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Salt Inducible Kinase induces cytoplasmic Histone Deacetylase 4 to promote vascular calcification

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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 22 December 2016

Thank you for the submission of your manuscript to our journal. I am sorry for the unusual delay in getting back to you, which is due to the fact that we have still not received the final referee report. Given that the other two referees are in fair agreement, I will make a decision now, but please note that this is subject to change should the third referee have very strong and convincing reasons for it. The two reports are copied below, as well as comments from an external advisor.

As you will see, both referees acknowledge that the findings are potentially interesting, but that the study should be strengthened. Given the number and nature of the referee comments, I think that all of them should be addressed. I also think that the advisor's comments should be addressed too.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

The authors address the role of histone deacetylase 4 (HDAC4) in the regulation of vascular calcification. Prior studies by other groups have linked nuclear HDAC4 to enhancement of bone

formation through transcriptional mechanisms. Here, the authors provide evidence to support a role for cytoplasmic HDAC4 as a stimulator of vascular calcification. HDAC4 expression is shown to be upregulated in models of vascular calcification and in calcified human aortic valves. Furthermore, overexpression of WT HDAC4 (which is both nuclear and cytoplasmic) but not nuclear localized form of HDAC4 in vascular smooth muscle cells promotes expression of osteochondrogenic marker genes such as osteocalcin. The authors provide evidence to support a role for salt-inducible kinase (SIK) as an HDAC4 nuclear export kinase, and show that a SIK inhibitor suppresses vascular calcification, presumably by retaining HDAC4 in the nucleus. Finally, the authors reveal a novel interaction between HDAC4 and ENIGMA, a cytoplasmic protein that was previously shown to promote bone formation. Indeed, in the authors' hands, knockdown of ENIGMA in vascular smooth muscle cells also reduced osteochondral differentiation.

The findings are interesting and generally convincing. However, several gaps should be addressed prior to publication.

Specific points:

1. In Figure 1A, why was qPCR performed to assess expression of certain HDAC isoforms but not others? For completeness, the authors should quantify expression of HDACs 1 - 9. The authors should also confirm that HDAC4 protein expression is elevated during valve calcification.

2. For the overexpression studies in Figure 2, what is the degree of HDAC4 overexpression relative to endogenous HDAC4 expression?

3. The data in Supplemental Figure 1 are important for the story and should therefore be in the main body of the manuscript.

4. Validation of inhibitor data with siRNA knockdown of SIK isoforms would strengthen the findings with SIK inhibitors and HDAC4 subcellular localization. Granted, there are three SIK isoforms, which complicates the issue, but the authors should at least try the experiment.

5. As an extension of Figure 8, the authors should determine if ENIGMA knockdown reduces HDAC4-mediated induction of osteochondrogenic marker gene expression.

6. The authors should comment on the recent paper by Wein et al. (Nat Commun. 2016 Oct 19;7:13176), which links SIK and HDAC4 to bone formation but provides data that are counterintuitive relative to the present findings (the other paper shows that SIK inhibition promotes bone formation by retaining HDAC4 in the nucleus of osteocytes).

Referee #3:

The authors report that cytosolic HDAC4 promotes calcification in vascular smooth muscle cells (VSMCs), and this is most likely dependent on salt-inducible kinase (SIK). The authors provide cell culture, aortic ring culture, and in vivo experimental data supporting these findings. They identify the adaptor protein Enigma as a binding partner for cytosolic HDAC4 and suggest that this protein allows for cytosolic HDAC4-induced calcification in VSMCs, although exactly how this occurs is not clear.

This is a very interesting, well-developed study that investigates the cytosolic role of HDAC4 in VSMCs and its role in aortic calcification. While much is known about the nuclear roles of HDACs, the cytosolic roles of these proteins have not been clarified. It is intriguing that HDAC4 seems to be mainly cytosolic in calcified VSMCs. The initial observations and the experiments identifying SIK as a regulator of HDAC4 in VSMCs are strong, but the field would benefit from a more detailed investigation of how cytosolic HDAC4 increases calcification of VSMCs.

