Disruption of the nectin-afadin complex recapitulates features of the human cleft lip/palate syndrome CLPED1

Kendall J. Lough, Danielle C. Spitzer, Abby J. Bergman, Jessica J. Wu, Kevin M. Byrd and Scott E. Williams
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MS TITLE: Disruption of the nectin-afadin complex recapitulates features of the human cleft lip/palate syndrome CLPED1

AUTHORS: Kendall J. Lough, Danielle C. Spitzer, Abby J. Bergman, Jessica J. Wu, Kevin M. Byrd, and Scott E. Williams

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 a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript.

I note that between the reviewers several new experiments are proposed, but I am not of the opinion that all of them are essential. Rather, I recommend you focus first on the technical concerns (i.e. controls, etc.), which I do consider essential. Then, carefully consider the reviewers' other proposed additional experiments, using your own judgement on how to move forward.

Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

This manuscript from Lough et al., explores the function of junctional adhesion proteins afadin, Nectin-1, and Nectin-4, in the development of the mouse secondary palate. The authors apply a cutting-edge LUGGIGE technique for rapid gene perturbation in the palatal epithelium to study the in vivo consequences of loss, or disease-causing mutations in these genes. This method is powerful, and is extremely useful for the study of oral epithelial development. In this study, the authors demonstrate that the afadin/Nectin cell adhesion molecules play important roles in epithelial cell adhesion during secondary palate morphogenesis, and that their loss results in aberrant cell adhesion that results in a failure of secondary palate elevation and closure. This is a beautiful methodological demonstration and makes the important discovery of the importance of the afadin/Nectin cell adhesion complex in mouse palate epithelial development, supporting the long-known human genetic role of the Pvrl1/Nectin-1 gene in human orofacial clefting. This will be of interest to the craniofacial community, and to human geneticists who study orofacial clefting, as well as specialists in the area of these adhesion molecules. The analysis stops slightly short of mechanistic understanding, however, and additional experiments further characterizing the basis for the epithelial adhesion phenotype would greatly contribute to this manuscript.

Comments for the author

Major concerns:
• What are the mechanisms by which Afadin/Nectin are working in controlling palate epithelial development? The authors demonstrate that ectopic cell adhesions are a common feature of their loss of function models. Do these adhesions correlate with aberrant differentiation, or loss of periderm cells (K6+ cells), changes in apoptosis or proliferation? Are other genes implicated in this specific phenotype such as Jag2/Notch, Irf6, Grhl3 altered at sites of adhesion, or is Afadin/Nectin likely developmentally downstream of these factors?

• The CP phenotype in Nectin14402; Nectin1W185X mutants is very impressive and dramatic. It appears that two layers of MES have been generated in these mutants, and that mesenchyme is also labeled with RFP (Fig 4D). Is it possible that the epithelium in these embryos has undergone a pathological EMT? Or is the mesenchyme indeed transduced? Presenting further histological and IF analysis of this phenotype, including cell death and cell proliferation, would also greatly improve understanding of the more precise basis of this dramatic phenotype.

• Have “WT” embryos in Fig. 2E, 3B been transduced with a scramble shRNA-mRFP? I cannot see RFP1 expression in the wt panel in 2D. This is the appropriate control to ensure that the LUGGIGE protocol does not cause clefting on its own.

• Are levels of expression of Nectin1W185X similar to endogenous levels of Nectin-1 expression?

• As the Nectin14402; Nectin1W185X the embryos exhibit higher penetrance than Nectin-1; Nectin-4 compound knockdown, can the authors speculate/further explain why they consider this a dominant negative effect rather than dominantly acting mutation?(i.e. gain of function). What is the phenotype of Nectin1W185X embryos without Nectin-1 knockdown? Does the presence of wt Nectin-1 exacerbate, or reduce the frequency and severity of the Nectin1W185X phenotype?

Minor concerns
• Does the LUGGIGE method transduce the basal epithelium, or only the periderm? Given that transduction occurs early, before periderm differentiation, I would expect all embryos exhibiting peridermal label to also have basal epithelium labeled, reflecting their lineage relationship.

