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Degradation of IF1 controls energy metabolism during osteogenic differentiation of stem cells

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(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 25 January 2013

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reviews on your manuscript.

You will see that all reviewers appreciate the interest of your findings and support publication of your study in our journal. However, they also point out aspects of your study that would need to be further strengthened to make the claims fully conclusive. For instance, referee 1 recommends testing the effect of differentiation on additional subunits of the mitochondrial respiratory chain. S/he also suggests to try and identify the protease that is responsible for the degradation by means of a knock-down approach. This reviewer also finds it hard to understand how the amount of respiratory chain complexes can be changed without an accompanying change in mitochondrial structure and/or number. Along these lines, referee 3 also feels that the data on the quantification of the mitochondrial content in both osteocytes and hMSCs would need to be strengthened and this may also help to address the concern of reviewer 2 with regard to this data. In addition, referee 3 also raises a number of technical issues, including missing or inadequate controls, and states that in some cases, additional clarifications are needed. S/he also feels that it should be investigated biochemically whether IF1 acts as an ATPase or and ATP synthase. Finally, referee 2 ask for further elucidation of the mechanism by which IF1 regulates stem cell differentiation, but we feel that this is beyond the scope of the current study and would therefore not make this a prerequisite for publication. If you already have additional data to this regard, you are, of course, welcome to include them if you wish.

Given these positive evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 29,000 characters (including spaces and references). If you feel that the additional data requested by the reviewers would make the manuscript too long you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only. I would also recommend combining the results and discussion section, as this helps to avoid unnecessary redundancies.

We have also started encouraging authors to submit the raw data of biochemical and/or microscopical images to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

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I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

Stem cells resemble tumour cells in the respect that both have shut down mitochondrial activity and activated anaerobic glycolysis for energy production. The mechanism how stem cells perform these task is unclear. In this paper, Sanchez-Arago et al. provide convincing evidence that the ATPase Inhibiting Factor 1 (IF1) may be a key molecule for inactivating the respiratory chain. This is an exciting and important new finding in a highly interesting and emerging field, namely the metabolism of stem cells.

Major points of criticism:

1. In Fig. 2A, data for a selection of subunits of the respiratory chain are shown. However, some important information is missing, for example no subunit of complex III is shown and the information on complex IV is provided only by probing for nuclear encoded cytochrome C oxidase subunit IV. This subunit, however, was shown to be assembled even in rho 0 cells, so it does not

really reflect the levels complex IV in the inner membrane. I strongly suggest to probe such western blots for additional subunits for complex I, for one of the core subunits of complex III, and some of the mitochondrial encoded subunits of complex IV to make the picture more clear. Probably the authors have already done that and found deviations from simple expectations. However, I would like to see these results and have them in the paper, even if they would leave open some important questions for the moment.
2. The use of an inhibitor of proteases is not state of the art. Since the authors have the method of siRNA in their hand, I highly recommend to try to show which of the mitochondrial proteases is responsible for degradation of IF1 by using siRNA. The soluble proteases Lon and Clp and the inner membrane proteases AFG3L2 or YME would be likely candidates.
3. The authors show electron microscopic pictures, in which the "number" of mitochondrial sections is not different between osteocytes and hMSCs. Also, the abundance of cristae looks similar, which is confirmed by the NAO signal. This finding is extremely hard to reconcile with the fact that the inner membrane surface always correlates with the amount of OXPHOS complexes. Again, probing for other subunits of the respiratory chain may give surprising results, probably there is a partially assembled respiratory chain and in addition to IF1, absence of some subunits may also be important for its inactivation in stem cells.
4. Of course, blue native page gels (BN-PAGE) would help to answer this question as well, so I don't understand why the authors have not used this method. An alternative would be to quantitate cristae surface in electron microscopic pictures by quantitative morphometry, but this is an extremely tedious task and I am not insisting on that. At least the authors should discuss this surprising lack of correlation between mitochondrial morphology and respiratory chain complexes in a more self-critical way.

Minor points:

Abstract:
Is "activation of the degradation" not better than "activation of the degradation rate"? Shouldn't it better say: "We conclude that IF1 ... " or "Our data support that IF1...."
Also the main finding is not only that IF1 is a stemness marker, the main finding is that it is important for keeping stem cells in the quiescence state.

