Mutant p63 causes defective expansion of ectodermal progenitor cells and impaired FGF signaling in AEC syndrome


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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 August 2011

Thank you for the submission of your manuscript "Mutant p63 causes defective expansion of ectodermal progenitor cells and impaired FGF signaling in AEC syndrome" to EMBO Molecular Medicine.

We also finally received the report of the third reviewer, which you will also find below. You will be glad to see that this reviewer is also supportive and raises minor concerns.

In particular, the reviewers feel that the study should be strengthened by a better characterization of the stem cell population under investigation and more insight into the functional role of the mutant p63.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged differently with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.
Referee #1

General Remarks:
In the manuscript entitled "Mutant p63 causes defective expansion of ectodermal progenitor cells and impaired FGF signaling in AEC syndrome", Ferone and colleagues reported the generation of a mouse model for AEC syndrome. Most human AEC patients carry heterozygous, mis-sense point mutations that are mapped to the SAM domain of the p63 protein. The knock-in mouse model (p63 L514F) faithfully represents one of the genetic alterations in human patients. Indeed, the observed phenotypes also recapitulate a spectrum of disorders from the human patients. Thus, this work is highly significant and paves a new path to explore the molecular mechanisms underlying AEC syndrome. That being said, several issues should be addressed in the potential revision.

1. Although the L514F mutation has been previously described and examined (Hay and Wells, 1976; McGrath et al., 2001; Huang YP et al., 2005; Koster MI et al., 2009), it is not clear if p63 L514F simply functions dominantly to interfere with the WT p63 copy (Koster MI et al., 2009) or alternates the splicing and stability of p63 (Huang YP et al., 2005). With the possession of the knock-in allele, the authors should examine p63 mRNA splicing by Northern and p63 protein expression by Western. This is particularly important because the authors found that whereas p63+/− heterozygous is completely normal the p63 L514F/+ shows significant defects, likely associated with the reduced p63 level. A straightforward prediction would be that in the mutant mice p63 protein expression is significantly reduced, comparing to both WT and p63+/−.

2. The authors observed "reduced number of hair follicles" (figure 1f and s2d) in the mutant. However, it was not clear if the reduced HF development was due to the lack of HF fate specification or the lack of HF growth. If it was the lack of specification, the potential perturbation to the Wnt/BMP/Shh pathways should be examined. To this end, the authors should examine the density of HF by whole-mount during embryonic development e.g. from E14-E18, in addition to their current analysis. Hair germs can be visualized by the staining of PCad/Lef1/Sox9 and compared to the WT.

3. It is interesting that the minor defects in BrdU incorporation in the mutant epidermis disappeared around E18.5. The authors should provide data to compare the expression level of p63 in the E13.5 epidermis when the defects were apparent and at E18.5 when the defects were largely absent. In addition, since the authors identified the FGF pathway likely contributes to the proliferation defects, a comparison of the FGF signaling at E13.5 and E18.5 should be performed to validate the finding.

4. The authors argued that it's the reduction of clonogenic cells but not their intrinsic self-renewal ability that contributes to the hypoplasia in vivo. This would predict that the size of the colonies is similar but the number of holoclones is reduced with the mutant cells. However, in fig.4A, the mutant cells generally form smaller colonies than the WT cells. It suggests that under the initial condition the mutant cells were still defective in their self-renewal ability. The authors should carefully examine the level of p63/FGF expression between WT and mutant cells during the initial and later passages (when they didn't see difference anymore). That could provide additional insights to whether the dosage of p63/FGF is responsible for the transient defects.

5. The characterization of the "epidermal stem cells" based on a6hi/CD71dim was highly questionable (pg.12 and fig4C). Among three cited papers, Tani et al., 2000 and Tumbar et al., 2004 studied mouse skin whereas Terunuma et al., 2007 studied human skin. Importantly, both mouse papers specifically examined hair follicle bulge stem cells but not interfollicular epidermal stem cells with a6hi/CD71dim. Tani et al., even pointed out that they didn't observed differential expression of CD71 at the basal layer of the epidermis. Therefore, a6hi/CD71dim is unlikely to identify interfollicular epidermal stem cells. There is also no evidence so far that bulge stem cell directly contributes to the development of epidermis during embryonic development. Thus the reduced a6/CD71 population could not explain the thinner epidermis in the mutant. In addition, in Terunuma's study in the human skin, they didn't observe any growth advantage of the a6hi/CD71dim population over the overall population until three months. It also argues against the causative role of the reduced a6/CD71 population for the mutant phenotypes. Finally, because the
authors didn't describe in details how they isolated the epidermal cells it is difficult to evaluate if the a6/CD71 population was a contamination from hair follicles, whose stem cell population is likely depleted in the mutant skin based on the reduced K15 and Sox9 levels (fig 5). In any case, the authors should re-do the analysis for the interfollicular epidermal stem cells.

6. Fig. 5G (colony formation assay with Fgfr2b KO cells) is identical to Fig. 4A (colony formation assay with the mutant cells)! And there is no Fig. 5H.

7. In Fig. 7C, it appears that with or w/o KGF treatment, the mutant keratinocytes showed significant defects in BrdU incorporation. This result seems contradictory to the results in Fig3E where no BrdU incorporation defects were observed after E18.5 (even at E13.5 the difference was quite small); and the results in Fig. 4A-B where very small difference in the colony formation was observed in the initial test and subsequently disappeared. The authors should address why the cultured keratinocytes showed much significant defects in proliferation/BrdU incorporation.