• From IF in Figure 2, it does not appear that Nectin-1 specifically localizes to the periderm-basal epithelial junction, and indeed many periderm-basal epithelial junctions show in Figure 2A lack apparent Nectin-1 localization, not just those that have been transduced with the shRNA-mRFP. It appears that Nectin-1 localization can be seen in the apical edge of periderm cells, but it is somewhat difficult to make out for certain which cells are basal in this image; including p63 IF in this analysis would greatly strengthen this point. It is also very difficult to see from Fig. 2B,C that

Nectin-4 is enriched in the periderm, though it is clear that periderm-delivery of shRNA has knocked it down.

Reviewer 2

Advance summary and potential significance to field

This paper investigates the roles of Afadin (Afdn) and Nectins in craniofacial development in mice. The authors use lentiviral ultrasound-guided (LUGGIGE) knockdowns of Afdn1 and Nectins 1 and 4 in oral epithelial cells to reveal requirements in palate development particularly cell adhesion required for palate fusion. Previous studies have implicated NECTIN1 in cleft lip and palate in humans, but mouse knockouts have been uninformative due to early lethality and/or a lack of suitable Cre drivers to disrupt function in early oral epithelia. The results here support the argument that the requirements for Afdn and Nectins are prior to the onset of Krt14-Cre expression, since earlier knockdown with LUGGIGE leads to clear clefting.
Mosaic knockdowns lead to loss of Nectin1 protein with basal cell transfection while Nectin4 protein is lost with periderm transfection, revealing potential cell autonomous requirements in different epithelial layers. Finally, the authors argue for a dominant negative mechanism with truncating Nectin1 mutations based on the higher penetrance, novel palate and limb phenotypes, and disrupted subcellular localization.

Comments for the author

While the data are novel and convincing, they are largely descriptive and do not delve much further into the mechanism of Afdn/Nectin function. The effectiveness and extent of mosaicism of the lentiviral knockdowns also need further documentation.

1) The authors should validate the efficiency of the LUGGIGE knockdowns. They mention in Methods that they have performed qPCR on transduced keratinocytes, but this is not included in the Results. These CT values should be reported.
They should also quantify the mosaicism of LUGGIGE knockdown, perhaps using a dose-dependent analysis reporting the percentage of cells expressing the reporter fluorophore at different lentiviral concentrations.

2) Krt14-Cre has been used to show requirements for other epithelial factors involved in CL/P including several involved in adhesion. The authors argue that this reflects an earlier role for Afdn-Nectins, prior to the onset of Krt14 expression. But are there other explanations? One useful additional experiment would be to confirm the results with Krt8-Cre or Krt18-Cre to remove Nectin4 in periderm.

3) The authors argue cell-type specific requirements for Nectin1 and Nectin4 in basal and peridermal cells, respectively. How does this relate to the expression patterns of these two Nectins? They should include in situ or immunohistochemical results supporting this argument, or refer to the evidence in the literature.

4) Nectin4 knockdown causes CP at very low penetrance 1/5. Is this significant?

5) Authors should refrain from the use of the word “mutant” when referring to their LUGGIGE method, since shRNA transduced embryos are knockdowns.

Reviewer 3

Advance summary and potential significance to field

This paper focuses on the nectin-afadin complex, which has previously been implicated in cleft lip/palate-ectodermal dysplasia syndrome 1 (CLPED1) or OFC7. While mutations in nectin genes are suggested to be causative, there has been no gene-function data in mouse models clearly linking

these genes to CLPED. This is largely due to early lethality of the afadin knockout (by E9.5 or so) and potential functional redundancy of the nectin genes, of which there are four.

In the first part, authors use lentiviral constructs in combination with genetic mutants to demonstrate that loss of afadin in the palatal epithelium leads to a failure in palatal shelf elevation, likely due to intraoral adhesions, consistent with a role in ectodermal dysplasia. They then use lentiviral shRNA constructs to demonstrate additive effects of knocking down multiple nectin genes, again, something that would be difficult to do (e.g. long-term and expensive) using classical genetic crosses. Finally, they express human nectin gene variants and show that these variants have pathological effects on palatal closure (as well as syndactyly, which is not seen in the LOF expts.)

All together, this paper is well-written and definitively demonstrates the crucial role of the afadin-nectin complex in palatogenesis.

