and E.

Figure 3: Panel B’ does not appear to be the magnified boxed-region in B. Has it been further dissected to produce the image in panel B’? Is the orientation different? Is the joint obscured in panel B? If so, additional labeling of bones in panels A and B would help to clarify.

Figure 3: “BrDU” in the legend and panel E should be changed to “BrdU”.

Figure 4: The authors should label major bones and structures in all panels, in particular, the structures described in the text (Lines 190-192).

Lines 193-194: The authors state “...indicative of the cartilage outgrowth seen in medial views (Figure 3B).” However, Figure 3B is a low magnification ventral view. Furthermore, it is difficult to see the similarities between the two figure panels compared in the text (Figure 3B or B’ and Figure 4H).

Line 187 & Line 614. The manuscript describes the 2 nkx3.2 MO fish as surviving until adulthood at 3 months of age (Line 187). However, the Alcian-Alizarin dissections in Figure 4 performed on these fish were conducted on 2 month old fish, as described in the figure legend (60 dpf).

Figure 6G, I: There appears to be significantly more nkx3.2 RNA expression in nkx3.2 mutants than in WT. While mRNA degradation is not a feature of all mutants it seems odd here. Do most/all of the cartilage cells in the mutant overgrowth phenotype have a joint-like identity?

Figure 6: The authors should label Meckel’s cartilage and palatoquadrate.

Figure 6: Provide higher quality images for Panels H-I’ and N, O, L.

Figure 6: Increase the font sizes in panel P? Also, the entire model could be enlarged to fill the white space in panel P.

Line 284: Quantify “increased proliferation” in mutant versus WT.

Line 285: Is sesn1 truly “excluded” from the PZ or dramatically reduced compared to the HZ? Or “essentially excluded?”

Lines 365-366: Include primer sequences used to make the ptgdsb.1 probe template.

Line 374: Include manufacturer, catalog # for cytoseal?

Line 401: Why were pectoral fin skeletons dissected and used in the scRNA-seq? The main body of the text described heads only (Line 205).

Line 126: “prevent” might be a better word to use here, as “block” might have a connotation similar to “translation blocking” usually reserved for steric blocking of translation as for many morpholinos. Also it would be more accurate to say “mRNA” or “RNA” here in place of “protein” as translation of the RNA into protein.

Usage of the term “Adult zebrafish”: Typically, zebrafish are considered to be “adults” at around 2.5-3 months of age or when fertile. Were the 2-month fish described as “adults” in the manuscript fertile? Later in the manuscript (Line 187), the authors describe nkx3.2 MO-injected fish saying “only two survived until adulthood (3 months of age).” By their previous statements of 2 month old fish as “adults,” potentially more than 2 nkx3.2 MO fish may have survived to 2 months of age.

First revision

Author response to reviewers' comments

REVIEWER #1:

1.1 -The statement in the introduction that the pre-hypertrophic zone is identical with the columnar zone is incorrect. The proliferative zones of chondrocytes are subdivided into two zones comprising the zone of round proliferative chondrocytes and flattened stacked chondrocytes organized into columns which border and partially overlap with the prehypertrophic zone.
-According to the literature, nkx3.2 is expressed in proliferating and pre-hypertrophic chondrocytes of the growth plate (and not as stated in the text 'only' in the pre-hypertrophic zone).
Response:
We have revised the Introduction on lines 52-58: "In the growth plates of endochondral bones, chondrocytes are arranged into a resting zone containing stem cells (Mizuhashi et al., 2018; Newton et al., 2019), a zone of round proliferative chondrocytes, a zone of proliferative flattened chondrocytes that merges into a pre-hypertrophic zone, a hypertrophic zone in which chondrocytes enlarge and calcify, and a transitional zone where chondrocytes undergo apoptosis or transdifferentiate into osteoblasts (Giovannone et al., 2019; Jing et al., 2015; Kronenberg, 2003; Yang et al., 2014; Zhou et al., 2014)."

On lines 69-72, we revise our introduction of Nkx3.2 expression: "In chick and mouse, *Nkx3.2* is expressed in the proliferating and pre-hypertrophic chondrocytes of the growth plate but largely excluded from the hypertrophic zone and articular surfaces of joints (Church et al., 2005; Provot et al., 2006; Tribioli et al., 1997)."

1.2 General comment: please provide n-numbers of independent biological samples analyzed for all data shown.
Response:
We now provide n-numbers for each experiment in the main text or methods section. We also clarify on line 428 that "for all in situ hybridizations and RNAscope stains we saw similar patterns of expression in at least 3 individuals for each experiment."

1.3 In the manuscript by Miyahita et al. 2019, the nkx3.2el802 mutation is described to have a 20 bp deletion, while here it is referred to as a 14 bp deletion. If the original description was incorrect then this should be clearly pointed out here.
Response: We thank the reviewer for pointing out this error. The mutation is in fact a 14bp deletion, and we had corrected this in the Miyahita manuscript prior to its final publication in JEB.

1.4 The deletion in the TALEN-induced nkx3.2el802 mutation should affect transcriptional levels, as (according to Fig. 1A) it appears to delete the region around the transcriptional start site. Yet, based on the in situ hybridizations shown in Figure 6, this does not seem to be the case. The authors should comment on this.
Response:
The *nkx3.2* el802 mutation removes the 'A' of the ATG at the translation start site but does not delete the TSS (transcriptional start site). We have revised Fig. 1A to better illustrate that the TSS is unaffected by the mutation, and have updated the text to read: "predicted to prevent translation of *nkx3.2* mRNA".
We also note that we still see *nkx3.2* transcripts at roughly the same levels in wild types and mutants (Fig. 6), showing that transcription is not affected by the el802 mutation, and also that nonsense-mediated decay has not occurred (arguing against genetic compensation (see REV 2.2)).

1.5 FACS blots for cell sorting should be provided. In addition, it would be good if the authors could provide an image of a section of the double-labeled cells in the jaw region of 21 dpf heads. Were microdissected regions from different specimens or the different regions pooled for the analysis?
Response:
We have included the FACS plots with gating for cell sorting and representative images from the fli:GFP;sox10:DsRed double-positive heads in a new Figure S2. On Line 220, we now indicate that we pooled 5 animals per genotype for FACS.

1.6 Please provide the full names for abbreviated genes mentioned in the text.
Response:
Full names have now been provided for genes described in the text.

1.7 References for the features referred to in the paragraph entitled 'Single-cell RNA sequencing ….' need to be provided.
Response:
We now provide references for the features we describe at the end of this paragraph.