Inroduction:
Page 3: What do you mean by resourceful?
Page 3: ... high proliferative capacity, as well as the capacity for renewal. (These are two different things!!).
Page 4: But is not expressed in differentiated osteocytes (delete "it").

Results:
Page 4: I would not call this increase in glycolysis in osteocytes large, but it is certainly significant. And why label the figure with 7, 14, and 21 nmol lactate ???
Page 6 lower para: Contrary to findings in colon cancer cells.....I don't understand this sentence.
Page 8 upper para: Higher energy requirements osteocytes, shouldn't it then say: "which cannot be covered by OXPHOS alone",
... and the relevance of metabolic regulation in defining the flux of glycolysis - I don't understand this part of the sentence.
Page 8 lower para: There is considerable evidence that kinetically, IF1 is able to inhibit the ATPase activity, however not the ATP-synthase activity. That is why classically the IF1 has been postulated to be an inhibitor of ATPase, in order to block this deleterious action of the enzyme under certain conditions. Please discuss why you think that IF1 can be a significant inhibitor of the ATP-synthase, as convincingly shown by your study.
Page 9 upper para: "The enhanced degradation of IF1 in osteocytes ... " (turnover is the summary of synthesis and degradation)?
What is a "subrogate" process, I don't know this word - do you mean surrogate - does this make sense ?
Should it be better "...that renders ongoing the differentiated state", or "keeps the differentiated state "?
Last sentence: "The regulated degradation of IF1 has the consequence that it"....would that be a better final conclusion ?
Page 10 lower para: No, you used qPCR, and not qRT-PCR.

Referee #2:

Sanchez-Arago et al. demonstrated that IF1, which was expressed in hMSCs but not in differentiated cells, regulated the activity of aerobic glycolysis, as shown by induction of cell differentiation of hMSCs through knock down-mediated silencing of IF1. Authors claimed that IF1 is an important marker gene of stem cells.

Major point
I can understand that IF1 plays an important function for maintaining stemness in hMSCs through the regulation of energy metabolism. However, authors did not show enough data on mechanisms of IF1, which was involved in bioenergic differentiation. If authors address to mechanisms more, this manuscript will become more informative for readership. For example, I want to know which signaling pathway is key for regulating the IF1 expression, and which downsteam genes (are HIF genes involved?) function in regulating cell differentiation.

I think that this manuscript is written well, and data are convincible. Function of IF1 is important enough for publishing to the EMBO Report. I think that this manuscript will be suitable for the EMBO Report after revision.

Referee #3:

Sanchez-Arago and colleagues examine ATPase inhibitory factor 1 (IF1) and find that it is expressed in human mesenchymal stem cells, prostate stem cells, and colon stem cells where they claim it inhibits oxidative phosphorylation and then is silenced in differentiated cells. Accelerated IF1 degradation increases oxidative phosphorylation and accelerates cell differentiation. The authors claim that IF1 is a stemness marker.

Overall the paper is interesting but has several important shortcomings that need to be addressed or clarified:

1. In Figure 1A, add scale bars and magnification in the figure legend. In Figure 2D, expand the text that indicates the size of the scale bars. In Figure 4A, 4B add scale bars and magnification information.

2. Figure 1C suggests that the osteocyte electron transport chain (ETC) is more tightly coupled to complex V than in mesenchymal stem cells, whereas Figure 3B suggests that mesenchymal stem cell ETC is coupled. Can the authors explain these seemingly contradictory results?

3. In Figure 1D, the authors should show the basal level of oxygen consumption in addition to OSR and MRR and plot the ratio against these basal levels. This is because they show multiple subunits in the OXPHOS Complex (I, II, IV) are up-regulated during osteogenic differentiation without any drug perturbations (Figure 2A); therefore the basal level of oxygen consumption should be increased as well, unless something else is regulating ETC activity and the levels data cannot be interpreted as having an important role in oxygen consumption rate.