Comments for the author

I am satisfied with the data and presentation & fully support publication of this MS.

First revision

Author response to reviewers' comments

In the rebuttal below, reviewer comments have been organized into three categories: 1) those addressed by the addition of new experiments/data, 2) those addressed by textual edits, and 3) those which require additional experiments that we cannot fully address. In accordance with *Development's* COVID-19 policy (https://dev.biologists.org/content/news - COVID19), our revision plan was discussed in advance with the editorial staff, including guest editor John Wallingford.

Comments which have been addressed through new experiments and/or data

Reviewer 1, Major comment #3: "Have "WT" embryos in Fig. 2E, 3B been transduced with a scramble shRNA-mRFP? I cannot see RFP1 expression in the WT panel in 2D. This is the appropriate control to ensure that the LUGGIGE protocol does not cause clefting on its own."

Response: We thank the reviewer for this critical observation. The WT embryos referenced in the previous draft were uninjected littermates, which we used because they represented ideal age-matched intra-experimental controls. However, we agree that using embryos injected with a control lentivirus is the best way to test whether the LUGGIGE procedure can induce clefting in and of itself. To address this comment, we collected a large cohort of existing samples transduced with a non-targeting scramble shRNA together with the same H2B-mRFP1 reporter used for the Nectin1/4 studies. This includes10 total embryos, ranging in age from E15.5-E18.5, from 5 distinct litters. These embryos were evaluated by stereoscopic imaging to validate infection (via RFP fluorescence) and the presence or absence of cleft palate (as in Fig. 1C, 2D, and 3B). Importantly, we observed normal palatal fusion in each of these controls. We have added an example stereoscopic image of one such Scramble H2B RFP E16.5 animal to Fig. S1 (Fig. S1B) and have added the aforementioned data regarding CP in these controls to the text. Furthermore, using the same approach, we have evaluated 4 additional embryos injected with a different control H2B-mRFP1 lentivirus which lacks the scramble shRNA and instead contains a short 18bp non-hairpin sequence downstream of the U6 promoter that normally drives shRNA expression.
Again, none of these controls displayed cleft palate.

Reviewer 1, Major comment #2: "The CP phenotype in Nectin14402; Nectin1W185X mutants is very impressive and dramatic. It appears that two layers of MES have been generated in these mutants, and that mesenchyme is also labeled with RFP (Fig 4D). Is it possible that the epithelium in these embryos has undergone a pathological EMT? Or is the mesenchyme indeed transduced?

Presenting further histological and IF analysis of this phenotype, including cell death and cell proliferation, would also greatly improve understanding of the more precise basis of this dramatic phenotype."

Editor comment: *I do not recommend adding data that has not been replicated. Since this experiment falls within the realm of "more stuff" rather than "controls" I think you should leave this out.*
I'd ask you to indicate in your response to reviewers upon re-submission that you have this result and at the "advice of the editor" felt it best to not to include it. This way, we don't run afoul of the reviewer!

Response: We thank the reviewer for their detailed observation. It is believed that during PS fusion, periderm cells make first contact between opposing palatal shelves, but quickly migrate in both dorsally and ventrally to form "triangles" of periderm cells at the nasal and oral cavities, which are eliminated by apoptosis (1, 2). The midline epithelial seam (MES) forms as epithelial cells from both PS converge, while its subsequent dissolution and fragmentation is driven at least in part by extrusion of epithelial cells towards the oral and nasal cavity, which may contribute to the formation of the epithelial "triangles" (2, 3). Periderm removal from the MES is necessary for proper palatal fusion, and apoptosis of periderm cells and both extruded and non-extruded populations of basal cells is thought to contributed to MES dissolution (1, 4, 5). While an epithelial-mesenchymal transition (EMT) was initially thought to occur in basal cells during MES dissolution (6-9), more recent studies using genetic lineage tracing have conclusively disputed this claim. Furthermore, other studies have argued that cell death plays a more prominent role in this process (4, 10, 11).With regards to the mechanism that causes the residual MES phenotype that we observe in some Nectin14402; H2B-mRFP1-T2A-NECTIN1W185X-V5 transduced embryos (as well as some Nectin14402; Nectin42589 embryos), we think this is unlikely to be caused by lentiviral transduction of mesenchymal cells because no other mesenchymal cells outside the MES region are labeled with H2B-RFP. In addition, our preliminary analyses of E16.5 embryos stained with E-cadherin and K14 suggests that many of these cells still have some epithelial characteristics (displaying low levels of Ecad/K14 labeling), arguing against pathological EMT as a cause for the persistent MES. Moreover, we observe that many of these cells are undergoing apoptosis (cCasp3+), as is normally believed to occur during MES dissolution. However, since the MES has already been completely lost in WT littermates at this age, it is difficult to make quantitative comparisons about the level of apoptosis observed in NECTIN1W185X embryos. Nonetheless, based on the data we have on hand, we favor a model whereby the persistent MES is cause by a failure of either extrusion or migration. We have updated Fig. 4D to highlight the E-cadherin staining of these epithelial islands. We have also added appropriate discussion of this concept and our speculative model to the text on page 10, paragraph 2. We have decided to not include additional data regarding apoptosis at the advice of the editor.