1.8 The description of the ptgdsb.1 expression pattern is misleading as there appears to be a weaker signal in the articular region of the palatoquadrate element next to the joint in D as well.
Response:
We have revised the text to indicate the much lower levels of *ptgdsb.1* expression at the jaw joint (Line 260) and added quantification of *ptgdsb.1* signal at the joint in a new Figure 6Q panel.

1.9 Discussion: Difficult to imagine how supernumerary bones (pseudoepiphysies) would be caused by cartilage overgrowth. Here an additional 'segmentation event' seems to have occurred.
Response:
It is known that within the mammalian autopod (and fish fin), the number of joints and hence cartilage/bone segments is proportional to the length of the cartilage/bone field. Hence, cartilage overgrowth could lead to a bigger field of cells that is partitioned into additional segments. We now explain this better in the Discussion (Lines 279-283): "The supernumerary bones and lengthening of the wrist and digits in SMMD could reflect analogous cartilage overgrowth in humans as the number of cartilage segments in the digits appears to be proportional to cartilage length, with joint-derived signals such as Wnt9a inhibiting the formation of the next joint within a certain distance (Hartmann and Tabin, 2001). Hence, an increase in cartilage size would result in increased numbers of digit bones."

1.10 Is defective ossification also observed in the fish spine, as it is seen in humans and mice? The spine phenotype in human patients is described as kyphoscoliosis, the phenotype in fish resembles more kyphosis, in contrast to the sidewise bending observed in scoliosis (as such I find the title a bit misleading, as the data do not really provide any new insights into the human SMMD phenotype).
Response:
With our higher resolution uCT analysis in Figure 2, we now demonstrate defective ossification in the fish spine, particularly in the cranial region. The caudal vertebral phenotype involves a sideways bending, consistent with a scoliosis phenotype, and the additional finding of shortened cranial vertebrae also mirrors the SMMD phenotype. This is now better described in the text.

1.11 As the authors discuss the expression pattern of various markers in the bidirectional growth plate of zebrafish, it would be good to include a drawing of such a growth plate as part of the diagram in Fig. 6P and to state, for readers that may not be so familiar with zebrafish, that this exists in the Ch element. Yet, this bony element is not affected in the mutant.
Response:
We have added a drawing of the Ch growth plate as suggested (Fig. 6S). We also clarify on Line 251 that we analyzed expression in the "bidirectional ceratohyal growth plates". We agree that it was unexpected that the ceratohyal was not affected in the mutants, which could reflect redundancy with other genes (but likely not *nkx3.1* which was not detected in our scRNAseq data) or more subtle defects below our level of detection.

1.12 Comments regarding figures and legends: -
Figure 1: A: please explain what the blue regions and the white boxed regions are (coding versus non- coding parts of the exons?)
C: what are the bright yellow cells to the right of the arrowhead in the mutant?
D: Position of ch bone should be pointed out in the wildtype as well.
E: Label for os and pts are very small and difficult to see. Arrows pointing to the position of the fusion of the two bones may be helpful.
Response:
We now show non-coding parts of the exons in white boxes and coding parts in blue boxes; this is explained in the figure legend. We have shifted the arrowhead in Fig. 1C mutant to better designate the missing jaw joint. In 1D, the Ch bone is now labeled in wild type. In 1E, we have enlarged the OS and PTS labels and added an arrow to designate the fused joint between these elements in mutants.

Figure 2: legend appears to be mixed up (e.g.: A,A',B,B'D,D',E, E' are microCT images and not A-D); arrows are mentioned in the legends but not present in the figure itself. What are the differences between images shown in C/C' and F/F' – magnification? If so then the size bars are missing. What is the frequency of this very abnormal spine curvature at the base of the fine shown in F/F' in the mutant? The alcian blue/alizarin red-stained images in Figure 4K look a lot less severe (which image is more representative?).
Response: We have replaced the image in Figure 4K with a more representative image that is more reflective of the majority of the s-shaped defects in the mutant caudal spine. This very abnormal spine curvature was seen in 11/11 animals by uCT and 5/6 by alcian/alizarin stain. Line 207-208 now reads: "5/6 *nkx3.2*^{el802} mutant animals displayed severe caudal tail curvature defects (Figure 4K)"

1.13 MicroCT images should be provided for the two mutant alleles and possibly also for the rare viable nkx3.2 morphants.
Response:
Due to the consistency of the phenotypes between the two alleles and the rarity of morphants, we have provided an updated and higher resolution analysis of the skeletal phenotype using the ua5011 allele, complemented by alcian/alizarin staining of the el802 allele and the rare viable nkx3.2 morphants. We note that the el802 allele was maintained at USC while the ua5011 allele was in maintained in Canada, where the microCT imaging was performed by the lab of Daniel Graf. Covid made it especially difficult to transfer el802 animals to Canada where we could repeat the microCT imaging in a consistent manner.

1.14 Figure 3: The boxes around the regions in A and B are difficult to see. What does the darker stained region (dark bluish) in A' correspond to? Is this kind of a sesamoid-like element?
Response:
A' dark stain shows the retroarticular (RA) cartilage in wildtypes, which we now label. It is indeed similar to a mammalian sesamoid bone. We have changed the box color in A and B to red to ease viewing.

1.15 Quantification of the results shown in C-F would be more representative and meaningful. What do the PCNA and BrdU stainings look like in the ceratohyal bone of the mutant?
Response:
In a new Fig. 3I, we have now performed a quantification of PCNA+ cells at 21dpf (in adjacent sections to nkx3.2 in situ expression to identify the joint region – e.g. Fig. 3G,H PCNA are adjacent sections to Fig. 6I,J *nkx3.2* expression). From this, we find a statistical six-fold increase in proliferative chondrocytes at the mutant jaw joint region. We also quantified PCNA+ cells in the ceratohyal growth plate and found no difference between wild types and mutants.

1.16 Figure 4: Alcian blue-stained sections may be better to represent the cartilage overgrowth in the jaw joint of the mutant. What happened to the palatoquadrate growth plate in the mutant?
Response:
We now provide H&E stained frontal sections through the fused jaw joint region which clearly show the jaw joint cartilage overgrowth phenotype compared to wild-type cartilage (new Figure 3C,D). On Lines 203-204, we explain that "in mutants, a continuous zone of cartilage fuses Meckel's cartilage to the palatoquadrate cartilage growth plate (Figure 4H)." We have also added the H&E staining protocol to the methods section (Lines 398-403).