Referee #2

General Remarks:
Ferone et al present a novel mouse model of AEC syndrome, which they have generated by targeted mutation of part of p63 known to be important for transcriptional activation. The model shows good recapitulation of the human disease and has been investigated well at the descriptive level. They show that this mutant p63 can impact on FGFR signaling in the mouse and that this underlies the stem cell compartment phenotype. By in silico studies, ChIP and reporter assays, they show FGFR2 and 3 to be regulated by p63 and show that the effect of mutant p63 on keratinocytes can be rescued by expression of FGFR2b.

The model is a good one but I think that the manuscript ought to show more mechanistic insight. There is a lot of literature regarding p63 and FGFR2 in the skin, and notably the link has been published in Wu et al 2003 (below).

In general, the quality of the data are good - particularly the descriptive images in Figs 1-3. The fact that mutant p63 is affecting Fgfr2 expression is convincing, but I think the data would be strengthened by expression of mutant as well as wt p63 constructs in their p63 null H1299 cell line luciferase reporter experiments. It would be good to see 1) that the mutant version does not activate expression to the same extent and 2) to show a dominant negative effect when co-transfected with wt. I think that it is key to show more mechanistic insight into how the mutant p63 exerts its effect. This is the really interesting point of the story and one is left no closer to understanding the mechanism by the end of the manuscript.

The quality of the written manuscript needs considerable improvement - I appreciate that the first and last authors may not be native English speakers but I would be surprised if "all authors critically revised the manuscript and approved its final version" given the number of typographical errors.

Specific points.

On P8 they mention that Ankyloblepharon couldn't be investigated in mutant mice as eyelids are normally fused til P15 - however, FGFR2b null mice have open eyes at birth. This should be discussed.

On P14 line 2 they mistakenly mention Fgfr2/- mice. Since these die around E4/5 they would not display any epidermal defects. Similar error in Fig 5G legend

On P15 they discuss p63 sites in FGFR2 and 3, and how these are potentially regulated genes. FGFR1 and 4 are not differentially expressed - however, have they checked these genes for p63 sites? If they could show that these do not have the sites then I think the argument would be strengthened.

P18 The order of the manuscript seems odd when it gets to P18 and then goes back to Fig 5

P19 In the discussion, I think that, given the literature, saying that they "demonstrate a previously
unsuspected functional link between p63 and FGF signaling in controlling progenitor cell expansion” is a little oversold. In the following paragraph, they say that Petiot et al reported unaffected proliferation in newborn skin - this is mistaken in two ways: 1) they say Fgfr2+/− not Fgfr2b+/− mice and 2) Petiot et al do report reduced proliferation in the knockout mice.

Fig 1 C. Would be clearer if letters were under the peaks in sequence data. 1 D. The primers representing the 400bp band are not indicated in the wt locus, unlike the other two bands.

Fig 4 B. Did they check the mutant clones for expression of the mutant form of p63? Otherwise, how do they know that these don’t just arise from cells which have shut down expression of the mutant allele either through mutation or, more likely, epigenetic modification. 4 C. The FACS data do not look particularly convincing to me. Unless they sort these selected cells and show them to have differential clonogenic potential I find it hard to just take the data as they are.

Fig 6 What are the controls for the heteronuclear RNA PCR (Fig 6) that show there is no amplification of genomic DNA contamination (i.e. that the DNase treatment worked)?

Fig 7 A. Would be good to include pERK. 7 B. would be good to include FGFR2 protein levels. 7 B/C. How about response to an unrelated growth factor such as EGF, to show the effect is FGF specific.

Sup Fig 3B FACS analysis needs clarifying as to which area is represented in the quantitation. I don't find the figure very convincing - especially given the massive difference in dead cell number in mutant cells - which as far as I can see is not discussed. What about quantitation of the staining in C - just including those 4 figures does not really add anything.

It might be nice for the reader (given that this is not a specialist journal) to have a cartoon of p63 showing sites of mutation in AEC and perhaps know what percentage of mutations are L514F. Perhaps referring to the relatively recent publication from Sathyamurthy et al. FEBS J. 2011. 278(15):2680-8. Structural basis of p63α SAM domain mutants involved in AEC syndrome.

Missing references:


- Expression of FGFR2 induced by TAp63α but inhibited by ΔNp63α. To quote "However, our study clearly shows that FGFR2 functions as a downstream target of p63, suggesting that the latter might be involved in limb development and/or skin formation through regulation of the FGF-signaling pathway"

No discussion of IKKa which also is a key target gene implicated elsewhere:


Or discussion of alternative mouse models:


Referee #3

General Remarks:
p63, a p53-related transcription factor, is a master regulator of epidermal development. Mice deficient in p63 lack all stratified epithelia, limbs and epidermal appendages. p63 comes in multiple isoforms: two distinct N-termini (deltaN and TA) can be associated with three different C-termini (alpha, beta, gamma) that are produced by alternative splicing. The different isoforms are thought to
have both overlapping and unique activities. Therefore, dissecting the exact functions of p63 has been very challenging. In humans, heterozygous mutations in p63 cause at least five distinct syndromes which nevertheless share several characteristics and are all classified as ectodermal dysplasias. One of them, AEC is characterized by cleft palate and a severe skin phenotype, and is due to mutations clustering in the SAM domain present in the longest alpha isoforms of p63.

The manuscript by Ferone et al. is the first one to describe the generation of a knock-in mouse model (p63+/L514F) for AEC, and represents a major breakthrough in the field. Phenotypic characterization of p63+/L514F heterozygous mice revealed that they phenocopy many aspects of AEC patients including skin, hair, tooth, and palate although the analysis was somewhat limited due to the perinatal lethality of mutant mice.