Major points:

1. Are osteocalcin or Sox9 direct targets of transcription factors regulated by HDAC4 in VSMCs?

2. Why does osteocalcin expression increase with increased HDAC4 expression, given that HDAC4 is generally a transcriptional inhibitor? (Figure 2A and Supplemental Figure 1A)

3. In Figure 3B/C it is difficult to determine if the cytosolic GFP is just background in the 3SA-infected VSMCs. Perhaps a western of nuclear vs cytosolic HDAC4 and HDAC4-3SA should be performed on the infected VSMCs. Also, representative pics of HeLa experiments (similar to Figure 3B) should be provided in the supplement.

4. The findings in Supplemental Figure 1 are downplayed by placing them in the supplemental data. These experiments seem to be critical as they demonstrate that cytosolic HDAC4 is required for the calcification process, and it is suggested that they be moved to Figure 3, and maybe Figure 3A can be moved to the supplement.

5. In Figure 6, the reversal of aortic calcification by HG-9-91-01 in vivo seems like a promising therapeutic approach. How does the dosage and treatment time compare to what might be used in humans? Perhaps off-target and side effects of the pan-SIK inhibitor should be mentioned in the discussion?

6. In Figure 8, Enigma protein levels in Enigma siRNA experiments should be determined. Can HDAC4 cytosolic localization be forced by overexpression of Enigma in VSMCs? Unaltered HDAC4 levels and localization after knockdown of Enigma suggests other proteins are involved in HDAC4-regulated calcification. Perhaps the role of osteocalcin should be investigated?

7. The authors might also include in the discussion the relative importance of cytosolic HDAC4 in regulating calcification of VSMCs versus decreased gene regulatory function. For example, does cytosolic HDAC4 allow for increased expression of calcification genes that enhance calcification (other than the markers reported herein), or is HDAC4 involved in another cytosolic mechanism that promotes calcification, such as by forming a matrix for calcium deposition?

Minor points:

1. In Figure 2, controls for Ad exps and siRNA exps should be included: protein levels and localization of HDAC4 in VSMCs and aortic rings need to be shown (FLAG immunostaining would be great for Ad exps).

2. What happens to calcification of aortic rings with the addition of HDAC4 in the presence of HPM?

3. Error bars are missing for Figure 3C, 4B, supplemental Figure 2A. There seems to be large variability in HDAC4 localization in response to 10uM HG-9-91-01 (Figure 4B versus Supplemental Figure2A), please comment.

Comments from external advisor:

The paper is very interesting and might provide new insights. But further investigations need to be conducted. I agree with your concern that it is not clear how HDAC4 regulates calcification in concert with ENIGMA. It is not surprising that HDAC4 regulates calcification in view of pervious reports about the interaction with Runx2. But here, the authors suggest a cytoplasmic role, which somehow contradicts the previous data and potentially provides a new conceptual thinking . It is indeed important to describe the cytoplasmic functions (because HDAC4 lives mostly in the cytoplasm and it seems to bind to acetylated proteins without deacetylating them) but so far this paper only gives a rough idea but not very deep insights. I think the authors need to substantiate their claims and in particular they need to show how HDAC4 regulates ENIGMA and - most importantly - they need to demonstrate thatHDAC4 and ENIGMA not only act in parallel but that they indeed depend on each other.

1st Revision - authors' response 19 March 2017

Please find enclosed the revised manuscript entitled “Salt Inducible Kinase induces cytoplasmic Histone Deacetylase 4 complex to promote vascular calcification” which we revised and are re-submitting for consideration of publication in EMBO Reports. We want to thanks the referees, external advisor, and you for the time and effort in the fair, yet critical review of our data. We have carefully read and addressed all the referees and external advisor comments, and performed all the requested experiment. These are now displayed in the revised manuscript, that includes 18

additional figure panels. A major point raised by both reviewers and external advisor was the interdependence of HDAC4 on ENIGMA for function. We agree with the referees and advisor that this point was not fully explored in the original manuscript, and have now conclusively addressed it. We show that overexpression of ENIGMA is not sufficient for induction of Osteocalcin and the osteochondral genes, and show that knocking down of ENIGMA significantly blunts the effects of HDAC4 overexpression, proving interdependence.