Reviewer 2, Major comment #1: The authors should validate the efficiency of the LUGGIGE knockdowns. They mention in Methods that they have performed qPCR on transduced keratinocytes, but this is not included in the Results. These CT values should be reported. They should also quantify the mosaicism of LUGGIGE knockdown, perhaps using a dose-dependent analysis reporting the percentage of cells expressing the reporter fluorophore at different lentiviral concentrations.

Editor comment: I think your response is totally reasonable, and do not think more experiments are required. I do think however that 1) there's no reason not to include the RT-PCR data and 2) the discussion here should be included in the paper to guide the thinking of any readers who have the same question as the reviewer.

Response: We appreciate the reviewer's consideration of our techniques. We have added the qPCR results to the text in the Results section (p. 7, bottom paragraph). These data were derived from the same cultured keratinocytes shown in Fig. S2A,B. While perhaps informative, qPCR is a less direct measurement of shRNA effectiveness than IF imaging in the specific tissue where the phenotype is observed, and our reference states as much.

Regarding the latter half of this comment: we appreciate the reviewer's comment regarding mosaicism of the LUGGIGE technique. We are somewhat confused, however, what the reviewer is specifically asking. Is the reviewer curious how varying degrees of transduction may effect

penetrance of CP? Or is the reviewer asking for clarity regarding the relationship between viral titer and transduction efficiency in the palatal epithelium? While we can analyze our in-hand samples to estimate transduction efficiency, it will be difficult to determine conclusively whether the transduction efficiency correlates with an increased likelihood of CP, particularly in groups where CP penetrance is extremely low (e.g. Nectin1 or Nectin4 single knockdowns). It should be noted that the LUGGIGE experiments were performed using parameters to maximize transduction efficiency (e.g., injecting between ~0.5-1.3 µl of virus into the amniotic space, using a virus with as high a titer as possible, typically >2x10$_9$ CFU/mL). Performing a "dose-dependent analysis reporting the percentage of cells expressing the reporter fluorophore at different lentiviral concentrations" would require a tremendous amount of work, and we are not sure how this would add to or alter any of our conclusions. We also would like to refer the Reviewer to our first publication on this technique, where the relationship between viral titer and in vivo epidermal transduction efficiency was directly demonstrated in Figure 2 (12). Moreover, we have also previously quantified LUGGIGE transduction efficiency of different regions of embryonic oral epithelia in Supplementary Figure 3, and demonstrate that it ranges between ~53-70% for E16.5 palatal epithelium, depending on the virus used (13). At the advice of the editor, this is now explicitly stated in the Results, p. 5: "This approach efficiently transduces surface epithelia of the embryo—including the epidermis and oral epithelia—achieving ~50-70% transduction of palatal epithelium in our hands…"

Comments which have been addressed by in-text alterations

Reviewer 1, Minor comment #1: "Does the LUGGIGE method transduce the basal epithelium, or only the periderm? Given that transduction occurs early, before periderm differentiation, I would expect all embryos exhibiting peridermal label to also have basal epithelium labeled, reflecting their lineage relationship."