The authors go on by analyzing more in detail the cleft palate and epidermal phenotype, and search for genes differentially expressed in the mutant vs. control skin. In vivo and in elegant in vitro experiments show that cleft palate is caused by reduced proliferation rather than defects in elevation or fusion of the palatal shelves. Analysis of keratinocytes suggested that the number of epithelial cells with high clonogenic potential was reduced. Interestingly, negative cell cycle regulators known to be repressed by p63 were not affected in mutant mice suggesting that the activator rather than repressor function of p63 was compromised. The remaining part of the manuscript is devoted to the identification of FgfR2 and FgfR3 as transcriptional targets of p63. It is proposed that downregulation of FgfR2/3 and thus impaired epithelial Fgf signaling in p63+/L514F heterozygous mice explains many of the defects observed in mutant mice and AEC patients. Data presented are convincing, and support authors’ conclusion. I enjoyed reading this interesting manuscript.

I have a few minor comments/questions.

1. RNA isolated from E14.5 wt and mutant epidermis was used to analyze expression of a number of candidate genes. However, it remained unclear to me how epidermis was separated and how the purity of the epidermal cell pool was tested?

2. It is proposed that the phenotype of the p63+/L514F mice and possibly also AEC patients is due to compromised epithelial Fgf signaling due to downregulation of FgfR2 and FgfR3 transcripts. AS a final piece of evidence it is shown that exogenously expressed FgfR2b is able to restore cell proliferation (BrdU incorporation) in p63+/L514F keratinocytes (Fig. 7C). I am somewhat puzzled by these data, as elsewhere in the manuscript it was stated that there was no difference in proliferation between mutant and wt neonatal keratinocytes (Fig. S5A). Were these experiments done in a different way or what explains the difference between data shown in Fig. S5A and the left part in Fig. 7C? Statistical analysis of data shown in Fig. 7C would also be useful.

3. Do the authors have any speculations why the similarities between Fgfr2b null and p63+/L514F mutants were more striking in some tissues (skin, palate) than in others (e.g. tooth where Fgfr2b has an early essential function)?

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1st Revision - Authors' Response 21 October 2011

We thank all referees for their very thoughtful and helpful comments that have allowed us to significantly improve the manuscript. Please find our answers to their comments below.

Referee #1:

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Referee 1: Although the L514F mutation has been previously described and examined (Hay and Wells, 1976; McGrath et al., 2001; Huang YP et al., 2005; Koster MI et al., 2009), it is not clear if p63 L514F simply functions dominantly to interfere with the WT p63 copy (Koster MI et al., 2009) or alters the splicing and stability of p63 (Huang YP et al., 2005). With the possession of the knock-in allele, the authors should examine p63 mRNA splicing by Northern and p63 protein expression by Western. This is particularly important because the authors found that whereas p63+/− heterozygous is completely normal the p63 L514F/+ shows significant defects, likely associated with the reduced p63 level. A straightforward prediction would be that in the mutant mice p63 protein expression is significantly reduced, comparing to both WT and p63+/−.
Reply: To gain more insights into the mechanisms by which the mutant elicits its function, we measured p63 at the mRNA and protein levels as suggested by the referee, and we have included these data in the manuscript (Page 7). At the mRNA level, total p63 expression is similar in mutant and in wild-type epidermis (Supplementary Figure S1B). In contrast, at the protein level, p63 is expressed at higher levels in mutant skin as compared to wild-type controls (Supplementary Figure S1C-D), in agreement with recent reports indicating that SAM domain mutants accumulate in the skin of AEC patients and show extended half-lives in vitro (Browne et al. J. Cell Science 2011, Marinari, J. Clin. Invest. 2010). The aberrant isoform described by Huang YP et al in 2005 was not detected (Supplementary Figure S1E), and no other aberrant isoforms were observed either at the RNA or the protein levels.

In the revised version of the manuscript, we also provide additional evidence that mutant p63 acts in a dominant negative fashion toward the wild-type as previously suggested by overexpression studies (Koster et al, 2009, Marinari et al., 2009, Lopardo et al. 2008). In p63L514F mutant epidermis a large number of p63 target genes is affected (Suppl. Table S1), suggesting a broad function of AEC mutants as dominant-negative. In addition as requested by referee 2, we show that p63L514F is unable to transactivate Fgfr2 promoter to the same extent of the wild-type, and that mutant p63 suppresses the Fgfr2 promoter transactivation elicited by wild-type p63 (Figure 4G). Thus, we provide novel data indicating that p63L514F functions as dominant negative by suppressing the transactivation activity of wild-type allele.

Referee 1: The authors observed "reduced number of hair follicles" (figure 1f and s2d) in the mutant. However, it was not clear if the reduced HF development was due to the lack of HF fate specification or the lack of HF growth. If it was the lack of specification, the potential perturbation to the Wnt/BMP/Shh pathways should be examined. To this end, the authors should examine the density of HF by whole-mount during embryonic development e.g. from E14-E18, in addition to their current analysis. Hair germs can be visualized by the staining of PCad/Lef1/Sox9 and compared to the WT.

Reply: To determine whether the reduced hair follicle development was due to lack of hair follicle fate specification, we performed whole-mount in situ hybridization at E14.5 for Wnt10b, Lef1, and EDAR, some of the earliest markers of the hair buds. These markers were expressed in the developing pelage hair placodes in a similar pattern to those in wild-type mice (Supplementary Figure S3D), indicating that hair follicle fate specification was not affected. In contrast a reduced number of more developed hair buds was observed not only at birth as reported in the original version of the manuscript, but also at E16.5 (Supplementary Figure 3C). A full characterization of the hair follicle development in AEC mutant is outside of the scope of the present manuscript, however these experiments indicate that hair development is delayed in AEC mutant mice due to reduced hair follicle growth, whereas fate determination is normal.