The revised manuscript now provides multiple lines of strong evidence for the cytoplasmic function of HDAC4 in driving vascular calcification, and mechanistically show that it requires phosphorylation by SIK and binding to the cytoskeletal associated protein ENIGMA. We also added data supporting a mechanosensing role for this cytoplasmic complex. Importantly, to the best of our knowledge, this is the first study that firmly establishes a functional cytoplasmic role for class IIa HDACs in general, and specifically for HDAC4. It is also the first implication of HDAC4, SIK and ENIGMA, and the association between them, as mediators of vascular calcification.

Below please find a point by point address to the referees and advisor comments. All reference in our response is to the revised manuscript text and figures. We have colored our response below each comment in blue for the sake of ease and clarity. References in our response are shown at the bottom, under the heading References for referees.

POINT BY POINT RESPONSE

Referee #1:

The authors address the role of histone deacetylase 4 (HDAC4) in the regulation of vascular calcification. Prior studies by other groups have linked nuclear HDAC4 to enhancement of bone formation through transcriptional mechanisms. Here, the authors provide evidence to support a role for cytoplasmic HDAC4 as a stimulator of vascular calcification. HDAC4 expression is shown to be upregulated in models of vascular calcification and in calcified human aortic valves. Furthermore, overexpression of WT HDAC4 (which is both nuclear and cytoplasmic) but not nuclear localized form of HDAC4 in vascular smooth muscle cells promotes expression of osteochondrogenic marker genes such as osteocalcin. The authors provide evidence to support a role for salt-inducible kinase (SIK) as an HDAC4 nuclear export kinase, and show that a SIK inhibitor suppresses vascular calcification, presumably by retaining HDAC4 in the nucleus. Finally, the authors reveal a novel interaction between HDAC4 and ENIGMA, a cytoplasmic protein that was previously shown to promote bone formation. Indeed, in the authors' hands, knockdown of ENIGMA in vascular smooth muscle cells also reduced osteochondral differentiation.

The findings are interesting and generally convincing. However, several gaps should be addressed prior to publication.

Specific points:

1. In Figure 1A, why was qPCR performed to assess expression of certain HDAC isoforms but not others? For completeness, the authors should quantify expression of HDACs 1 - 9. The authors should also confirm that HDAC4 protein expression is elevated during valve calcification.

We thank the referee for his comments. We now provide in the revised manuscript an analysis of all HDACs. It should be noted that the absolute expression level of class IIa HADC5 and HDAC9 is comparatively low. Both these HDACs are upregulated several folds during vascular calcification, however we feel this representation may mislead readers, as the absolute level is low (compared with the other HDACs). We now show the expression of HDACs 1,2,3,4,6,7,8 in Fig 1C, and the expression of HDACs 5, 9 in Fig EV 1A. The relative expression of all HDACs, showing the low level of expression of HDAC5 5,9 is shown in Fig EV 1B. The revised manuscript now reads:

"We examined the expression of different HDACs upon induction of VSMCs calcification, and observed unchanged expression of HDACs 1,2 and 7, a small but significant upregulation of the class I HDAC3 (1.16 fold), an upregulation of the class I HDAC8, the class IIb HDAC6, and a 2 fold up-regulation of the class IIa HDAC4 levels (Fig 1C). The expression of the class IIa HDAC5

and HDAC9 was also significantly upregulated upon induction, but their absolute level was low (Fig EV1A and B)."

We tried extracting proteins from calcified valves or calcified aortic rings using several approaches and protein extraction buffers, however we got very poor yields, most probably because of the calcified and fibrotic nature of these tissues. Instead we have analyzed the HDAC4 protein levels from VSMCs. This analysis shows a two-fold elevation in HDAC4 protein level, that is consistent with the similar degree of upregulation of HDAC4 mRNA. This concordance between mRNA and protein levels for HDAC4, strengthen our mRNA analysis from aortic rings and human valves. The analysis is now presented in Fig 1D with quantification. We also analyzed the protein level of HDAC4 with control or HDAC4 siRNA (Fig EV2D), and see a concordance between the degree of HDAC4 mRNA and protein knockdown. The revised manuscript now reads: "We also analyzed HDAC4 protein level, and noted a similar significant 2-fold increase in its level (Fig 1D)."

