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IRF6 is a mediator of Notch pro-differentiation and tumour suppressive function in keratinocytes

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1st Editorial Decision 10 June 2011

Thank you very much for submitting your research paper on the characterization of IRF6 as direct Notch-target gene in keratinocyte differentiation for consideration to The EMBO Journal editorial office.

Having received consistent comments from two expert scientists, I am able to reach a decision on your study to facilitate efficient proceedings. Ref#1 encourages stronger evidence for the causality of IRF6-regulation by Notch in SCC, whereas ref#2 is mostly concerned with the statistical significance of the proposed findings. Conditioned on addressing these major concerns and some minor issues that are clearly expressed in the reports, we would be delighted to receive a revised version of your paper for final assessment.

Please be reminded that it is EMBO_J policy to allow a single round of revisions only and that the final decision on acceptance or rejection depends on the content and strength of the revised version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

General comment:
This manuscript by Restivo and colleagues describes pivotal roles of the Notch/Interferon Regulatory Factor 6 (IRF6) axis in the regulation of keratinocyte differentiation and tumor suppression. Using molecular and genetic approaches, the authors demonstrate that the Notch/CSL pathway positively regulates the expression of IRF6 gene by directly binding to its promoter region and this up-regulation is required for the pro-differentiation and tumor suppressive functions of Notch signaling in the keratinocytes. This manuscript is well crafted. Experiments are elegantly designed and carefully executed, and in general support the conclusions drawn. As the authors suggest, the findings in this manuscript are definitively intriguing, and if the data would be solidified, would be of great interest to a wide variety of readers of the EMBO journal. One fundamental issue, however, should be addressed before publication.

Major point:
1. Although the authors postulate the implication of the IRF6 down-modulation in SCC tumorigenesis, the supporting data appears to be little bit descriptive. While the tissue microarray analysis show an inverse relationship between expression of Notch1/IRF6 versus EGFR/IRF7, it is not clear to what degree down-modulation of IRF6 contribute SCC tumorigenesis. It could be possible that such lower expression of IRF6 reflects differentiation status of each SCC sample. At least, the authors should take advantage of space limitations to present more thorough discussion about this issue.

Referee #2:

In this manuscript Restivo et al describe one of the mechanisms through which Notch signalling induces differentiation, and acts as a tumor suppressor pathway, in human and mouse epidermis. It has been previously shown that IRF6 is necessary for proper epidermal development, but the molecular mechanisms regulating IRF6 activity are currently unknown. The authors provide clear data showing that IRF6 is a direct target of Notch, and show that IRF6 is necessary to promote differentiation and suppress proliferation of basal keratinocytes. In addition they provide evidence that suggests that IRF6 may be an important tumor suppressor pathway in human squamous cell carcinomas.

The manuscript is well writen, the conclusions are clear and the data confirming these conclusions are adequate. However, there are several issues that should be addressed before the manuscript is apt for publication.

Specific comments:

1. It would be helpful if the authors cite and discuss the works of other groups which have also described in detail the functions of Notch over epidermal homeostasis (Blanpain et al., 2006; Williams et al., 2011; Wang et al., 2008; Ambler and Watt, 2010; Collins and Watt, 2008; Estrach et al., 2008; Lowell et al., 2000) with respect to their own findings. Otherwise the introduction and discussion sections appear too biased towards their own findings.

2. Fig 1A: The immunofluorescence of IRF6 shown in Fig1A shows a similar pattern of expression than that of Notch1. The colocalization is not entirely clear from the picture shown, and the staining shown for IRF6 should be enhanced (as it is it almost appears as a non-specific staining). In addition, although IRF6 acts as a transcription factor it is not nuclear in the epidermis. This is in clear contrast to the stainings shown in figure 9B (normal skin) in which IRF6 appears uniquely nuclear. Do the authors have any explanation for this? These stainings should be enhanced, and the lack of nuclear localization, if real, should be discussed in depth. The authors should provide an alternative method to describe the expression of IRF6 in the epidermis (i.e. RT-qPCR of sorted alpha6 integrin bright, dim and low populations, western of IRF6 in these populations, or an alternative method)

3. The error bars shown in Fig1B are rather small. This is surprising given the fact that the authors are working with primary human keratinocytes cultures which always show some variability from

experiment to experiment inherent to primary cultures. This makes one think that the error bar shown is from the triplicate of the qPCR rather than from the average of three independent results. Have the authors repeated these experiments three independent times? This same comment on the error bar is to be applied to every experiment shown in this work (i.e. the error bars are suspiciously small in all experiments considering they are working with primary cultures). They did use the same batch of keratinocytes for all their experiments (not ideal), or did they perform the experiments with keratinocytes isolated from three independent donors (desirable). This is not indicated neither in the main text, the figure legends, or the materials and methods. Here and throughout the entire manuscript the authors should clearly indicate how many times each experiment was performed. All experiments should be performed with keratinocytes obtained from at least two donors to avoid dealing with donor specific phenotypes.