Referee 1: It is interesting that the minor defects in BrdU incorporation in the mutant epidermis disappeared around E18.5. The authors should provide data to compare the expression level of p63 in the E13.5 epidermis when the defects were apparent and at E18.5 when the defects were largely absent. In addition, since the authors identified the FGF pathway likely contributes to the proliferation defects, a comparison of the FGF signaling at E13.5 and E18.5 should be performed to validate the finding.

Reply: As pointed out by the referee we observe reduced epidermal cell proliferation during development but not at birth. Interestingly, a strikingly similar proliferation defect in development but not at E18.5 is observed in the Fgfr2b** mice. Quoting from Petiot et al. Development 2003: "A significant reduction in the number of BrdU-positive cells was observed in the basal layer of the interfollicular epidermis of Fgfr2-IIIb−/− mice from E14.5 to E16.5, a period that normally correlates with the development of stratification. At E18.5, the difference in the number of proliferating keratinocytes was no longer significant.” Thus Fgfr2b expression is required for
epidermal cell proliferation during development, whereas at birth other mitogenic signaling are likely to be sufficient to sustain cell proliferation.

Concerning the quantitative comparison of p63 expression and FGF signaling at E13.5 and E18.5, protein extracts from E13.5 epidermis are technically very challenging to obtain. Using a limiting amount of antibodies we observed that p63 is significantly more expressed in mutant versus wild-type epidermis both at E13.5 and E18.5 (Suppl. Figure S1D), suggesting that p63 levels are not differentially regulated in mutant epidermis during development. Measurement of FGF signaling during development has not been possible since anti-phospho-FRS2a antibodies do not work by immunofluorescence.

Referee 1: The authors argued that it’s the reduction of clonogenic cells but not their intrinsic self-renewal ability that contributes to the hypoplasia in vivo. This would predict that the size of the colonies is similar but the number of holoclones is reduced with the mutant cells. However, in fig.4A, the mutant cells generally form smaller colonies than the WT cells. It suggests that under the initial condition the mutant cells were still defective in their self-renewal ability. The authors should carefully examine the level of p63/FGF expression between WT and mutant cells during the initial and later passages (when they didn’t see difference anymore). That could provide additional insights to whether the dosage of p63/FGF is responsible for the transient defects.

Reply: We thank the referees for giving us the opportunity to clarify and improve the stem cell characterization. We agree with referee 1 that in Figure 4A the mutant colonies were smaller than wild-type, and—to avoid any confusion—we have changed this Figure with a more representative experiment showing that there are some large colonies in the mutant (Figure 6A). Importantly, quantitative data in the lower panels (now Figure 6B) are the average of 3 independent experiments, and demonstrate that while the % of colonies obtained in mutant cells is only slightly decreased (left panel), the difference being not statistically significant, the number of holoclones is significantly decreased with a p-value<0.01 (right panel). We provide the entire set of data at the end of this reply (Table 1 for referee).

As shown in Suppl. Fig.S9B, mutant primary cells have a normal proliferation rate under growing conditions. To assess the proliferation rate under clonogenic conditions, we measured the overall cell proliferation 1-week after plating, and found that proliferation is decreased in mutant cell cultures (Suppl. Fig.10A) consistent with a reduced number of clonogenic cells. Importantly, however cell proliferation occurs at the same rate in individual mutant and wild-type holoclones 3-weeks after plating (n=20) (Suppl. Fig.10B), even thought Fgfr2b expression is still reduced in mutant cells as compared to their wild-type counterparts (Suppl. Fig. S10C).

Interestingly, similarly to the AEC mutant cells, Fgfr2b<sup>−/−</sup> epidermal cells generate a reduced number of holoclones (Figure 6C) that can be passaged at a somewhat reduced but still high frequency (Figure 6D), suggesting that under clonogenic conditions in a fully supplemented medium the absence of Fgfr2b does not significantly affect the ability of stem cells to proliferate or self-renew.

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re-do the analysis for the interfollicular epidermal stem cells.

Reply: We thank the referee for making this point and we apologize for the misinterpretation of previous published data. We fully agree that according to the published literature a6br/CD71dim population is unlikely to identify interfollicular epidermal stem cells in mouse skin. As suggested by the referee, we checked whether we have contamination of hair follicle bulge cells in our epidermal preparation. As shown for adult epidermis isolated with trypsin (Jensen et al, Nature Protocols 2010), we found that bulge stem cell markers are uniformly distributed in epidermis and dermis separated by our dispase protocol (details in Materials and Methods of the revised version), suggesting that the a6br/CD71dim population is likely to derive from the bulge. In light of these findings we fully agree with the referee that the reduced a6br/CD71dim population is unlikely to explain the thinner epidermis in the mutant, and therefore we deleted this statement and entirely revised this chapter of the manuscript (page 18-19 of the Result Section).