2. For the overexpression studies in Figure 2, what is the degree of HDAC4 overexpression relative to endogenous HDAC4 expression?

We analyzed the over-expression of HDAC4 relative to the endogenous levels by western blot. Ad-HDAC4 overexpression results in approximately 1.6-fold increase for the 'low' adenovirus concentration and 2.5 fold increase in HDAC4 protein level for the 'high' concentration. The results are now presented in Fig EV2A with quantification.

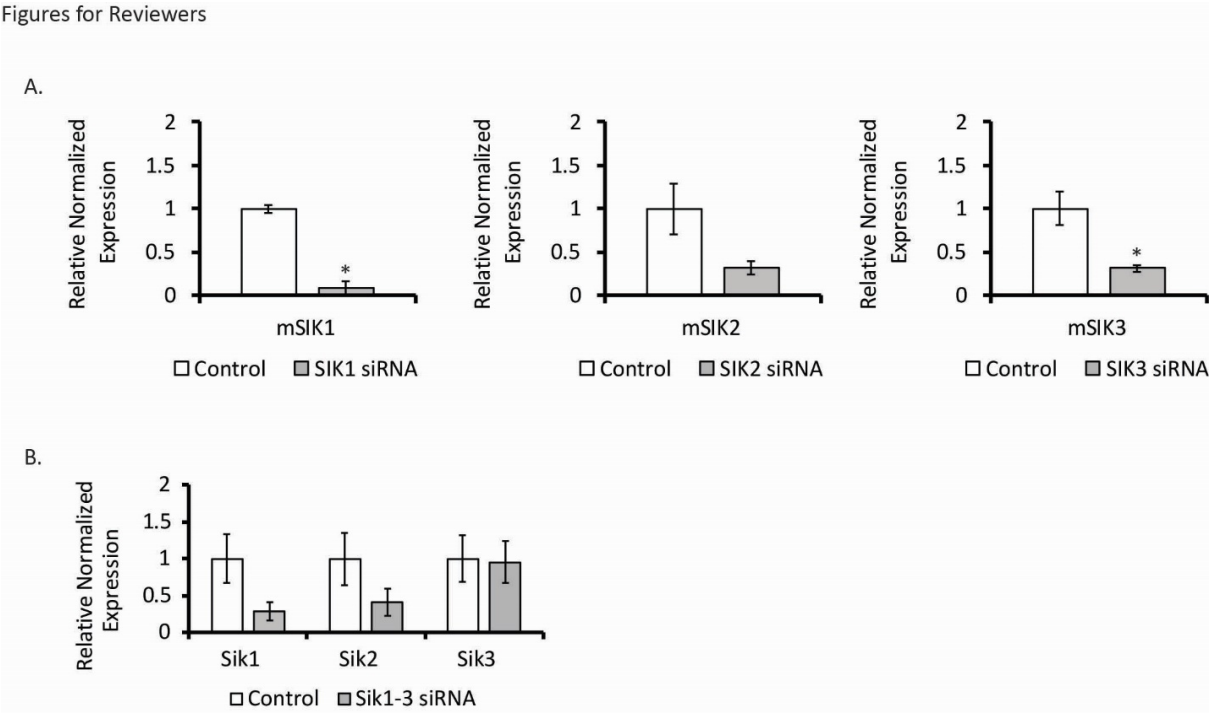
3. The data in Supplemental Figure 1 are important for the story and should therefore be in the main body of the manuscript.

We thank the referee for his comments. The data are now presented as Fig 3C and D.

4. Validation of inhibitor data with siRNA knockdown of SIK isoforms would strengthen the findings with SIK inhibitors and HDAC4 subcellular localization. Granted, there are three SIK isoforms, which complicates the issue, but the authors should at least try the experiment.