4. In Figure 1E, cell death should be presented. A more than 50% decrease of ATP may indicate cell death as a cause. Then the conclusion might change to osteocytes are more sensitive to oligomycin-induced cell death because of deprivation of ATP generated from Complex V.

5. In Figure 2A, tubulin is underloaded in osteocytes; the lower protein level of a couple of glycolysis enzymes could be because of underloading. A balanced loading control is shown in Figure 3A, for example. The authors also showed basal level of glycolysis is not different in hMSC and osteocytes in Figure 1C despite these differences in enzyme levels- are the authors claiming another reason for the similarity in glycolysis levels with different levels of glycolytic enzyme

abundances? In addition to these comments, the authors' should normalize for tubulin abundance to allow comparisons of levels of glycolysis and OXPHOS enzyme levels.

6. A key is needed for Figure 2A plots- dark box = hMSC and light box = Ost.

7. In the text several places in the paper, the authors claim credit for showing that stem cells do not increase mitochondrial mass or numbers by proliferation but instead these measures remain relatively constant and mitos mature in their functions as they differentiate. These ideas were demonstrated and reported before for pluripotent stem cell differentiation (Birket, et al J Cell Sci 124:348-358, 2011; Zhang et al EMBO J 30:4860-4873, 2011) and needs to be cited and discussed in the context of the authors' current findings throughout the text.

8. Figure 2C- cardiolipin can vary between mitochondria sources and is not a good method for quantification of mito content. Instead, the authors could use citrate synthase activity assays (Zhang et al EMBO J 30:4860-4873, 2011) or quantify by Western blot for porin (VDAC).

9. The quantities beta-F1-ATPase/Hsp60 ratio as a measure of bioenergetic competence in Figure 2E and beta-F1/GAPDH ratio as a measure of mitochondrial capacity in Figure 2A are not generally used in the field and are not intuitive measures of complex V function; if they are to be used as the authors' intend, the rationale for these ratios need to be clearly explained and or referenced to support their use.

10. Its difficult to believe that hMSCs and osteocytes have only 6 mitos per cell (Figure 2D). The use of EM to quantify mito numbers in this manner is too sensitive and underestimates their number. Rather, confocal microscopy with a mito-vital fluorescent dye, such at TMRM or even mitotracker, should provide a more reasonable result. Pluripotent stem cells imaged and counted in this way show many more granular-appearing mitos than these more differentiated cells.

11. In Figure 3C, the authors used OSR to indicate ATPase activity. It is controversial about whether IF1's role is to inhibit ATPase activity or ATP synthase activity, (Laura Sánchez-Cenizo, JBC, 2010), (Michelangelo Campanella, Cell Metabolism, 2008). Therefore, it's important for the authors to demonstrate IF1's role biochemically as an ATPase or ATP synthase in the context of the cells used in their study. Also it's been shown that IF1 is highly expressed in more oxidative neuronal cells as compared to being lowly expressed in glycolytic astrocytes (Michelangelo Campanella, Cell Metabolism, 2008). Finally, the IF1/beta-F1-ATPase ratio has been shown to be low in human pluripotent stem cells compared to differentiated cells, such as human embryonic fibroblasts (Zhang et al EMBO J 30:4860-4873, 2011). How do the authors reconcile their findings with these prior studies?

12. Page 6 in the text- the authors' state that IF1 is a major switch for energy metabolism from Figure 3D data, which shows a ~25-30% ATP effect from siRNA against IF1, compared to the ~60-70% ATP effect for blocking complex V in Figure 1E- from this, can't one conclude that IF1 is just one part of the controlling "switch" in energy metabolism that the authors are reporting? It seems doubtful that degrading IF1 with differentiation is akin to turning off and on a single switch for metabolic change, such as a room light.

13. In Figure 3F, the densitometry ratios of IF1/b-actin in untreated hMSC and osteocytes do not seem correct when looking at the Western blots versus the ratio plots. They should not look the same from the data in the Westerns.

14. Figure 3H shows the change in CD44 and osteopontin expression as an indication of differentiation, but it would be more powerful to show cell images, as seen in Figure 1A, to conclude that this is more than 2 biomarker changes under these conditions.