Response: LUGGIGE transduces both periderm and basal cells of the palatal epithelia. While we believe our IF figure panels (specifically Fig. S1A,C) demonstrate this, we have stated this more explicitly in the text as well (page 5, final paragraph).

Reviewer 2, Major comment #4: "Nectin4 knockdown causes CP at very low penetrance 1/5. Is this significant?"

Response: Using the statistical comparisons described in the methods section, these results are not significant (Nectin4 2589, p = 0.5; Nectin4 2632, p = 0.3846). These comparisons are always made between the experimental (knockdown) embryos and ONLY their uninjected littermates (control n = 6 for 2589 and n=8 for 2632). If compared to all CD1 embryos in the study (n > 80) then the results do become statistically significant. We have added "n.s." labels to all genotypes that do not fit the significance criteria currently described in the methods section.

Reviewer 2, Major comment #5: "Authors should refrain from the use of the word "mutant" when referring to their LUGGIGE method, since shRNA transduced embryos are knockdowns."

Response: We thank the reviewer for noticing our oversight and have removed all inappropriate use of the term mutant.

Reviewer 1, Minor comment #2: From IF in Figure 2, it does not appear that Nectin-1 specifically localizes to the periderm-basal epithelial junction, and indeed many periderm-basal epithelial junctions show in Figure 2A lack apparent Nectin-1 localization, not just those that have been transduced with the shRNA-mRFP. It appears that Nectin-1 localization can be seen in the apical edge of periderm cells, but it is somewhat difficult to make out for certain which cells are basal in this image; including p63 IF in this analysis would greatly strengthen this point. It is also very difficult to see from Fig. 2B,C that Nectin-4 is enriched in the periderm, though it is clear that periderm-delivery of shRNA has knocked it down.

Reviewer 2, Major comment #3: "The authors argue cell-type specific requirements for Nectin1 and Nectin4 in basal and peridermal cells, respectively. How does this relate to the expression patterns of these two Nectins? They should include in situ or immunohistochemical results supporting this argument, or refer to the evidence in the literature."

Response: We believe these two reviewer comments are referring to the following portion of the text (Results, p. 9): "Previous studies have shown that nectin-1 and nectin-4 localize to the interface between palatal basal epithelium and the periderm (Richardson et al., 2017), but have not established which cell populations express each nectin. At E14.5, transduction of basal cells, but not periderm, with the Nectin14402 H2B-mRFP1 lentivirus was sufficient to ablate nectin-1 accumulation at this interface (Fig. 2A). In contrast, in Nectin42589 H2B-mRFP1-transduced palatal epithelium, nectin-4 protein loss was seen with periderm-specific infection (Fig. 2B,C). These data strongly suggest that nectin-1 is expressed by basal cells while nectin-4 is enriched in periderm cells."

The Richardson study cited here (4) is to our knowledge the only report of nectin-4 protein expression in oral epithelia, and this study showed similar patterns of expression for nectin-1 and nectin-4 at the basal-periderm border in E13.5 palatal shelf epithelium. While Nectin1 is a direct target of the basal- specific p63 transcription factor in both epidermal and palatal epithelium—and is lost in p63 knockouts—Nectin4 is regulated by Irf6, not p63, and is still expressed in p63 knockouts (4, 14, 15). While this does not prove that Nectin1 and Nectin4 are expressed in different cell populations, it does demonstrate that they are subject to distinct modes of transcriptional regulation. In further searching the literature, we came across a recent Development paper from the Trevor Williams lab (16) which performed single-cell RNA-seq on E11.5 murine lambdoidal junction tissue (site of primary palatogenesis), and reported that Nectin4 (Pvrl4) is highly enriched in periderm while largely absent from palatal epithelium (Table 1 and Figure 5). These studies are now discussed on p. 8 of the Results section.