We thank the referee for the comment. We show that all three SIKs are expressed in VSMCs (Fig EV4B). We attempted to knockdown SIK1, SIK2, SIK3, and while we could knockdown each SIK individually (A), we failed to knock them simultaneously (B). (see image for reviewer below). We do not understand why the siRNA for SIK3 worked when it was administered alone, but not when co-administered with siRNA for SIK1 and SIK2, but this result has been consistent with several attempts and different siRNAs. This interaction may result from some technical interaction between the siRNA molecules or the ability of cells to take up and use different siRNAs simultaneously. Since in the triple knockdown attempts we achieved only about 30% knockdown of SIK1, 40% knockdown of SIK2 (not statistically significant), and 0% knockdown of SIK3 (not statistically significant), we were left with substantial SIK activity, and did not see significant changes in Osteocalcin or other gene expression.

Figures for Reviewers



We want to note that knockdown of the upstream kinase LKB1 did result in significant effects of downregulation of Osteocalcin, Runx2 and Sox9 expression (Fig EV4C). We also show that other pan-SIK inhibitors WH-04-023 and MRT67307 result in similar effects to HG-9-91-01 (Fig EV4A). Together these results strengthen our data and support the claim that SIK control HDAC4 localization and vascular calcification in VSMCs.

5. As an extension of Figure 8, the authors should determine if ENIGMA knockdown reduces HDAC4-mediated induction of osteochondrogenic marker gene expression.

We overexpressed HDAC4 together with control or ENIGMA siRNA. While HDAC4 overexpression was sufficient to induce osteochondral gene expression, the concomitant knockdown of ENIGMA significantly blunted this ability (Fig 8E). These data show that HDAC4 and ENIGMA depend on each other to drive vascular calcification.

6. The authors should comment on the recent paper by Wein et al. (Nat Commun. 2016 Oct 19;7:13176), which links SIK and HDAC4 to bone formation but provides data that are counterintuitive relative to the present findings (the other paper shows that SIK inhibition promotes bone formation by retaining HDAC4 in the nucleus of osteocytes).

We thank the referee for his comment. Wein and colleagues studied the mechanisms of PTH effects on bone, in the context of osteoporosis [1]. In their paper, they show that in bone PTH inhibition of *SOST* (sclerostin), a WNT antagonist, requires HDAC4 and HDAC5.

As noted in our manuscript's introduction vascular calcification and osteoporosis often coexist in the same patient, and vascular calcification is often more pronounced in patients with bone loss. At the molecular level, several papers have shown that while PTH, the subject of Wein study, promotes mineralization in the skeleton, it suppresses vascular calcification (reviewed in [2]).

Wein showed that compound deletion of both HDAC4 and HDAC5 led to a skeletal phenotype characterized by severe trabecular osteopenia, implying that HDAC4 is a positive regulator of bone growth. This data agrees with the study of Obri [3] that showed that osteoblast specific knockout of either HDAC4 or HDAC5 results in bone loss, and agrees with our data showing that HDAC4 is a positive regulator of vascular calcification. Wein suggests that HDAC4/5 bone promoting function results (in part) from repression of sclerostin expression in the nucleus. Sclerostin is a glycoprotein that is mainly secreted by osteocytes, and it decreases bone formation by inhibiting the terminal differentiation of osteoblasts. Although sclerostin is an established negative regulator of bone

mineralization, its potential role in vascular biology and arterial health is less clear. Sclerostin is up-regulated in calcifying vascular smooth muscle cells [4]. Patients with aortic valve calcification showed significantly higher sclerostin serum levels as compared to healthy controls, and immunohistochemical analysis showed positive sclerostin staining in calcified valves, in contrast to negative staining for sclerostin in non-calcified valves [5]. The serum sclerostin levels in patients with chronic kidney disease, a condition that is associated with very high levels of vascular and valve calcification, are several times higher than in healthy subjects[6]. Loss-of-function or loss-of-expression mutations in sclerostin result in the bone-thickening diseases sclerosteosis or Van Buchem disease, respectively, characterized by abnormal bone growth without vascular calcification. Together these data show that sclerostin is up-regulated in vascular calcification (and not repressed like it is in bone growth) and strongly suggest that in contrast with bone, sclerostin does not inhibit, and may actually promote, vascular and valve calcification. Since PTH suppress vascular calcification, and sclerostin is up-regulated during vascular calcification and does not appear to suppress it, the nuclear role of HDAC4 as a repressor of sclerostin expression is most likely not relevant for the promotion of vascular calcification.