It would be very interesting to determine whether the interfollicular stem cell compartment is reduced in AEC mice as suggested by the referee. The stem cells of the interfollicular epidermis have long been thought to reside in the basal layer, however their identity is still elusive, and there are no definitive markers for these cells (reviewed in (Fuchs, 2009; Watt & Jensen, 2009)). Jensen and Watt have recently published a cell sorting protocol based on a triple selection (Itga6+/ CD34-/Sca1-) or on a double selection (Lrig+/CD34-) to enrich for adult interfollicular stem cells (Nature Protocols, 2010). However CD34 is not a reliable marker for the bulge stem cell population at the neonatal stage, and the lethal phenotype observed at birth in our mice has prevented us from doing this analysis (space constraints has prevented us from adding this information). In spite of these technical limitations we have shown that there is a reduction of the epithelial stem cell compartment of the skin, as indicated by reduction of K15 and Sox9 expression, and of the a6br/CD71dim population. In addition we report a reduced number of clonogenic cells. While none of these assays is definitive, all reveal a general reduction in the epithelial stem cell population of the skin. Importantly, in spite of the quite different structure of the human skin, expression of Krt15 and Sox9 is also observed in AEC patient skin.

Referee 1: Fig. 5G (colony formation assay with Fgfr2b KO cells) is identical to Fig. 4A (colony formation assay with the mutant cells)! And there is no Fig. 5H.

Reply: The two Figures look very similar but they are completely independent experiments. To clarify this point we inserted the actual % in each figure.

Referee 1: In Fig. 7C, it appears that with or w/o KGF treatment, the mutant keratinocytes showed significant defects in BrdU incorporation. This result seems contradictory to the results in Fig3E where no BrdU incorporation defects were observed after E18.5 (even at E13.5 the difference was quite small); and the results in Fig. 4A-B where very small difference in the colony formation was observed in the initial test and subsequently disappeared. The authors should address why the cultured keratinocytes showed much significant defects in proliferation/BrdU incorporation.

Reply: We agree that this a confusing issue. As pointed out by the referee, in vivo we observe defective proliferation only during development but not at birth. This is not surprising since the exact same phenotype is observed in the Fgfr2b−/− epidermis (Petiot et al Development 2003), suggesting that Fgfr2b is not required for epidermal cell proliferation at birth and other growth factor receptors may be sufficient. Similarly in vitro mutant keratinocytes proliferate to a similar extent of the wild-type (and have similar levels of phospho-ERK) under growing conditions in the presence of the several growth factors contained in the medium (Supplementary Figure S9B). In contrast, in the absence of growth factor reduced expression levels of Fgfr2b and Fgfr3b results in a reduced response to KGF (Figure 5C). We now clarify this point in the text (Page 16 in the Result Section). We also added EGF treatment as an additional control (Supplementary Figure. 6A), demonstrating that mutant keratinocytes do not have a general defect in ERK signaling activation but rather a specific FGFR-dependent defect reduction in FGF-ERK signaling and proliferation.
Referee #2: Ferone et al present a novel mouse model of AEC syndrome, which they have generated by targeted mutation of part of p63 known to be important for transcriptional activation. The model shows good recapitulation of the human disease and has been investigated well at the descriptive level. They show that this mutant p63 can impact on FGFR signaling in the mouse and that this underlies the stem cell compartment phenotype. By in silico studies, ChIP and reporter assays, they show FGFR2 and 3 to be regulated by p63 and show that the effect of mutant p63 on keratinocytes can be rescued by expression of FGFR2b.

The model is a good one but I think that the manuscript ought to show more mechanistic insight. There is a lot of literature regarding p63 and FGFR2 in the skin, and notably the link has been published in Wu et al 2003 (below).

In general, the quality of the data are good - particularly the descriptive images in Figs 1-3.

Referee 2: The fact that mutant p63 is affecting Fgfr2 expression is convincing, but I think the data would be strengthened by expression of mutant as well as wt p63 constructs in their p63 null H1299 cell line luciferase reporter experiments. It would be good to see 1) that the mutant version does not activate expression to the same extent and 2) to show a dominant negative effect when co-transfected with wt. I think that it is key to show more mechanistic insight into how the mutant p63 exerts its effect. This is the really interesting point of the story and one is left no closer to understanding the mechanism by the end of the manuscript.

Reply: We are very grateful to the referee for this comment and we agree that more mechanistic insights need to be provided. First of all we performed the luciferase reporter experiment suggested by the referee. As predicted we found that p63L514F is unable to transactivate Fgfr2 promoter to the same extent of the wild-type, and co-expressing wild-type and mutant p63 suppresses the Fgfr2 promoter transactivation. Previous overexpression studies in vitro have shown that AEC mutants have dominant negative activities towards wild-type Ad-63a (Koster et al, 2009; Lopardo et al, 2008; Marinari et al, 2009). In the present version of the manuscript, we also provide gene expression profiling data showing that in p63+/-L514F a large number of p63 target genes are affected beside Fgfr2 and Fgfr3, suggesting a broad dominant-negative function elicited by AEC mutants. Thus, we provide novel data indicating that p63L514F functions as dominant negative by suppressing the transactivation activity of the wild-type allele.

Referee 2: The quality of the written manuscript needs considerable improvement - I appreciate that the first and last authors may not be native English speakers but I would be surprised if "all authors critically revised the manuscript and approved its final version" given the number of typographical errors.

Reply: We apologize for the written quality of the first version of the manuscript. We made all possible efforts to improve the revised version.

Referee 2: On P8 they mention that Ankyloblepharon couldn't be investigated in mutant mice as eyelids are normally fused til P15 - however, FGFR2b null mice have open eyes at birth. This should be discussed.

Reply: We discuss this point in the Discussion Section (page 22).

Referee 2: On P14 line 2 they mistakenly mention Fgfr2-/- mice. Since these die around E4/5 they would not display any epidermal defects. Similar error in Fig 5G legend

Reply: Thank you. We checked the entire manuscript for these mistakes and corrected them all.