15. On page 7 of the text, the authors said that IF1 could be a stem cell marker. How does that apply to pluripotent stem cells which are also glycolytic but have a low IF1/beta-F1-ATPase ratio?

ANSWERS TO REVIEWERS

Referee #1:

-We thank the reviewer for appreciating the novelty and relevance of our contribution in the field of the metabolism of stem cells.

Major Points:

1) Following the reviewer's indication we have now determined the expression level of the additional subunits of the respiratory chain complexes requested. Specifically, we have analyzed the expression of NDUFS3 from complex I, Core 2 from complex III and of two mitochondrial-encoded subunits from complex IV (COXI and COXII). The results obtained are presented in new Figures 2A and Figure S2A. The Results (see page 5) and Supplemental section have been modified accordingly. We should mention that we also analyzed the expression of the mitochondrial-encoded ND4 (Abcam, ab130496) from complex I but unfortunately the supplied commercially available antibody didn't work. Overall, the data shows that differentiation of hMSCs triggers an increase in the expression of most protein subunits of the respiratory chain.

2) Following the reviewer's indication we have done the screening experiments aimed at the identification of the protease involved in the degradation of IF1. We have knocked-down by siRNA the expression of the four proteases requested (Lon, Clp, AFG3L2, YME) and of three additional ones (HtrA2, PMPCB, SPG7) in hMSCs and colon cancer HCT116 cells (see new Figure S5). Silencing of the proteases was verified by western blotting (Lon, Clp, HtrA2). When the commercial antibodies available provided non-reliable signals in the blots (anti-Spg7, Abcam, ab96213; anti-PMPCB, Abcam, ab103343; anti-AFG3L2, Abcam, ab68023) or didn't arrive on time (Yme1L1, Thermo Scientific, PA5-24808) the silencing was verified by RT-qPCR. The knock-downs attained were in the 80%-40% range. Unfortunately, under these conditions the data obtained showed no major impact on the accumulation of IF1 in the cell lines studied (new Figure S5), what might be explained by (i) the partial silencing attained, (ii) over-lapping functional activities of the proteases, (iii) off-targeting effects in response to partial silencing of the proteases, (iv) the time-window of siRNA studies when compared to AEBSF experiments and/or (v) because none of the proteases investigated is responsible for the degradation of IF1, suggesting that the control of the degradation of IF1 is more complex that originally anticipated. The Results (see page 7) and Supplemental section have been modified accordingly to incorporate these findings.

3) In agreement with this comment of the reviewer (see also comment 10 of R#3) we have stained osteocytes and hMSCs with MitoTracker Red to check out the abundance and morphology of mitochondria in live cells. The results illustrate that hMSCs have punctiform organelles whereas osteocytes preferentially show thread-like mitochondria (new Figure 2D). This change in mitochondrial morphology was not accompanied by significant differences in fluorescence intensity further supporting that mitochondrial changes in morphology might result from the functional differentiation of the organelles. Hence, we reanalyzed by electron microcopy putative differences in mitochondrial ultrastructure between hMSCs and osteocytes. Remarkably, we found a large percentage of mitochondria with abnormal structure (onion-like) in hMSCs when compared to osteocytes, what emphasizes the presence of immature organelles in these cells (new Figure 2E). We thank the reviewers for their comments because as a result we have documented an event that we over-looked in our previous submission. These findings have been incorporated in new Figure 2 and the Results (see page 5) and Supplemental section have been modified accordingly.

4) As suggested by the reviewer we have run BN-PAGE to investigate a putative impairment on the assembly of respiratory complexes in hMSCs. The results obtained revealed no main differences in respiratory complexes between hMSCs and osteocytes. These results have been incorporated in the new Figure S2B and the Results (see page 5) and Supplemental section have been modified accordingly. Hence, these findings support that hMSCs do not have an impairment on the assembly of the respiratory chain further stressing that osteogenic induction promotes the bioenergetic differentiation of pre-existing abnormal mitochondria of hMSCs (see major point 3).

Minor points:

Abstract: In agreement with the reviewer's suggestions the revised Abstract incorporates the three indicated comments.