We have tried several different antibodies and staining conditions in an attempt to replicate the beautiful results on nectin-1 and nectin-4 expression on palatal shelves reported in Figure 5 of Richardson et al (4). However, the images presented in Figure 2A-C and Supplementary Figure 3B represent the best we were able to achieve in cryosections (note that it appears that Richardson et al used FFPE with antigen retrieval). We agree that basal- and/or periderm-specific markers would enhance this panel. However, we lack images which include these stains (e.g. p63 to label basal cells or K8 to distinguish periderm) and are currently unable to perform additional staining and microscopy to generate new images. We have added appropriate discussion related to that above to page 8, paragraph 2, and have changed our language regarding nectin-4 localization to highlight that periderm specific shRNA delivery was sufficient to knockdown Nectin4, without specifying periderm enrichment.

Comments requiring additional experiments that cannot be easily addressed experimentally

Editor comment: I feel that all of these are in the realm of "more stuff" and are not needed for publication, especially in light of the current circumstances.

Response: We thank the editor for his understanding and have attempted to discuss and address each of these comments in the text, as indicated.

Reviewer 1, Major comment #1: "What are the mechanisms by which Afadin/Nectin are working in controlling palate epithelial development? The authors demonstrate that ectopic cell adhesions are a common feature of their loss of function models. Do these adhesions correlate with aberrant differentiation, or loss of periderm cells (K6+ cells), changes in apoptosis or proliferation? Are other genes implicated in this specific phenotype such as Jag2/Notch, Irf6, Grhl3 altered at sites of adhesion, or is Afadin/Nectin likely developmentally downstream of these factors?"

Response: Intraoral adhesions were only observed in the Afdn knockdown embryos, and with limited penetrance. In these examples, we did observe absence of periderm cells (as visualized by K8+ stain) between contacting epithelia (see Fig. 1H, I). Given the variability in the presentation of this phenotype and the difficulties of definitively demonstrating that these are intraoral adhesions rather than delays in shelf elevation (which are not mutually exclusive), we decided to interpret these results by stating, "These data suggest that afadin loss delays palatal shelf elevation, which may cause - or be attributed to - the formation of intraoral adhesions between the PS and the tongue epithelium, leading to frequent CP."
Addressing the reviewer's rather broad comment in full would require a large cohort of additional samples from ages E13.5-E15.5, controlling for the degree of lentiviral infection, and serial

sectioning and staining of every sample to monitor the % periderm coverage through developmental time. These techniques would then need to be repeated using a variety of additional stains (e.g. Jag2, Irf6, cleaved Caspase3, etc.) to fish for pathways that might be affected by loss of afadin. Our intention with this figure is to highlight 1) afadin is required for palatogenesis, and 2) loss of afadin delays shelf elevation – and we believe this language reflects an appropriate interpretation of the data. Describing the underlying molecular and cellular mechanisms would require an additional figure (or two, depending on results), and we are already at the maximum of four primary figures for the Research Report format. While these proposed experiments would be highly informative, we feel that they are beyond the scope of this manuscript.

Reviewer 1, Major comment #4: "Are levels of expression of Nectin1W185X similar to endogenous levels of Nectin-1 expression?"

Response: We acknowledge that how NECTIN1$_{W185X}$ levels compare to endogenous nectin levels—arguably, not just nectin-1, but other nectin-1 binding partners as well—could likely impact its ability to dominantly interfere with nectin binding in trans. Unfortunately, adequately addressing this question would require substantial additional experiments, which under normal conditions would be both time- consuming and costly, but under current conditions (where the flow cytometry core is closed indefinitely) are impossible. To accomplish this, we would need to perform new lentiviral injections with the Nectin14402; H2B-mRFP1-T2A-NECTIN1W185X-V5 construct and isolate transduced (RFP+) and non- transduced (RFP-) palatal epithelial cells by flow cytometry, then compare endogenous nectin-1 protein levels in the RFP- population to NECTIN1W185X levels in the RFP+ population by western blot. Because of the small size of the MEE, we would likely need to collect a large number of embryos—likely from multiple experiments—and pool them to generate sufficient protein for detection by WB. Moreover, to directly compare expression levels between exogenous and endogenous nectin-1, we would need to identify a new antibody that recognizes both full-length and truncated nectin-1 protein, since the antibody we are currently using does not recognize the NECTINW185X protein (see Figure 4E, lack of colocalization between nectin-1 and V5). Such an antibody would have to be raised against the extreme N-terminus and would need to cross-react with mouse and human proteins, and we have been unable to locate an antibody that fits these criteria. Finally, even if we were able to quantitatively compare NECTIN1W185X levels to endogenous nectin-1 levels, we would still not know how this affected expression of nectins-1/2/4, which is also important to consider.