4. Why is there no statistical analysis in Fig. 2C, 2D, 2F, Fig. 3B, 3C (right panel), 4B, 5B, 5C, 5D, 5E, 6A, 6B, 6C, 6D (middle panel), and Supp Fig 1? Were these experiments performed three independent times? For all these experiments the authors should state how many times they repeated the experiment, whether they used the same batch of keratinocytes (which in my opinion, if so, it would weaken the overall conclusions), and provide p-values for all the results.

5. In figure 5D there seems to be an increase in the levels of Hey1 in IRF6-depleted cells. Considering this fold change is not that different from the ones in which the authors describe an effect for IRF6, it is important to determine whether this is a statistically significant difference?

6. It is not clear why were the changes in proliferation determined in primary keratinocytes by measuring BrdU incorporation, and in SCCs by the levels of the transcript of Ki67.

7. In figure 8 the authors should show a staining for K5 or K14 (or any other basal marker such as alpha6, beta4, or beta1 integrins) to clearly delineate where the cysts are located. In its present form it is not clear where the cysts are located making hard to interpret the results.

1st Revision - Authors' Response 26 July 2011

We thank the reviewers for the overall favorable opinion and appreciation of the findings. We have addressed their concerns as follows :

Referee #1.

The main concern of this reviewer is that down-modulation of IRF6 in SCC cells is a consequence, rather than cause, of their low level of differentiation. We have addressed this issue in two ways. Experimentally, we now show (Fig. 3C) that, in SCC cells expressing the activated Notch1 protein in a tamoxifen-inducible form (SCC13-rNERT cells), induction of Notch activity under conditions of protein synthesis inhibition results in induction of IRF6 but not differentiation marker expression. This provides direct experimental support to the overall conclusion that IRF6 is a primary Notch target gene in keratinocytes, which is in turn involved in control of differentiation. Thus, the decreased IRF6 levels in SCC cells can be explained by compromised Notch signaling, with differentiation being a secondary cause. This does not rule out the likely possibility of an amplification mechanism, whereby differentiation is also reinforcing IRF6 expression and function. Positive feedback loops of this kind are very often at the basis of important cell fate decisions. Of relevance to the present situation, expression of Notch1 receptor as well as Jagged 1/2 ligands are under positive control of Notch pathway activation in keratinocytes as in other cellular systems. As recommended by the reviewer, we now consider these possibilities in the discussion (p. 15, starting 7th line from the bottom).

Referee #2.

1. We apologize for having limited overview of Notch signaling in keratinocytes to previous papers immediately related to our present work. We have rectified the problem. In the introduction, we now give a much broader overview, taking into consideration papers from other laboratories, including the most recent ones on this topic (p. 3, second paragraph, p. 4, first paragraph) and, in the discussion, we also place our findings in the more general context of work in the field (p. 14, first 5 lines; p. 16, 11th line from the bottom).

2. Fig. 1A : We have improved the quality of the IRF6 immunofluorescence images and indicated the dermal-epidermal junction by dotted lines to help with the interpretation of the pictures. The analysis shows clear IRF6 positive staining in the epidermis and absence of staining in the underlying dermis, with IRF6 being more highly expressed in the suprabasal layers in concomitance with the Notch1 protein. As for the question of subcellular localization, we have included high magnification images (Fig.1A, lower panels), showing that, while the IRF6 (red) and Notch1 (green) signals are mostly cytoplasmic, IRF6 can also be detected in the nuclei of some cells of the suprabasal differentiated cells (as indicated by arrows). For IRF6, we confirm the results by additional immunofluorescence / confocal imaging analysis of human skin, utilizing slightly different conditions (Suppl. Fig. 1).

We note that the quality of IRF6 staining that we have obtained with our affinity-purified antibodies is comparable to that previously shown by others, with antibodies raised against the same epitopes (Ingraham et al., 2006, Fig. 3q; Richardson et al., 2006, Fig. 4e).

Concerning the immunohistochemical analysis shown in Figure 9B, the pattern of IRF6 staining (as detected by the red chromogenic reaction) is largely cytoplasmic. The nuclear signal (blue) is due to hematoxylin counterstaining. We now specify this in the figure legend (p. 38, 8th line from the bottom).