Referee 2: On P15 they discuss p63 sites in FGFR2 and 3, and how these are potentially regulated genes. FGFR1 and 4 are not differentially expressed - however, have they checked these genes for...
p63 sites? If they could show that these do not have the sites then I think the argument would be strengthened.

Reply: There are no p63-binding regions in the Fgfr1 and Fgfr4 genomic locus spanning 50Kb upstream and downstream genes. Since the data are publicly available (GEO Series accession number GSE17611), we added this information as data not shown (Result Section, Page 14).

Referee 2: P18 The order of the manuscript seems odd when it gets to P18 and then goes back to Fig 5

Reply: We agree with the referee’s comment and we completely revised the order of the text and figures by placing the experiments on stem cells at the end of the Result Section.

Referee 2: P19 In the discussion, I think that, given the literature, saying that they "demonstrate a previously unsuspected functional link between p63 and FGF signaling in controlling progenitor cell expansion" is a little oversold.

Reply: We change this sentence in “a novel functional link between p63 and FGF signaling in controlling progenitor cell expansion”. We truly think that this is a novel finding.

Referee 2: In the following paragraph, they say that Petiot et al reported unaffected proliferation in newborn skin - this is mistaken in two ways: 1) they say Fgfr2/-/- not Fgfr2b/-/- mice and 2) Petiot et al do report reduced proliferation in the knockout mice.

Reply: We have corrected the manuscript text, which now reads Fgfr2b/-/- instead of Fgfr2/-/- . We agree that Petiot et al report a defective proliferation during development, but not at birth. Citing the original paper: “At E18.5, the difference in the number of proliferating keratinocytes was no longer significant.”

Referee 2: Fig 1 C. Would be clearer if letters were under the peaks in sequence data. 1 D. The primers representing the 400bp band are not indicated in the wt locus, unlike the other two bands.

Reply: We made the suggested changes to improve the quality of Figure 1.

Referee 2: Fig 4 B. Did they check the mutant clones for expression of the mutant form of p63? Otherwise, how do they know that these don't just arise from cells which have shut down expression of the mutant allele either through mutation or, more likely, epigenetic modification.

Reply: We thank the referee for this interesting suggestion. We checked the mutant clones, and found that the mutant allele is still expressed at similar levels compared to the wild-type at the third passage in culture (Supplementary Figure S10D).

Referee 2: 4 C. The FACS data do not look particularly convincing to me. Unless they sort these selected cells and show them to have differential clonogenic potential I find it hard to just take the data as they are.

Reply: As suggested by the referee we tested the clonogenic potential of a6b/CD71dim sorted cells and found that they are significantly more clonogenic than unsorted cells (Supplementary Figure S10F).

Referee 2: Fig 6 What are the controls for the heteronuclear RNA PCR (Fig 6) that show there is no amplification of genomic DNA contamination (i.e. that the DNase treatment worked)?

Reply: Thank you for this comment. To control for genomic DNA contamination, we measured Fgfr2 in samples in which the Reverse Transcriptase was not added (Figure 4C).
Referee 2: Fig 7 A. Would be good to include pERK.

Reply: Consistent with normal cell proliferation under growing conditions (in a medium containing EGF and several other growth factor), pERK is unaffected in spite of reduced FGF signaling (Figure 5A).

Referee 2: 7 B. would be good to include FGFR2 protein levels.

Reply: The anti-Fgfr2 antibody recognizes a carboxyl-terminal epitope that is deleted in the exogenous Fgfr2b-C3 alternative splice variant (Cha et al, JBC 2009), thus we cannot check for Fgfr2 expression in Fgfr2b-C3 expressing cells. To ensure that the level of FGF signaling activation elicited by Fgfrb-C3 is similar to the endogenous one upon KGF stimulation, we measured pFRS2a (Figure 5B).

Referee 2: 7 B/C. How about response to an unrelated growth factor such as EGF, to show the effect is FGF specific.

Reply: Thank you for this suggestion. We measured ERK phosphorylation in response to EGF as control (Suppl. Fig. S9A). EGF induces ERK phosphorylation to a similar extent in wild-type and in mutant keratinocytes in the presence or in the absence of exogenous Fgfr2b, supporting the notion that defects in Ras/ERK signaling occur only upon FGF stimulation.

Referee 2: Sup Fig 3B FACS analysis needs clarifying as to which area is represented in the quantitation. I don't find the figure very convincing - especially given the massive difference in dead cell number in mutant cells - which as far as I can see, is not discussed.

Reply: We apologize for the confusing figure. We performed 3 experiments with a total of 12 independent biological samples, and the samples shown in the Figure are the only ones in which a massive cell death was observed, most likely due to harsh conditions during keratinocyte isolation. Importantly, the % of apoptotic cells was consistent in all samples. We now show a more representative Figure and the average of all these experiments (Suppl. Figure 5B), demonstrating a small difference between wild-type and mutant samples which did not reach statistical significance.

Referee 2: What about quantitation of the staining in C - just including those 4 figures does not really add anything.