Introduction: We have modified the Introduction following the reviewer's suggestions. On page 3 we have deleted "resourceful". The sentence now reads "Adult human mesenchymal stem cells (hMSCs) are characterized by their multi-lineage differentiation potential (pluripotency) and their self-renewal capacity". On page 4, we have deleted "it."

Results:
Following the reviewer's suggestions on page 4 we have deleted the adjective "large". Moreover, the scale on new Fig. 1C has been relabeled from 0-25 nmol lactate/μg protein/h.

Following the reviewer's suggestion on page 6 (now beginning of page 7) the new sentence now reads "The regulated expression of IF1 does not affect the proliferation rate of hMSCs (Fig. S4) in contrast to data obtained in colon cancer cells [3] suggesting a cell-type specific variability in nuclear responses to IF1 signaling".

On page 8 we have incorporated the reviewer's suggestion. Moreover, we have reworded the last sentence to indicate that the flux of glycolysis is also controlled by allosteric regulation of key enzymes of this metabolic pathway.

Following the reviewer's suggestion on page 8 (lower paragraph) (now page 9), in the revised version we have discussed the role of IF1 as an inhibitor of the ATP synthase. The new sentences read: "Kinetic evidence indicates that IF1 inhibits the ATP hydrolase activity of the H^+-ATP synthase [1]. However, it is likely that binding of IF1 to the H^+-ATP synthase also depends on the mass-action ratio and hence when IF1 is expressed, as it is the situation in hMSCs, it can also inhibit the synthase activity of the enzyme. Indeed, silencing of IF1 in hMSCs resulted in a drop in glycolysis and a concurrent increase in ATP synthase activity. Conversely, its overexpression in different cell types has been shown to increase glycolysis and to inhibit the ATP synthase activity, mimicking the metabolic effects triggered by the ATP synthase inhibitor OL [2,3]".

On page 9 (now page 10) we have followed the reviewer's indication: turnover has been substituted by degradation, surrogate has been spelled correctly, and the alluded sentence now reads: "…as a surrogate process of the nuclear reprogramming that renders ongoing the differentiated state…"

To comply with reviewer's suggestion we have rephrased the last sentence of the Discussion, now it reads: "Overall, we show that IF1 is a stem cell marker that regulates energy metabolism of hMSCs. The regulated degradation of IF1 hinders self-renewal of stem cells to favor differentiation".

On page 10 (now page 11), we have corrected qPCR as requested.

Referee #2:
We thank the reviewer for his/her positive comments towards our work. The revised version of the manuscript now nicely documents the bioenergetic differentiation of mitochondria in hMSCs upon osteogenic induction. As previously mentioned, we are grateful to the reviewers for their valuable comments on this regard which have significantly improved the paper. As we have illustrated by the new experiments incorporated in the revised version of the paper the study of the regulation of IF1 is very complex. Unveiling the signaling pathways and of the mechanisms that regulate IF1 expression and function are at the core of the activities of our laboratory and we should mention that we have no evidence for the implication of HIF1α in its regulation.

Referee #3:
-We thank the reviewer for appreciating the significance of our work.

1) Following the reviewer's indications in this point: we have added the scale bars and magnification in new Figure 1A; in the new Figure 2D we have expanded the text that indicates the size of the scale bars and in new Figure 4A and 4B we have included the scale bars and the magnification information.

2) We agree with the reviewer that data on Figure 1C suggests that the osteocyte electron transport chain is more tightly coupled to complex V than in hMSCs. However, there is no contradiction with data on Figure 3B. In fact, Figure 3B supports that upon silencing of IF1 in hMSCs the electron transport chain becomes coupled to complex V. In other words, it supports that the presence of IF1

is hindering the ATP synthetic activity of the ATP synthase (see new paragraph included in this regard as a result of the request of R#1).

3) To satisfy the reviewer's concerns we have included in the new Figure 1D the basal rates of respiration in both hMSCs and osteocytes. As anticipated by the reviewer the basal level of oxygen consumption is significantly increased in the osteocytes when compared to hMSCs paralleling the changes observed in the expression of the proteins of respiratory complexes. The Results section (see page 4) has been modified accordingly to incorporate these findings.