As we indicate in the text (p. 6, 2nd line from the bottom; p. 17, bottom line and following page), our expression / localization findings are consistent with what reported in the literature in that : 1) In most systems, Notch1 nuclear localization is very difficult to demonstrate, consistent with the fact that, upon nuclear translocation, this protein is subject to rapid CDK8- and ubiquitin-dependent degradation (Kopan and Ilagan, 2009); 2) As previously reviewed (Bailey and Hendrix, 2008), IRF6, like other IRF family members, is also mostly localized to the cytoplasm, with its nuclear translocation (through an as yet uncharacterized mechanism) being quickly followed by its phosphorylation-dependent degradation.

As recommended by the reviewer, we have also used an alternative biochemical method to assess levels of IRF6 expression in human keratinocyte populations at various stages of differentiation. Keratinocytes from freshly dissociated human epidermis were separated on the basis of their rate of attachment to the substrate, which can enrich for undifferentiated keratinocytes with high self renewal potential (quickly adhering) versus cells at an intermediate or late stage of differentiation (adhering after longer time or failing to adhere) (Dazard et al., 2000; Jones and Watt, 1993). Immediate RNA preparation, without culturing, followed by real time RT-PCR analysis showed markedly higher expression of IRF6 in the differentiating versus proliferative compartments, which paralleled the up-regulation of differentiation marker expression (keratin 1 and loricrin) and the down-regulation of basal layer integrin ß4 expression (Fig. 1B).

3. As the reviewer noted, the error bars of real time RT-PCR analysis in Fig. 1 and all subsequent figures are usually small. This is because, as indicated in the text (p. 22, bottom lines), they correspond to triplicate measurement values within individual representative experiments. It would be impossible to pool data from different experiments since, as the reviewer is aware, there is intrinsic variability of results obtained with primary cells, especially of different origin. As the reviewer requested, for each panel we now state, in the corresponding figure legends, how many times and with how many different batches of keratinocytes we performed the experiment.

In fact, we fully agree with the reviewer on the importance of validating the results with cells of different origin and utilizing multiple conditions. Our paper is based on this principle, as all

conclusions are derived from combined results with different strains of primary human keratinocytes (as we now specifically indicate in the legends), primary keratinocytes and skin of both human and mouse origin, and multiple cancer cell lines and clinically occurring tumors. We note that the paper is based on results of several years of work, with multiple people involved, so that experimental repeats are often based on slightly different conditions and, for this reason, even more significant.

4. Statistical analysis of the results has now been performed for all requested experiments, with P values indicated in the corresponding figure legends.

5. In Fig. 5D, there is indeed a slight increase in Hey1 and Hey2 expression levels in cells with IRF6 depletion following activated Notch1 expression. This is in full agreement with our main conclusion that IRF6 plays a selective role in mediating the Notch effects on differentiation, while, for induction of canonical Notch targets, IRF6 is not required and/or, if anything, may negatively control it. This would also be consistent with our analysis of the epidermis of IRF6 mutant mice, in which there is a slight but statistically significant increase of Hes1 and p21 expression (Fig. 5E). This possibility is now indicated in the text (p. 11, starting 11th line from the bottom).

6. Ki67 is a well accepted marker of proliferation and, even in cultured cells, its expression can be used to assess levels of proliferation. We had used this method as an alternative to BrdU labeling with SCC cells as a way to assess the proliferate state of the same cells that were examined for expression of IRF6 and various differentiation-related genes. For consistency, we have extended use of this method to evaluate the proliferative state of HKCs plus/minus IRF6 knock down, under basal condition and in co-culture with Jagged 1 expressing fibroblasts (Fig. 6F).

7. Fig. 8. As requested by the reviewer, we have now included results of K14 staining (low magnification) in order to unequivocally delineate the location and size of the intradermal lesions formed by cells plus/minus IRF6 knockdown.

Cited literature

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Ingraham, C.R., Kinoshita, A., Kondo, S., Yang, B., Sajan, S., Trout, K.J., Malik, M.I., Dunnwald, M., Goudy, S.L., Lovett, M.*, et al.* (2006). Abnormal skin, limb and craniofacial morphogenesis in mice deficient for interferon regulatory factor 6 (Irf6). Nat Genet.

Jones, P.H., and Watt, F.M. (1993). Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell *73*, 713-724.

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2nd Editorial Decision 16 August 2011

The manuscript has been accepted after being reviewed by referee # one, who had the following comment:

This study presents a detailed dissection of pivotal roles of the Notch/Interferon Regulatory Factor 6 (IRF6) axis in the regulation of keratinocyte differentiation and tumor suppression. The main finding of this study is that the Notch/CSL pathway positively regulates the expression of IRF6 gene by directly binding to its promoter region and this up-regulation is required for the pro-differentiation and tumor suppressive functions of Notch signaling in the keratinocytes. In this revised manuscript the authors have addressed my comments regarding the previous version, and this paper is now suitable for publication in EMBO Journal.