Reply: We agree with the referee that more quantitative data need to be provided. We counted active caspase 3 positive cells in skin samples of 20 independent newborn mice. Apoptosis was a very rare event in both wild-type and mutant samples, and mutant samples had a highly variable degree of apoptosis. However, in spite of the low rate of apoptosis (<2 cells/mm), given the high number of samples that we examined, we were able to detect a statistically significant higher apoptotic index in mutant compared to wild-type skins. A similar quantitative analysis was not possible at E16.5 given again a very low apoptotic index and the limited number of embryos due to the lethal phenotype of AEC mutant in heterozygosity. We revised the text accordingly: “Apoptosis was a very rare event in p63<sup>+/L514F</sup> epidermis as determined by staining for active caspase 3 at E16.5 and P0 (Figure 3E and Supplementary Figure S5A), or by Fluorescent Activated Cell Sorting (FACS) analysis with annexin V antibodies of freshly isolated keratinocytes (Supplementary Figure S5B). However, the apoptotic rate was modestly increased in mutant versus wild-type epidermis. Thus, in p63<sup>+/L514F</sup> epidermis, hypoplasia is associated with a transient reduction of cell proliferation during embryonic development and a modest increase in apoptosis, whereas terminal differentiation is not significantly affected.”

Referee 2: It might be nice for the reader (given that this is not a specialist journal) to have a cartoon of p63 showing sites of mutation in AEC and perhaps know what percentage of mutations are L514F. Perhaps referring to the relatively recent publication from Sathyamurthy et al. FEBS J. 2011. 278(15):2680-8. Structural basis of p63<sup>##x03B1##</sup>; SAM domain mutants involved in AEC syndrome.
Reply: Thank you for this suggestion. We included a cartoon in Suppl. Fig. 1A. Since AEC syndrome is extremely are, we haven’t been able to determine the percentage of each single mutation.

Referee 2: Missing references:

Expression of FGFR2 induced by TAp63α but inhibited by ΔNp63α. To quote "However, our study clearly shows that FGFR2 functions as a downstream target of p63, suggesting that the latter might be involved in limb development and/or skin formation through regulation of the FGF-signaling pathway"

Reply: Thank you for pointing this work out. Wu et al reported that overexpression of Tap63alpha and DNp63alpha by Adenoviral transduction in Saos2 cells that are devoid of p63 elicits an opposite effect on Fgfr2 expression, with Tap63alpha inducing Fgfr2 expression and DNp63 alpha inhibiting it. There was no indication in Wu et al. whether this effect might be direct or whether a similar regulation was found in a more physiological context. We now quote this work in the discussion (page 23).

Referee 2: No discussion of IKKα which also is a key target gene implicated elsewhere: Marinari et al (2009) Journal of Investigative Dermatology 129, 60-69. IKKα Is a p63 Transcriptional Target Involved in the Pathogenesis of Ectodermal Dysplasias

Reply: We now quote this work in the discussion as one of the examples in which AEC mutant has been suggested to play a dominant negative function (page 23). We do not know discuss IKKα further, since its expression is unaffected in mutant epidermis and skin.


Reply: Compared to DeltaNp63 knockdown mouse, our model has the advantage or closely mimicking AEC syndrome at the genetic level. However, as requested by the referee we now discuss the alternative mouse model in the Discussion Section (page 22) as follows: “A previously described mouse model for AEC syndrome obtained by conditional ΔNp63 knockdown in epidermis is affected by skin erosions and by focal disruption of the basement membrane (Koster et al, 2009), which was not observed in p63+/-L514F newborn mice.”

Referee #3

p63, a p53-related transcription factor, is a master regulator of epidermal development. Mice deficient in p63 lack all stratified epithelia, limbs and epidermal appendages. p63 comes in multiple isoforms: two distinct N-termini (deltaN and TA) can be associated with three different C-termini (alpha, beta, gamma) that are produced by alternative splicing. The different isoforms are thought to have both overlapping and unique activities. Therefore, dissecting the exact functions of p63 has been very challenging. In humans, heterozygous mutations in p63 cause at least five distinct syndromes which nevertheless share several characteristics and are all classified as ectodermal dysplasias. One of them, AEC is characterized by cleft palate and a severe skin phenotype, and is due to mutations clustering in the SAM domain present in the longest alpha isoforms of p63.

The manuscript by Ferone et al. is the first one to describe the generation of a knock-in mouse model (p63+/-L514F) for AEC, and represents a major breakthrough in the field. Phenotypic characterization of p63+/-L514F heterozygous mice revealed that they phenocopy many aspects of AEC patients including skin, hair, tooth, and palate although the analysis was somewhat limited due to the perinatal lethality of mutant mice.

The authors go on by analyzing more in detail the cleft palate and epidermal phenotype, and search
for genes differentially expressed in the mutant vs. control skin. In vivo and elegant in vitro experiments show that cleft palate is caused by reduced proliferation rather than defects in elevation or fusion of the palatal shelves. Analysis of keratinocytes suggested that the number of epithelial cells with high clonogenic potential was reduced. Interestingly, negative cell cycle regulators known to be repressed by p63 were not affected in mutant mice suggesting that the activator rather than repressor function of p63 was compromised. The remaining part of the manuscript is devoted to the identification of FgfR2 and FgfR3 as transcriptional targets of p63. It is proposed that downregulation of FgfR2/3 and thus impaired epithelial Fgf signaling in p63+/L514F heterozygous mice explains many of the defects observed in mutant mice and AEC patients. Data presented are convincing, and support authors’ conclusion. I enjoyed reading this interesting manuscript.

I have a few minor comments/questions.

Referee #3

1. RNA isolated from E14.5 wt and mutant epidermis was used to analyze expression of a number of candidate genes. However, it remained unclear to me how epidermis was separated and how the purity of the epidermal cell pool was tested?