4) Following the reviewer's suggestion we have studied the potential cell death triggered by oligomycin treatment in hMSCs and osteocytes. These data has been included as new Figure S1. The results obtained reveal no significant effect of oligomycin in cell death in any of the cells studied excluding the possibility that the drop in ATP observed in osteocytes after OL treatment could result from an enhanced cell death. The Results (see page 4) and Supplemental section have been modified accordingly to incorporate these findings.

5) Yes, as indicated above (see Minor points of R#1) the similar rates of glycolysis observed between osteocytes and hMSC suggest that the higher energy requirements of the differentiated cells cannot be covered by OXPHOS alone and need additional supply from the activation of glycolysis. Since osteocytes show the down-regulation of the cellular content of glycolytic enzymes we suggest that the activation of glycolysis in these cells result from allosteric activation of any of the key enzymes that regulate glycolysis. These comments have been introduced in the revised version of the paper (see page 8).
We should mention that selecting a "loading" control is not an easy task in this sort of experiments because during differentiation changes in the expression of proteins involved in the cytoskeleton, mitochondria and energy metabolism occur. In any case, we should emphasize that irrespective of the method used to normalize the expression of the proteins the results obtained are essentially the same. For reviewer's information we present the two alternatives we have used for assessing protein expression:
A.- Method used in the paper: The expression of the protein is normalized relative to the signal obtained for the same protein in HCT116 cells which were included in all experiments for inter-gel comparisons. The value obtained was further expressed as fold change of the value obtained in hMSCs.
B.- Normalized by tubulin expression.

	Method A		**Method B**	
	hMSCs	*Osteocytes*	*hMSCs*	*Osteocytes*
GAPDH	1.0 ± 0.01	0.3 ± 0.04	5.0 ± 0.02	2.2 ± 0.37
LDH-A	1.0 ± 0.01	0.4 ± 0.03	3.0 ± 0.01	1.7 ± 0.12
PKM2	1.0 ± 0.03	0.3 ± 0.01	3.5 ± 0.19	1.8 ± 0.12

6) Following the reviewer's indication the color code used is now indicated in the figure legend which in the revised version of the paper is new Figure S2A.

7) Following the reviewer's indication we have now quoted and commented the alluded references in the Results and Discussion sections of the revised version (see page 5 and page 9, beginning and end of page)(new references 9 and 15).

8) We do not fully support the reviewer's argument. Nonyl acridine orange (NAO) has been widely used to analyze the mitochondrial content or to measure changes in mitochondrial mass. Unlike many other mitochondrial probes is known to bind cardiolipin regardless of the energetic state of mitochondria. In any case, to satisfy the reviewer's concern, we have now determined the protein expression of the voltage-dependent anion channel (VDAC/porin) by western blotting. The results obtained (new Figure 2A and Supplemental Figure S2A) revealed no significant differences in its expression between hMSCs and osteocytes, thus reinforcing the lack of significant changes in the mitocondrial content. Moreover, and as suggested by the reviewer in the following point, staining of mitochondria with MitoTracker Red further reinforce the absence of differences in mitochondrial mass between hMSCs and osteocytes (see new Figure 2D).

9) Following the reviewer's indication the two ratios have been explained in detail in the Supplemental information and appropriately referenced both in the main text (new reference 8).

10) As indicated above (see main point 3 of R#1) we agree with the reviewer's comment. Following his/her indications we have carried out the experiment requested and stained mitochondria in osteocytes and hMSCs with MitoTracker Red to check the number and morphology of mitochondria. The results illustrate that hMSCs have punctiform organelles whereas osteocytes preferentially show thread-like mitochondria (new Figure 2D). This change in mitochondrial morphology was not accompanied by significant differences in fluorescence intensity further supporting that mitochondrial changes in morphology might result from the functional differentiation of the organelles. Hence, we reanalyzed by electron microcopy putative differences in mitochondrial ultrastructure between hMSCs and osteocytes. Remarkably, we found a large percentage of mitochondria with abnormal structure (onion-like) in hMSCs when compared to osteocytes, what emphasizes the presence of immature organelles in these cells (new Figure 2E). We thank the reviewers for their comments because as a result we have documented an event that we over-looked in our previous submission. These findings have been incorporated in new Figure 2 and the Results (see page 6) and Supplemental section have been modified accordingly.