However, it should be noted from our similar previous experiments with lentiviral-mediated "rescue" in embryonic epidermis using this PGK promoter, that expression levels were moderate, and comparable to endogenous levels for the relatively non-abundant protein LGN (Williams et al, Nature 2011; Supplementary Fig. 5)(17). Thus, we feel it is unlikely that NECTIN1W185X levels far exceed endogenous Nectin-1 levels.

Reviewer 1, Major comment #5: "As the Nectin14402; Nectin1W185X the embryos exhibit higher penetrance than Nectin-1; Nectin-4 compound knockdown, can the authors speculate/further explain why they consider this a dominant negative effect rather than dominantly acting mutation? (i.e. gain of function). What is the phenotype of Nectin1W185X embryos without Nectin-1 knockdown? Does the presence of wt Nectin-1 exacerbate, or reduce the frequency and severity of the Nectin1W185X phenotype?"

Response: We acknowledge that the experiments suggested by the reviewer could be helpful in determining whether the NECTIN1$_{W185X}$ mutation acts in a gain-of-function vs dominant-negative manner. However, we would like to point out that we made no claims about how the Nectin1$_{4402}$; H2B-mRFP1-T2A-NECTIN1$_{W185X}$-V5 phenotype compares to the Nectin1$_{4402}$; Nectin4$_{2589}$ phenotype, but rather compared the former to Nectin$_{4402}$ alone (Results, p. 12). In fact, there is no statistical significance in the penetrance of palatal phenotypes between the Nectin14402; NECTIN1$_{W185X}$ group (7/7; Fig. 4C) and Nectin1$_{4402}$; Nectin4$_{2589}$ compound knockdown (12/12; Fig. 3F) cohorts. To the contrary, the compound knockdown embryos displayed complete palatal clefting more frequently than W185X mutants (11/12 vs 4/7, respectively). However, the W185X mutants did display a significantly higher frequency in both cases when compared to Nectin1 knockdown alone. This is the key observation (among others, listed in the penultimate paragraph of the Results & Discussion section) that lead us to conclude this mutation operates as a dominant negative rather

than loss-of-function. The reviewer is correct, however, that, strictly speaking, a dominant negative acts by impairing the function of the wild-type allele. Here, we postulate that it may dominantly interfere with other nectins, rather than nectin-1 itself. We have altered our language to refer to this as a "dominant interfering" mutation, and to acknowledge that we cannot rule out that it could have other gain-of-function activities: "It should be noted, however, that we have not formally ruled out the possibility that the NECTIN1$_{W185X}$ mutation could confer gain-of-function activity, which could be tested by overexpression on a WT rather than Nectin1 knockdown background" (Results, p. 11). In the current environment, we do not have the ability to generate a new W185X lentiviral construct lacking the Nectin14402 hairpin, generate high titer virus, and perform LUGGIGE experiments to test this directly.

Reviewer 2, Major comment #2: "Krt14-Cre has been used to show requirements for other epithelial factors involved in CL/P, including several involved in adhesion. The authors argue that this reflects an earlier role for Afdn-Nectins, prior to the onset of Krt14 expression. But are there other explanations? One useful additional experiment would be to confirm the results with Krt8-Cre or Krt18-Cre to remove Nectin4 in periderm."

Response: We appreciate the reviewer's consideration of the contrast between LUGGIGE and traditional genetic strains. It is important to note, however, that both Krt14-Cre and LUGGIGE (when delivered at E9.5, as was done in all experiments here) drive gene expression that originates in basal layer cells, which is passed on to all descendants of basal layer progenitors, including suprabasal cells and periderm. Therefore, there is no difference in the cell types affected, only in the timing. This has been previously demonstrated by our group in Supplementary Figure 4 (12). Given that the K14-Cre; Afdn fl/fl animals did not develop CP, but still had complete protein loss by ~E16.5 (data not shown in this manuscript, but see Lough, et al. 2019) (18), we conclude that loss of Afdn prior to Krt14-Cre expression is necessary to induce CP. However, the reviewer does raise the interesting question of whether afadin and/or nectins are required and function in the periderm, a point which we do not address in the text. In principle, this could be addressed either by using periderm-specific Cre driver lines—as the reviewer suggests—or by performing lentiviral injections at a later age (similar to experiments published by the Nah lab) (19). However, we do not have either of these Cre lines in our colony, and the Krt18CreER line is cryopreserved (https://www.jax.org/strain/017948). Even if these mice were immediately available, it would take two generations to generate mice that could be analyzed for CP. Similarly, a LUGGIGE-based approach is currently not feasible given our restricted access to the University and its core facilities. While we agree that this is a very interesting question, we argue that this is well beyond the scope of this short research report paper.