1.17 Figure 5: The legends next to the UMAP projection graph are too small to read. Also the labels of the x- and y-axes are too small. The same is true for the labels in C and D (not readable on the printed version). C) The organization is not self-explanatory; where are the top 5 enriched genes per cluster. Please explain color-coding; yellow corresponds to high levels of expression?
Response:
We have substantially revised the sizing and organization of Figure 5 to increase legibility and added legends for levels of expression (yellow high vs magenta low). We also added color coding immediately adjacent to gene names to facilitate matching of the top enriched genes with each cluster.

1.18 Figure 6: What does the DIG-in situ hybridization for nkx3.2 in the mutant look like?

Fluorescent images in H-I' and N-O are of low resolution (pixels are visible at higher magnification). No convincing upregulation of ptgdsb.1 or sesn1 in the fluorescent images visible. Does the blue staining correspond to DAPI or HOECHST as a nuclear stain? If so, how do the authors explain that the nkx3.2 signal is not localized to the nucleus?
Response:
We have now performed colorimetric in situ at 21dpf for *nkx3.2* in mutant animals (revised Figure 6B) and include improved and higher resolution images for *ptgdsb.1* and *sesn1* in G-J and O-P. Quantification of the fluorescence intensity for *ptgdsb.1* and *sesn1* in the jaw joint region confirms upregulation of these transcripts in mutants (new Figure 6Q). In the legend for Fig. 6G-J, we now clarify that "DAPI labels nuclei in blue." For *nkx3.2* mRNA signal, only nascent and unspliced transcripts will be nuclear, with spliced transcripts in the cytoplasm where translation occurs.

REVIEWER #2:

2.1. Can the authors comment on why the morphant is lethal but the mutants are not? Are the genetic backgrounds similar? Could nkx3.2 mutations reported here elicit genetic compensation? At the very least it seems like the authors could comment on whether nkx3.1 is expressed in their scRNA-seq data and if so, if it is upregulated in the mutants.
Response:
The genetic backgrounds are similar (largely Tubingen but most common wild-type zebrafish strains are notorious for being highly polymorphic) in the mutants and morphants and should not account for this difference. The lethality is probably due to general toxicity seen with morpholinos (off-target effects) resulting in a failure to inflate the swim bladder (Line 198). nkx3.1 was not detected in the scRNA-seq data, either controls or mutants (Line 350).

2.2. The authors state that both alleles are likely "complete LOF alleles (L127-128)" but little data or justification of this prediction is provided. Could el802 initiate translation at a downstream ATG? Would the nature of the el802 allele predict that this mutation would not induce genetic compensation (if so, this could be argument against compensation occurring). The ua5011 allele seems like it would result in NMD so one might predict compensation.
Response:
In Figure 6, we observe largely normal expression of *nkx3.2* in el802 mutants, arguing against the nonsense mediated decay (NMD) required for genetic compensation. This is consistent with our current understanding of NMD which requires the presence of a premature stop codon (PTC) at least 50nt upstream of the last exon-exon junction. nkx3.2 is a two-exon gene and the ua5011 allele is a PTC in the last exon. The el802 allele destroys the ATG and hence there is no translation. Nonetheless, we now refer to both alleles as "severe" as opposed to "complete" LOF on Line 130.

On Lines 348-354, we also discuss how genetic compensation is unlikely to be an issue for our mutant interpretation: "However, we did not detect cartilage overgrowth within mutant zebrafish endochondral bones, despite expression of *nkx3.2* in these cartilage templates. This cannot be attributed to compensation by *nkx3.1* (which we failed to detect in our scRNAseq analysis) or a broader genetic compensation (as we saw no evidence of the nonsense-mediated decay of *nkx3.2* transcripts required for transcriptional adaptation). Hence, Nkx3.2 function in zebrafish appears to be preferentially important for regulation of joints and joint-associated cartilage."

2.3. The authors reference earlier papers suggesting that nkx3.2 upregulates sox9 and runx2, while repressing col10a1. The authors present data showing col10a1 is down not up in the mutant jaw joint, which is convincing. Can the authors comment on whether sox9 or runx2 are present and if so different by genotype in the scRNA-seq data?
Response:
On Lines 244-245, we describe that *sox9a* and *runx2b* expression are unaltered in mutant scRNAseq data: "However, many other regulators of chondrocyte biology, such as *sox9a* and *runx2b*, were unchanged in mutants."

2.4. Can the authors add segregation numbers to address lethality in the mutant alleles – i.e. were homozygous mutants recovered at the expected 25% frequency? Line 181 states "in marked contrast to the viability of most jaw-joint-less nkx3.2 mutants" which makes it sound like some

mutants die. The discrepancy between the morphant and mutants is an important point, so presenting some kind of observed genotype ratios (or numbers for percent of mutants that die) would help clarify.
Response:
In contrast to the swim bladder problems in morphants, mutants are recovered as adults but we did note sub-viability when raised in the same tank as WT (perhaps due to food competition). To grow enough mutants for the analyses described here, we would perform fin-clips at 14dpf and segregate mutants and wildtypes into separate tanks and perform later analyses on age and size-matched animals. In the 14 dpf fin clips, we recover mutant animals at approximately the expected 25% frequency. The Methods is now updated to reflect the extra husbandry considerations for these mutants. Line 372-378: "*nkx3.2* mutants inflate their swim bladders and are recovered at expected rates for analyses at 7-21dpf (39/161: 24.2% mutant). When raised with wild types, mutant adults are recovered at lower rates (6/90: 6.6% mutant) perhaps due to food competition with phenotypically normal clutch-mates. To raise sufficient numbers of mutants to adulthood for experimental purposes, genotyping was performed at 14 dpf. Mutants and wild types were then raised at similar densities in different tanks and size matched for downstream analyses at the indicated ages with no survival defects noted."

2.5. Can PCNA and/or BrdU results in Figure 3C-H somehow be quantified? Doing so, as well as some kind of statistical test if possible, would increase confidence in the claims of expanded cell proliferation across the jaw joint in the mutant.
Response:
In new Fig. 3I, we have now added quantification of PCNA staining in mutants versus controls. We used *nkx3.2* expression in adjacent sections to determine the fused joint region in mutants (e.g. Fig. 3G,H PCNA are adjacent sections to Fig. 6I,J *nkx3.2/sesn1* expression). From this, we found a statistically significant six-fold increase in chondrocyte proliferation in mutant jaw joint regions (see also response to REV 1.15).