11) Most of the studies regarding the biochemistry of IF1 were done *in vitro* with recombinant proteins and by sedimentation equilibrium analysis. These studies investigated the ATP hydrolytic activity of F1-ATPase (see Gledhill et al., 2007 for review). We have studied the effect of IF1 *in vivo*. In fact, data in our previous contributions (JBC 285, 25308-25313, 2010; Mol. Cell 45, 731-742, 2012) conclusively showed that the overexpression of IF1 inhibits the synthase activity of the H^+-ATP synthase and promotes aerobic glycolysis. Moreover, and in response to IF1 overexpression we have documented that the mitochondrial membrane potential ($\Delta\Psi m$) increases concurrently with ROS production in different cancer and non-cancer cells. Our findings are in absolute agreement with similar findings obtained by the overexpression of IF1 in mouse embryonic fibroblasts (Shen et al., Cell Death Diff. 16, 603-612, 2009). Our previous data (JBC 285, 25308-25313, 2010) obtained in cells that naturally have a high content of IF1, such as HeLa and T47D argue against the IF1 overexpression data reported by Campanella et al., in HeLa cells (Cell Metab. 8, 13-25, 2008). We have demonstrated that these cells (HeLa, T47D,...) are already highly glycolytic showing no response in the metabolic flux of glycolysis or on cellular ATP levels to oligomycin treatment, questioning their use as model systems to investigate the role of the overexpression of IF1 in cellular adaptive responses. A different situation is offered by experiments in which IF1 is being silenced in the cells. In this regard, we do not question or argue against the studies by Campanella et al, 2008 and Shah et al., (Nature 491, 608-612, 2012) because the inhibition of the synthase activity of the ATP synthase by the mass-action ratio is not incompatible with the pH dependent regulation of the hydrolase activity of the ATP synthase by IF1 in situations of mitochondrial dysfunction. The expression of IF1 varies greatly depending upon the cell type analyzed (JBC 285, 25308-25313, 2010) hence, cell type specific differences might explain the different content of IF1 between pluripotent stem cells and differentiated embryonic fibroblast. Paradoxically, neurons and other normal human tissues with a high metabolic demand overexpress IF1, suggesting that an additional mechanism of regulation of the activity of IF1 operates in these tissues that remains to be investigated (see also point 15 for additional information regarding this point).

12) In agreement with the reviewer's suggestion in the revised version of the paper we now suggest that IF1 is part of the switch that controls energy metabolism upon osteogenic induction of hMSCs (see page 6-7).

13) In answer to this comment of the reviewer we should mention that the values reported in Figure 3F are the mean of the calculated IF1/β-actin ratio of four different experiments and basically illustrate what is being shown in the western blots.

14) We partially agree with the reviewer's comment. However, analysis of cell morphology did not provide as clear evidence of the influence of IF1 silencing on differentiation as the molecular data (Figure 3H). In fact, acceleration of differentiation was only half-way as reveal by the osteopontin/CD44 ratio.

15) As above commented the expression of IF1 varies greatly depending upon the cell type analyzed and cell type specific differences might explain the different content of IF1 between pluripotent and mesenchymal and other stem cells. Moreover, the mechanism that controls the metabolic switch during differentiation of pluripotent stem cells seems to be mediated by the repression of UCP2 expression. A comment in this regard has been included in the revised version of the paper (see page 9). It reads: “However, other mechanisms might also operate since it has been reported that human pluripotent stem cells have a low content of IF1 and their differentiation is triggered by repression of UCP2 [9]”.

2nd Editorial Decision 10 May 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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REFEREE REPORTS:

Referee #1:

The authors have performed a tour-de-force now to satisfy most of the requests, even most of the completely exaggerated requests of Reviewer # 3. This beautiful work should now be published.

Referee #3:

The statement in the abstract that "Activation of IF1 degradation acts as the switch that regulates energy metabolism during differentiation" needs to be modified to be concordant with the text on page 6-7 that states "These results suggest that IF1 is part of the switch that controls energy metabolism upon osteogenic induction of hMSCs (Fig. 1)".