Reply: At E14.5 the epidermis is a very thin tissue composed by a bilayer of cells and thus separation of epidermis and dermis is not feasible by enzymatic dissociation or by dissection. The epidermis therefore was not separated. For this reason in the manuscript we refer to E14.5 skin. Some of the analyzed genes are predominantly expressed in the epidermis, including Fgfr2b, Fgfr3b and miR-34a. However, others such as p21Cip1 and p16INK4a are likely to be expressed in both compartments of the skin. We cannot formally rule out the possibility that small differences in their epidermal expression might be masked by the dermal component. For this reason, we also measured their expression in isolated keratinocytes and found no difference in their expression.

Referee #3. 2. It is proposed that the phenotype of the p63+/L514F mice and possibly also AEC patients is due to compromised epithelial Fgf signaling due to downregulation of FgfR2 and FgfR3 transcripts. As a final piece of evidence it is shown that exogenously expressed Fgfr2b is able to restore cell proliferation (BrdU incorporation) in p63+/L514F keratinocytes (Fig. 7C). I am somewhat puzzled by these data, as elsewhere in the manuscript it was stated that there was no difference in proliferation between mutant and wt neonatal keratinocytes (Fig. S5A). Were these experiments done in a different way or what explains the difference between data shown in Fig. S5A and the left part in Fig. 7C?

Reply: We agree with the referee that this a confusing issue. Mutant keratinocytes proliferate to a similar extent of the wild-type ones under growing conditions in the presence of the several growth factors contained in the medium (Supplementary Figure S9A). In contrast, in the absence of growth factors, reduced expression levels of Fgfr2 and Fgfr3 results in a reduced proliferative response to KGF (Figure 5C), indicating that the defect is due to selective impairment of the FGF signaling and that this defect is observed only in the absence of other growth factors. We also added EGF treatment as an additional control, showing that activation of the MAP kinase pathway is normal in mutant keratinocytes (Suppl. Figure S9A), supporting the notion that mutant keratinocytes do not have a general proliferation defect but rather have a specific defect in FGF signaling. While dispensable for keratinocyte proliferation, we propose that FGF signaling may be required for cell proliferation at a specific time during development.

Referee #3. Statistical analysis of data shown in Fig. 7C would also be useful.

Reply: Figure 7C is a representative experiment of a set of three independent experiments. A student’s t-test performed between the ratio of mutant and wild-type cells expressing exogenous Fgfr2b and treated with KGF, versus the ratio of mutant and wild-type under basal conditions, revealed a statistically significant difference (p=0.005). We added this information in Legend to Figure 5C.

Referee #3. 3. Do the authors have any speculations why the similarities between Fgfr2b null and
p63+/L514F mutants were more striking in some tissues (skin, palate) than in others (e.g. tooth where FgfR2b has an early essential function)?

Reply: This is an interesting question. Some defects observed in embryos lacking Fgfr2b are not observed or observed at much lesser extent in p63+/L514F embryos. We speculate that in some tissues p63 may not be the main regulator of Fgfr2b expression or reduced Fgfr2b levels may be still sufficient to allow normal development. Conversely, other p63 target genes affected by AEC mutant may also contribute to the phenotype. In the revised version we present gene expression analysis of several p63 target genes that are affected in mutant skin and keratinocytes (Supplementary Table S1).

Table 1 for referees. Clonogenicity assays (Figure 6B).

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Exp 3
Thank you for the submission of your revised manuscript "Mutant p63 causes defective expansion of ectodermal progenitor cells and impaired FGF signaling in AEC syndrome" to EMBO Molecular Medicine.

We have now received the enclosed reports from the reviewers who were asked to re-assess it and you will be glad to see that they recommend publication. Before we can proceed to the official acceptance of your manuscript, please address the point below:

- Please see below for our instructions on how to mention p values. Please mention the actual p values in each case.

- Data of gene expression experiments described in submitted manuscripts should be deposited in a MIAME-compliant format with one of the public databases. We would therefore ask you to submit your microarray data to the ArrayExpress database maintained by the European Bioinformatics Institute for example. ArrayExpress allows authors to submit their data to a confidential section of the database, where they can be put on hold until the time of publication of the corresponding manuscript. Please see http://www.ebi.ac.uk/arrayexpress/Submissions/ or contact the support team at arrayexpress@ebi.ac.uk for further information. Please provide the accession number in the figure legend or material and methods part of the manuscript.

- We have noticed that your manuscript contains data, which are not shown. Please either include these data in the Supporting information or delete the reference to them.

Revised manuscripts should be submitted within two weeks of a request for revision. I look forward to seeing a revised form of your manuscript as soon as possible.

Statistical analysis
The description of all reported data that includes statistical testing must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the actual P value for each test (not merely 'significant' or 'P < 0.05').

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<td></td>
</tr>
<tr>
<td>+/-L514F</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>tot</td>
<td>14</td>
<td>9</td>
<td>67</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>15.6</td>
<td>10.0</td>
<td>74.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):
The manuscript by Ferone et al. is the first one to describe the generation of a knock-in mouse model (p63+/L514F) for the human AEC syndrome, one of the entities produced by mutations in p63 gene, and represents a major breakthrough in the field.

Referee #1 (Other Remarks):
In the revised manuscript by Ferone et al., the authors have addressed all the points raised in my previous review.

Referee #2 (Comments on Novelty/Model System):
This revised study is a significantly improved both in terms of clarity/organisation and analytical rigour.

Referee #2 (Other Remarks):
The authors have addressed all my concerns from the original submission and present a much improved study.

Referee #3:
I'm pleased that the authors take a considerable effort to address my concerns for the original manuscript. I now support the publication of the interesting and important manuscript in its current form.