2.6. The methods and results should both state more clearly which mutant allele was used for the scRNA- seq.
Response:
We now state in both the methods and results that the *nkx3.2*^{el802} allele was analyzed.

2.7. Do the fish mutants have spleens?
Response:
We attempted to identify the spleen in a few adult wild types and mutants but were unsuccessful in capturing suitable images of the spleen even in controls. As our manuscript is focused on skeletal biology in the mutants, we have chosen not to further analyze potential spleen defects here.

2.8. Line 29, wording makes it ambiguous whether rare mutations are GOF or LOF in humans, it might be clearer to add "Homozygosity for rare inactivating mutations … "
Response:
Thank you for the suggestion. The Line 29 wording has been modified as suggested (Line 30 in revised manuscript).

2.9. Lines 421-422 How were "Fin and gill chondrocytes" identified and removed?
Response:
In new Figure S3, we now show the marker genes that allowed us to identify and remove gill and fin cells from the single cell sequencing analysis. Methods (Lines 465-469): "In order to focus the analysis on the craniofacial chondrocytes affected in *nkx3.2* mutants, cells of the gill were identified based on the expression of *ucmaa* and *ppp1r1c* (to be described elsewhere) and fin cells based on expression of posterior *hox* genes (*hox9-13*) (Figure S3) (Ahn and Ho, 2008). We then excluded gill and fin cells from downstream analysis using the subset function."

2.10. The font sizes in Figures 5 and 6 (especially in model in Figure 6) are so small they are very hard to read.
Response:
We have increased the font sizes and reorganized these figures to make them easier to read.

REVIEWER #3:

3.1 Lines 147-149, Fig 2. Details in the µCT images of the skeletal defects in nkx3.2 mutants are hard to see. Please provide higher-resolution insets, particularly for the vertebral fusions. Please also indicate the number of animals that have the os-pts fusions and spinal defects described.
Response:
We now provide higher resolution µCT images, including insets, in revised Fig. 2. In the main text, we indicate that 6/6 mutants have os-pts fusions and 11/11 have spinal defects.

3.2 Lines 160-164, Fig 3C-D: Include quantitation for PCNA expression. At minimum the number of animals examined that display the general pattern of PCNA expression should be included.
Response:
In new Fig. 3I, we now quantify PCNA staining in mutants versus controls (n=3 each genotype as shown in figure). We used *nkx3.2* expression by RNAscope analysis in adjacent sections to determine the fused joint region in mutants (e.g. Fig. 3G,H PCNA are adjacent sections to Fig. 6I,J *nkx3.2*/*sesn1* expression). From this, we found a statistically significant six-fold increase in chondrocyte proliferation in mutant jaw joint regions (see also response to REV 1.15). We have updated the Methods (Lines 479-481) to reflect the serial sections to be compared: "The regions selected for proliferation analysis were identified by wild- type joint morphology and/or by performing RNAscope staining for *nkx3.2* in adjacent 5 µm sections (e.g. Figure 3G/H PCNA staining versus Figure 6I/J *nkx3.2* expression)."

3.3 Lines 165-166, Fig 3D: Provide evidence for continuous PCNA+ cells spanning the lower and upper jaw. For the joint region between Meckels and the adjacent cartilage there are few or no PCNA+ cells. Patterns of PCNA expression seem identical at the jaw joint and in Meckels between WT and mutant (Fig. 3C,D). Line 171: Provide quantifications for the "increased numbers" of PCNA+ cells described in the text and definitive statements about the numbers of labelled cells in WT versus mutant.
Response:
Based on *nkx3.2* expression in adjacent sections (e.g. Figure 3G/6I, 3H/6J), we quantified PCNA+ cells at the fused jaw joint region, not throughout Meckels cartilage. As described above, quantification of mutants versus controls revealed a six-fold increase in proliferative chondrocytes (PCNA+ cells per total DAPI+ cells in the *nkx3.2*+ region).

3.4 Lines 168-169, Figure 3E-F: Quantify the number of BrdU cells at the jaw joint in WT and presumptive jaw joint regions in mutants. If chondrocytes hyper-proliferate at the jaw joint in nkx3.2 mutants causing cartilage overgrowth, the number of BrdU+ cells in WT and mutant jaw joints should be striking. How much of the region displayed in panel F will give rise to what is observed in panel B'? I am wondering because it appears that there is just a single BrdU+ cell at the presumptive jaw joint in the nkx3.2 mutant (panel F) while most BrdU+ cells appear to be in regions that are largely Meckels and adjacent cartilages.
Response:
As described above, we chose to quantify proliferative cells using PCNA, and not BrdU, as it captures more chondrocytes in the cell cycle. Expression of *nkx3.2* in adjacent sections (e.g. Figure 3G/6I, 3H/6J) allowed us to only quantify PCNA+ cells in the presumptive jaw joint region.

3.5 Lines 197-199: The authors state that the absence of scoliosis in the 2 surviving nkx3.2 MO fish is evidence of a post-embryonic role for NKX3.2 in spine development by comparison to their nkx3.2 mutants. However, N=2 and perhaps given the high lethality in MO-injected fish (only 2/105 surviving) they may have developed scoliosis but died before 3 months of age for any number of reasons. Did the authors observe any spine defects in the other 103 MO-injected fish prior to their deaths (at late embryonic, larval, or other stages)? Additionally, because MO may be titrated-out by 2 dpf, it is still possible that nkx3.2 may play a role in ensuring proper spine development after 2 days, but still "embryonically." When do the authors first observe spine and/or tail defects in their genetic nkx3.2 mutants?
Response:
We have repeated the MO injections to look for earlier spinal defects following MO injections. This resulted in the generation of 2 additional surviving 3 mpf morphant animals, both lacking spinal defects. Now with n=4 adult morphants with clear jaw fusion and lacking spinal defects, we are

confident in the absence of scoliosis following morpholino injection, especially given that 11/11 genetic mutants developed scoliosis. We also did not observe any spinal defects in the non-surviving morpholino-injected fish, which failed to inflate their swim bladders and died by ~7dpf (Line 198). We first observe abnormal spinal curvature in genetic mutants at 1mpf (new Figure S1).