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Second decision letter

MS ID#: DEVELOP/2020/189241

MS TITLE: Disruption of the nectin-afadin complex recapitulates features of the human cleft lip/palate syndrome CLPED1

AUTHORS: Kendall J Lough, Danielle C Spitzer, Abby J Bergman, Jessica J Wu, Kevin M Byrd, and Scott E Williams
ARTICLE TYPE: Research Report

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

Reviewer 1

Advance summary and potential significance to field

This manuscript from Lough et al., explores the function of junctional adhesion proteins afadin, Nectin-1, and Nectin-4, in the development of the mouse secondary palate. The authors apply a cutting-edge LUGGIGE technique for rapid gene perturbation in the palatal epithelium to study the in vivo consequences of loss, or disease-causing mutations in these genes. This method is powerful, and is extremely useful for the study of oral epithelial development. In this study, the authors demonstrate that the afadin/Nectin cell adhesion molecules play important roles in epithelial cell adhesion during secondary palate morphogenesis, and that their loss results in aberrant cell adhesion that results in a failure of secondary palate elevation and closure. This is a beautiful methodological demonstration and makes the important discovery of the importance of the afadin/Nectin cell adhesion complex in mouse palate epithelial development, supporting the long-known human genetic role of the Pvrl1/Nectin-1 gene in human orofacial clefting. This will be of interest to the craniofacial community, and to human geneticists who study orofacial clefting, as well as specialists in the area of these adhesion molecules.

Comments for the author

The authors have responded to reviews as advised by the editor, and in accordance with current Development policies.

Reviewer 2

Advance summary and potential significance to field

This paper investigates the roles of Afadin (Afdn) and Nectins in craniofacial development in mice. The authors use lentiviral ultrasound-guided (LUGGIGE) knockdowns of Afdn1 and Nectins 1 and 4 in oral epithelial cells to reveal requirements in palate development particularly cell adhesion required for palate fusion. Previous studies have implicated NECTIN1 in cleft lip and palate in humans, but mouse knockouts have been uninformative due to early lethality and/or a lack of suitable Cre drivers to disrupt function in early oral epithelia. The results here support the argument that the requirements for Afdn and Nectins are prior to the onset of Krt14-Cre expression, since earlier knockdown with LUGGIGE leads to clear clefting.
Mosaic knockdowns lead to loss of Nectin1 protein with basal cell transfection while Nectin4 protein is lost with periderm transfection, revealing potential cell autonomous requirements in different epithelial layers. Finally, the authors argue for a dominant negative mechanism with truncating Nectin1 mutations based on the higher penetrance, novel palate and limb phenotypes, and disrupted subcellular localization.

Comments for the author

This manuscript is improved and I appreciate the thoughtful responses of the authors. I have no further concerns.

Reviewer 3

Advance summary and potential significance to field

This paper focuses on the nectin-afadin complex, which has previously been implicated in cleft lip/palate-ectodermal dysplasia syndrome 1 (CLPED1) or OFC7. While mutations in nectin genes are suggested to be causative, there has been no gene-function data in mouse models clearly linking these genes to CLPED. This is largely due to early lethality of the afadin knockout (by E9.5 or so) and potential functional redundancy of the nectin genes, of which there are four.

This paper is a clever demonstration of using the mouse embryo to understand human gene variants. All together it will add to our understanding of the mechanisms underlying human cleft lip/palate and ectodermal dysplasias.

Comments for the author

I am satisfied with the authors' response to reviewers.