3.6 Figure 4, lines 616-617. Please provide higher magnification images of panels H and I, preferably at cellular resolution. In both panels, there appears to be alizarin staining between the Meckel's cartilage and the palatoquadrate, though this region is much reduced in the mutant compared to the MO-injected example. Also, while there is more alcian blue staining at this region in the mutant, there seems to be some light blue alcian staining in the region indicated by the arrowhead in panel I as well. Can the authors be sure that there is no delay in ossification of the palatoquadrate in mutants compared with knockdowns resulting in the observed phenotype?
Response:
While we cannot entirely rule out delayed ossification in mutant animals, the mutant and morphant animals were size and age-matched for this analysis. We have also now examined 2 additional morphant animals and continue to see the same differences in the alcian/alizarin staining compared to mutants.

3.7 Lines 238-239: The authors state that "In mutants, col10a1a was not expressed in the missing jaw joint region, consistent with lack of col10a1a reads in the mutant chondrocyte cluster in the single-cell data (Figure 6C)". However, it is impossible to correlate position (jaw joint region) with mutant chondrocyte cluster 2 from the single-cell data. Cells from this cluster could be derived from any col10a1a-low chondrocytes from multiple cartilages in the head.
Response:
We agree and have deleted the phrase "consistent with lack of col10a1a reads in the mutant chondrocyte cluster…" and now simply refer to the obvious lack of *col10a1a* by in situ in the fused jaw joint region (Figure 6D).

3.8 Line 242: The authors state that ptgdsb.1 expression is excluded from the jaw joint in WT, but in Figure 6F', there clearly are ptgdsb.1(+) cells at the jaw joint. Also, the authors state that ptgdsb.1 is expressed at "...lower levels in Meckels cartilage of the lower jaw..." but this region (Figure 6D) appears to overlap with the expression domain of nkx3.2 (Figure 6A), which marks the jaw joint. What delineates the "jaw joint" from the "lower levels in Meckels cartilage" here? There appear to be some cells with both ptgdsb.1 and nkx3.2 spots in panel F.
Response:
We agree that there are some cells with both ptgdsb.1 and nkx3.2 spots and have revised the text to more accurately describe ptgdsb.1 expression. Lines 257-260: "In wild types, *ptgdsb.1* was expressed in the late hypertrophic zone in the upper jaw palatoquadrate and ceratohyal cartilages, in the proliferative zone of the ceratohyal growth plate, at modest levels in the lower jaw Meckel's cartilage, and at lower levels in jaw joint articular cartilage, particularly on the upper jaw side of the joint (Figure 6E, G, K, M)."

We also quantify jaw joint region *ptgdsb.1* expression in mutants versus controls in new Figure 6Q, which now supports a clear upregulation of *ptdgsb.1* in the fused jaw joint region of mutants.

3.9 Line 243: The authors state that sesn1 is excluded from the jaw joint in WT, but it looks like there is some sesn1 expression in cells that have nkx3.2+ spots (Figure H, H'). Please confirm or deny using the color picker tool of an image editing program or some other image analysis method. The authors should clarify how they classify cells as being in the "jaw joint region" or not, as well as how they define the presence or absence of expression in this and the case above. Is there a way to quantify the level of expression in jaw joint cells? If so, it would probably be more accurate to say that ptgdsb.1 and sesn1 expression is increased at the jaw joint in mutant versus WT.
Response:
We now more accurately describe *sesn1* expression at the wild-type jaw joint starting on Line 261: "Whereas *sesn1* was expressed on either side of the wild-type jaw joint, it was expressed at much lower levels at the articular surface, with only rare *sesn1*+/*nkx3.2*+ double-positive cells detected; in the ceratohyal growth plate *sesn1* was expressed in all regions except the proliferative zone (Figure 6I, O)." We have also performed quantification of the fluorescence intensity for ptgdsb.1 and sesn1 in double stains with nkx3.2 (to select the jaw joint region as ROI in FIJI). This quantification demonstrates upregulation of both these transcripts at the jaw joint (Figure 6Q).

3.10 Lines 278-280: Similarly, clarify what is meant by "...generally excluded from the jaw joint, although there does appear to be weak co-expression of ptgdsb.1 and nkx3.2 on the lower jaw side..." As discussed above, where there appears to be co-expression when the authors claim there is none, they claim a "general" exclusion but then follow-up with an invalidating statement that there is some co- expression. Please provide further analysis and clarification in the results portion of the text to reflect these discrepancies.
Response:
We apologize for this confusion and have modified the Results to better reflect *ptgdsb.1* and *sesn1* expression at the joint (see 3.8 and 3.9 above). In the Discussion, we now provide a more accurate description of gene expression at the wild-type jaw joint: "In wild-type fish, *col10a1a* expression is generally excluded from the jaw joint, although there does appear to be co-expression of *nkx3.2* in some cells expressing low levels of *ptgdsb.1* and *sesn1* at the joint.".

3.11 Lines 291-292: The authors frame the difference in expression levels of nkx3.2 in the jaw versus the growth plate as possibly "reflect[ing] slower growth of the subarticular zone relative to the expansive growth of the endochondral cartilage template" but provide no references for this distinction. Their data (Figure 3C,D) also do not support this statement as no quantitation of number of proliferative cells at the jaw joint versus ceratohyal growth plate in WT was performed. There appear to be similar numbers of PCNA(+) cells in the Meckels cartilage part of the jaw joint and the ceratohyal in WT.
Response:
By quantifying PCNA+ cells in a new Fig. 3I, we now show that the level of proliferation is four-fold higher in the wild-type growth plate than the jaw joint. This quantification supports our statement that proliferative growth is likely much higher in the growth plate than the joint subarticular zone. Further, this quantification now allows us to reveal that proliferation in the mutant jaw joint region increases to levels seen in the
wild-type ceratohyal growth plate, supporting our statement that Nkx3.2 functions in part to limit proliferation at the jaw joint region.

3.12 The resolution of the μCT images in Figure 2, in particular panels A', D, E', E, E' is quite low. It is hard to appreciate what is being displayed here.
Response:
We have included higher resolution images in Figure 2, along with additional magnified images of 3 select regions with abnormal phenotypes.

3.13 The authors should update the Miyashita et al. 2019 BioRx citations to reflect the accepted manuscript status at the Journal of Experimental Biology.
Response:
The reference has been updated with the published citation.

3.14 Figure 1: The authors should consider labeling some major cartilage elements and bones in panels B-E in addition to areas of interest such as the jaw joint and presumptive jaw joint region referred to later in the text (Lines 156-157).
Response:
We have now included additional labels for major cartilage elements and bones in panels B-E.

3.15 Lines 596-597: The figure legend describes arrows to indicate spinal abnormalities, but there are no arrows in any of the panels (A-D). Similarity, the legend describes arrows for panels (E-F), but there are none.
Response:
We have substantially revised Figure 2 and modified the text and legends accordingly.

3.16 Figure 2: How consistent are the tail defect morphologies in nkx3.2 mutants? While the images in Figure 2F, F' closely resemble their counterparts in E, E', the E and F image sets were acquired from different fish (E is ua5011 and F is el802). Were the F examples chosen because they were most similar to E or does this morphology reflect the vast majority of tail defects in nkx3.2 mutants?
Response:
Severe spinal curvature in the tail region was seen in 11/11 ua5011 mutants (Fig. 2) and 5/6 el802

mutants. We have added these numbers to the main text and updated Figure 4 with a representative image of tail spine defects. Line 207-208: "*nkx3.2*^{el802} mutant animals all displayed spinal defects with 5/6 animals showing severe caudal tail curvature defects (Figure 4K)."

3.17 Figure 2 legend: Please specify that panels with the prime symbol indicate dorsal views. Consider changing "60 dpf" in Figure 1 to "2 mpf" to be consistent with the labelling in Figure 2 or change Figure 2 labels to "30 dpf" and "60 dpf."
Response:
Figure 2 was completely revised and includes a new higher resolution analysis of 3 regions in the spine exclusively at 60 dpf. As suggested, we have changed this figure legend from 2 mpf to 60 dpf. We have indicated in the figure legend that the prime symbol indicates dorsal views in A' and F'.

3.18 Figure 3: The authors should label some of the major bones/skeletal elements in all panels. This would be particularly helpful when visually comparing panels A and B considering the altered morphologies of most jaw bones in nkx3.2 mutants. They should also consider adding labels for "lower jaw," "upper jaw," and/or Meckel's cartilage, palatoquadrate to panels C and E as the authors refer to all in the text and/or legend but these are difficult to follow for a reader not familiar with zebrafish anatomy.
Response:
Labels have been added for clarity in Figure 3, panels A-J and legend. LJ: lower jaw; UJ: upper jaw; M: Meckel's cartilage; PQ: palatoquadrate; CH: ceratohyal; JJ: jaw joint.

3.19 Figure 3 legend: The authors should indicate in the legend that panels A-F are ventral views. They should also describe in the legend the nuclear stain in panels C-F and include a colored label for it as for "PCNA" and "BrDU" in panels C and E.
Response:
The legends have been revised as suggested and labels for the Hoescht nuclear stain have been added.

3.20 Figure 3: Panel B' does not appear to be the magnified boxed-region in B. Has it been further dissected to produce the image in panel B'? Is the orientation different? Is the joint obscured in panel B? If so, additional labeling of bones in panels A and B would help to clarify.
Response:
The legends have been revised to indicate that the inset image is a further dissection of B. The joint is obscured in panel B due to the misorientation of the lower jaw towards the viewer, which motivated us to dissect out this region and re-image as now shown in the insets. We have now added labels to panels A and B to clarify the relative positioning of the lower and upper jaws. UJ: upper jaw; LJ: lower jaw.

3.21 Figure 3: "BrDU" in the legend and panel E should be changed to "BrdU".
Response:
Corrected.

3.22 Figure 4: The authors should label major bones and structures in all panels, in particular the structures described in the text (Lines 190-192).
Response:
Labels have been added for clarity in Figures 1, 3, 4 and 6. Where appropriate, these include: LJ: lower jaw; UJ: upper jaw; M: Meckel's cartilage; PQ: palatoquadrate; CH: ceratohyal; JJ: jaw joint; RA: retroarticular.

3.23 Lines 193-194: The authors state "...indicative of the cartilage outgrowth seen in medial views (Figure 3B)." However, Figure 3B is a low magnification ventral view. Furthermore, it is difficult to see the similarities between the two figure panels compared in the text (Figure 3B or B' and Figure 4H). ***Response:***
We agree that these views may be difficult to reconcile for the reader. We have therefore revised the figure legend to reflect that Fig 3B is a ventral view and removed the statement comparing these views. The cartilage overgrowth is now highlighted in both dissected ventral views and new H&E-stained sections through the fused jaw joint (Figure 3B,D). Line 203-205 now reads "In

mutants, a continuous zone of cartilage fuses Meckel's cartilage to the palatoquadrate cartilage growth plate (Figure 4H).

3.24 Line 187 & Line 614. The manuscript describes the 2 nkx3.2 MO fish as surviving until adulthood at 3 months of age (Line 187). However, the Alcian-Alizarin dissections in Figure 4 performed on these fish were conducted on 2 month old fish, as described in the figure legend (60 dpf).
Response:
We thank the reviewer for pointing out this error. We have now raised two additional MO animals to 3mpf to complement the original two 2mpf MO animals. We now describe in the text that all 4 MO animals lack cartilage overgrowth and spine defects despite lacking a jaw joint and having a locked-open mouth.

3.25 Figure 6G, I: There appears to be significantly more nkx3.2 RNA expression in nkx3.2 mutants than in WT. While mRNA degradation is not a feature of all mutants, it seems odd here. Do most/all of the cartilage cells in the mutant overgrowth phenotype have a joint-like identity?
Response:
In contrast to the increased expression of *sesn1* and *ptgdsb.1* we do not see a consistent increase in *nkx3.2* expression across all mutants. Most of the chondrocytes at overgrowth site have an abnormal joint-like identity characterized by co-expression of *nkx3.2* with high *ptgdsb.1* and *sesn1*. We have revised the text to reflect this shared co-expression. Line 264: "In *nkx3.2* mutants, *ptgdsb.1 and sesn1* were upregulated across most chondrocytes within the fused jaw joint region, which we identified by co- expression of *nkx3.2* (Figure 6E-J)."

3.26 Figure 6: The authors should label Meckel's cartilage and palatoquadrate.
Response:
We have now added labels for M and PQ in Figure 6.

3.27 Figure 6: Provide higher quality images for Panels H-I' and N, O, L.
Response:
We have now repeated the RNAscope experiments and provide higher quality images.

3.28 Figure 6: Increase the font sizes in panel P? Also, the entire model could be enlarged to fill the white space in panel P.
Response:
We have increased the font sizes and enlarged the model as suggested, in addition to diagramming zones in the ceratohyal growth plate in a new Fig. 6S.

3.29 Line 284: Quantify "increased proliferation" in mutant versus WT.
Response:
The proliferation is now quantified in Figure 3I.

3.30 Line 285: Is sesn1 truly "excluded" from the PZ or dramatically reduced compared to the HZ? Or "essentially excluded?"
Response:
We have repeated the *sesn1* in situ for the ceratohyal and confirm that it is nearly absent from the PZ (Figure 6O). We have revised the text to reflect the very low level of sesn1 seen in the proliferative zone, particularly as compared to the high levels of expression in the rest of the ceratohyal. Line 263: "in the ceratohyal growth plate *sesn1* was expressed at high levels in all regions except the proliferative zone (Figure 6I, O)." and Line 322-324: "In the bidirectional growth plates of zebrafish ceratohyal (Figure 6S), we observe a central zone of *nkx3.2*-low, *sesn1*-low, *ptgdsb.1*-positive chondrocytes that correlate with the zone of proliferation (Figure 3C,E; (Giovannone et al., 2019)."

3.31 Lines 365-366: Include primer sequences used to make the ptgdsb.1 probe template.
Response:
Primer sequences are now included.

3.32 Line 374: Include manufacturer, catalog # for cytoseal?
Response:
These details are now provided in the Methods.

3.33 Line 401: Why were pectoral fin skeletons dissected and used in the scRNA-seq? The main body of the text described heads only (Line 205).
Response:
We have now included details in the Methods as well as Figure S3 that outline the gene expression profiles used to identify fin and gill cells in the single cell sequencing dataset. We then removed these cells from the analysis to better focus on the craniofacial cartilages affected in mutants.

3.34 Line 126: "prevent" might be a better word to use here, as "block" might have a connotation similar to "translation blocking" usually reserved for steric blocking of translation as for many morpholinos. Also it would be more accurate to say "mRNA" or "RNA" here in place of "protein" as translation of the RNA into protein.
Response:
We have revised to: "prevent translation of *nkx3.2* mRNA" (Line 128).

3.35 Usage of the term "Adult zebrafish": Typically, zebrafish are considered to be "adults" at around 2.5- 3 months of age or when fertile. Were the 2-month fish described as "adults" in the manuscript fertile? Later in the manuscript (Line 187), the authors describe nkx3.2 MO-injected fish saying "only two survived until adulthood (3 months of age)." By their previous statements of 2 month old fish as "adults," potentially more than 2 nkx3.2 MO fish may have survived to 2 months of age.
Response:
As described earlier, we have now raised an additional 2 MO animals to 3 mpf, to complement our previously analyzed MO animals at 2 mpf. We did not check the fertility of these MO animals as our priority was to sacrifice these precious animals for facial skeletal analysis. However, we do note that our nkx3.2 mutant animals at similar stages were fertile despite loss of the jaw joint.

Second decision letter

MS ID#: DEVELOP/2020/193409

MS TITLE: Zebrafish Model for Spondylo-Megaepiphyseal-Metaphyseal Dysplasia Reveals Post-Embryonic Nkx3.2 Requirements in the Skeleton

AUTHORS: Joanna Smeeton, Natasha Natarajan, Arati Naveen Kumar, Tetsuto Miyashita, Pranidhi Baddam, Peter Fabian, Daniel Graf, and 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 suitability for publication in Development with two supporting publication whereas the third considers that you have not presented sufficient insight into how Nkx3.2 functions to justify publication in Development. While I don't expect you to be able to fully address this concern, I would like to give you an opportunity to respond to the points raised and consider whether there is anything else you would like to add to further strengthen your study. Please attend to all of the reviewers' 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.

Reviewer 1

Advance summary and potential significance to field

In the manuscript, the authors report that rare surviving Nkx3.2 morphants do not show ectopic cartilage formation in the region of the missing jaw. On this basis, they conclude that Nkx3.2 has

an additional postembryonic requirement restricting proliferation and dampening stress response in the joint adjacent cartilage. The latter is based on their findings from the single-cell analysis.

Comments for the author

I am completely satisfied by the revisions made by the authors and have no further comments.

Reviewer 2

Advance summary and potential significance to field

Smeeton and colleagues investigate the roles of nkx3.2 during skeletal development in zebrafish. The authors report two genetic alleles of nkx3.2 which reveal new roles for nkx3.2 in regulating post-embryonic skeletal development. nkx3.2 mutants display jaw joint loss in larvae, phenocopying the previously reported morphant phenotype, as well as ectopic cartilage outgrowths around their jaw joints, scoliosis, and skull defects. In the jaw joint, ectopic proliferation was observed across the jaw joint region. Using single-cell RNA-sequencing, the authors discover stress pathways are upregulated in mutant chondrocytes, including components of the prostaglandin and mTORC1 pathways. All three of the post-embryonic mutant skeletal phenotypes resemble phenotypes seen in human SMMD patients, suggesting this mutant could be a new model for this human disease.

Comments for the author

Smeeton and colleagues have thoroughly addressed all of the reviewer comments. The improved microCT data in Figure 2, the improved H&E histological sections and proliferation quantification in Figure 3, and the numerous text additions have significantly improved this manuscript which should be of great interest to readers of Development.

Reviewer 3

Advance summary and potential significance to field

The authors present a new zebrafish genetic model for SMMD. They attempt to distinguish between embryonic and post-embryonic roles of Nkx3.2, contrasting the previously known role in specifying the jaw joint in zebrafish, which they verify here with genetic mutants, with newly described cartilage overgrowth and spinal defects presented in the current study. These observations are complemented by a single-cell RNAseq analysis of mutant cranial chondrocytes showing an apparent increase in mitotic (correlative evidence for hyperproliferation) and stress response genes.

Comments for the author

The authors have improved the manuscript by addressing most of the comments with regards to data quality and quantitation. However, some major concerns remain:

1) Many aspects of the mutant phenotype are consistent with mouse models or observations of SMMD patients and therefore do not really further our understanding of nkx3.2 function or SMMD. Since the role of nkx3.2 in specifying the jaw joint is not conserved in mammals it is unclear how these findings relate to other joints, mouse models of SMMD or SMMD patients

2) The manuscript is largely descriptive and while some observations are interesting (e.g. cartilage overgrowth, stress-response gene upregulation in mutants), the authors do not propose any hypotheses to explain how Nkx3.2 regulates proliferation and there are no experiments to test this role or the potential role of stress-response genes in the joint.

Other comments/corrections:
Fig 4. There is still Alizarin-red bone that separates Mc from pq in mutants. This does not look like a “continuous zone” as described in the text (lines 205-206);
Related to the point discussed in 3.6 of the authors response to reviewer comments in reference to cartilage across the Mc and pq of mutants in contrast to MO-

injected.

Line 299: “zebrafish ceratohyal (Fig. 6S),” There is no figure 6S.

Second revision

Author response to reviewers' comments

1 These observations are complemented by a single-cell RNAseq analysis of mutant cranial chondrocytes showing an apparent increase in mitotic (correlative evidence for hyperproliferation) and stress response genes.

Response: A strength of our study is that we show not just correlation but also direct evidence for hyperproliferation, including BrdU (Fig. 3E,F) and PCNA (Fig. 3-J) staining. Quantitation of PCNA in Fig. 3K shows a ~500% increase in proliferation at the mutant jaw joint region (p=0.009) and a ~33% increase in proliferation at the mutant growth plate (p=0.07). This strengthens our finding of greatly increased mitotic cells in the mutant scRNAseq analysis.

2 The authors have improved the manuscript by addressing most of the comments with regards to data quality and quantitation.

Response: We are glad the revision has satisfactorily addressed the original comments regarding the quality and quantitation of data. We have therefore focused on better highlighting the novelty and impact of our study in this second revision.

3 Many aspects of the mutant phenotype are consistent with mouse models or observations of SMMD patients and therefore do not really further our understanding of nkx3.2 function or SMMD. Since the role of nkx3.2 in specifying the jaw joint is not conserved in mammals it is unclear how these findings relate to other joints, mouse models of SMMD or SMMD patients.

Response: While it is true that the scoliosis and neurocranium defects are similar between mouse and our new zebrafish mutants, our report is the first to model the skeletal overgrowth phenotypes of SMMD patients. We have therefore substantially revised the manuscript throughout to better highlight what we have learned from zebrafish about possible causes for skeletal overgrowth in SMMD patients. In particular, we show that skeletal overgrowth is associated with increased cartilage proliferation and upregulation of genes of the mTORC1 and stress-response pathways. While we acknowledge that the overgrowth being studied is in the jaw joint and not the limbs (which fish lack), quantitation of PCNA in the growth plates in Fig. 3K also reveals a trend toward increased chondrocyte proliferation (~33% increase, p=0.07), suggesting that Nkx3.2 may also restrict chondrocyte proliferation within the endochondral types of bones affected in the limbs of SMMD patients.

e.g. Abstract: “Whereas *nkx3.2* knockdown zebrafish and mouse *Nkx3.2* mutants display embryonic lethal jaw joint fusions and skeletal reductions, respectively, they lack the skeletal overgrowth seen in SMMD patients. Here we report adult viable *nkx3.2* zebrafish mutants displaying cartilage overgrowth in place of a missing jaw joint, as well as severe dysmorphologies of the facial skeleton, skullcap, and spine.”

4 The manuscript is largely descriptive and while some observations are interesting (e.g. cartilage overgrowth, stress-response gene upregulation in mutants), the authors do not propose any hypotheses to explain how Nkx3.2 regulates proliferation and there are no experiments to test this role or the potential role of stress-response genes in the joint.

Response: We would like to point out several novel insights from our study that the reviewer may have overlooked and that we feel will appeal to a broad readership.

a. We provide the first single-cell atlas of skeletogenic cells in zebrafish. In Fig. 5, we describe 11 cell types that include multiple types of chondrocytes, osteoblasts, perichondral progenitors, and mesenchyme. This should be a useful resource for many others working in the zebrafish skeletal system.

b. Previous studies have shown a role for zebrafish Nkx3.2 only in the jaw joint, making it unclear what relation Nkx3.2 function in fish had to mammals. By showing expression in growth plates of fish endochondral bones, and common mutant phenotypes in the spine and cranial bones, we establish the first shared requirements of Nkx3.2 between fish and mammals.

c. We show an unexpected expression of prostaglandin synthases in the proliferative zone of the wild-type growth plate, suggesting new roles for these pathways in regulating chondrocyte proliferation.

d. We have generated the first animal model displaying the type of skeletal overgrowth seen in the wrists and digits of SMMD patients, and we then go on to link this to abnormal chondrocyte proliferation and stress-response gene expression. This role of Nkx3.2 in restricting chondrocyte proliferation markedly contrasts with previous studies in chick and mouse claiming that Nkx3.2 functions mainly to inhibit hypertrophic maturation of chondrocytes.

We do agree that there are many exciting future directions toward understanding how Nkx3.2 might regulate proliferation (e.g. direct binding to enhancers of cell cycle genes) and the potential role of stress-response genes (e.g. phenotypic analysis of new mutants). However, we feel these are well beyond the scope of this study. Nkx3.2 genomic occupancy would require creation of high-quality zebrafish anti-Nkx3.2 antibodies, and generation and characterization of new stress-response pathway mutants would require substantial time and have uncertain feasibility due to genetic redundancy and pleiotropy. We do acknowledge these unanswered questions in the Discussion:

e.g. “The functions of prostaglandins in regulating chondrocyte biology, whether they are linked to proliferation, and why zebrafish would have D2 and mouse E2 subtypes remain outstanding questions.”

e.g. “In the future, it will be informative to determine how prostaglandins and other stress-induced pathways interact with mTORC1 signaling to regulate chondrocyte proliferation, and how Nkx3.2-mediated regulation of these pathways differentially fine-tunes stem cell- mediated chondrocyte expansion in joints versus growth plates.”

5 Fig 4. There is still Alizarin-red bone that separates Mc from pq in mutants. This does not look like a “continuous zone” as described in the text (lines 205-206); Related to the point discussed in 3.6 of the authors response to reviewer comments in reference to cartilage across the Mc and pq of mutants in contrast to MO-injected.

Response: We now also refer to the histology in Fig. 3D that clearly shows a fusion of lower jaw cartilage to the pq cartilage growth plate in mutants.

6 Line 299: “zebrafish ceratohyal (Fig. 6S),” There is no figure 6S.

Response: Fig. 6S is the diagram of the Ch growth plate that the reviewer may have missed.

Third decision letter

MS ID#: DEVELOP/2020/193409

MS TITLE: Zebrafish Model for Spondylo-Megaepiphyseal-Metaphyseal Dysplasia Reveals Post-Embryonic Roles of Nkx3.2 in the Skeleton

AUTHORS: Joanna Smeeton, Natasha Natarajan, Arati Naveen Kumar, Tetsuto Miyashita, Pranidhi Baddam, Peter Fabian, Daniel Graf, and 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.