We have added this information to the discussion section in brief: "It was recently shown that parathyroid hormone inhibition of SOST (sclerostin), a WNT antagonist, requires HDAC4 and HDAC5, and is dependent on the inhibition of SIK2 to promote bone growth [1]. In contrast with bone, parathyroid hormone suppresses vascular calcification[2], and sclerostin is upregulated in the calcification process [4]"

Referee #3:

The authors report that cytosolic HDAC4 promotes calcification in vascular smooth muscle cells (VSMCs), and this is most likely dependent on salt-inducible kinase (SIK). The authors provide cell culture, aortic ring culture, and in vivo experimental data supporting these findings. They identify the adaptor protein Enigma as a binding partner for cytosolic HDAC4 and suggest that this protein allows for cytosolic HDAC4-induced calcification in VSMCs, although exactly how this occurs is not clear.

This is a very interesting, well-developed study that investigates the cytosolic role of HDAC4 in VSMCs and its role in aortic calcification. While much is known about the nuclear roles of HDACs, the cytosolic roles of these proteins have not been clarified. It is intriguing that HDAC4 seems to be mainly cytosolic in calcified VSMCs. The initial observations and the experiments identifying SIK as a regulator of HDAC4 in VSMCs are strong, but the field would benefit from a more detailed investigation of how cytosolic HDAC4 increases calcification of VSMCs.

Major points:

1. Are osteocalcin or Sox9 direct targets of transcription factors regulated by HDAC4 in VSMCs?

The referee raise important yet complex questions about the transcriptional regulation of vascular calcification, the role and regulation Sox9, the role and regulation of Osteocalcin, and HDAC4 control of these processes.

As explained in details in our response to comment #2, our data strongly supports a cytoplasmic function of HDAC4. In the revised manuscript, we further show that HADC4 requires the cytoplasmic protein ENIGMA for function (Fig 8E), and that ENIGMA is attached to key cytoskeletal proteins (Fig EV6). Our data now shows that both SIK activity and binding to ENIGMA are required for HDAC4 cytoplasmic function as promoter of vascular calcification. Our proposed mechanism is also outlined in response to comment #2.

We will try to break down our response to the specific question raise by the referee about Sox9 and Osteocalcin:

1. What is the role and regulation of Sox9 in vascular calcification?

Some insights about the role of and regulation of Sox9 can be gained from bone and cartilage development, although as noted, vascular calcification is a distinct entity, and there are some major

notable differences between the development of vascular calcification and normal bone and cartilage development.

During limb bud development, multipotent mesenchymal cells that give rise to both chondrocytes and osteoblasts, express the transcription factor Sox9 before mesenchymal condensation occurs [7]. Sox9 marks therefore the mesenchymal progenitors that give rise to all osteoblasts. As far as we know it is not known what drives the initial upregulation of Sox9 in mesenchyme and what transcription factors act upstream of Sox9. Conditional deletion of Sox9 in the limb bud mesenchyme led to the absence of chondrocytes and osteoblasts [7], with failure of Runx2 expression, suggesting that Runx2 upregulation is dependent on the prior expression of Sox9. In contrast, when Sox9 was deleted in the neural crest cells that contribute to the craniofacial bones, the cells that normally form chondrocytes differentiated to osteoblasts, with upregulation of Runx2 [8], suggesting that in these cells Sox9 is not required for osteoblast formation or Runx2 expression, but in fact prevents it. The reason for the different outcomes for Sox9 deletion in the limb bud mesenchyme versus neural crest cells is not clear.

Sox9 and Runx2 are also important for the development of vascular calcification, but like bone, the relationship between these two factors is not clear. The transcription factor Runx2 is necessary but not sufficient for the development of vascular calcification. The complete elimination of Runx2 from smooth muscle cells blunted the development of vascular calcification [9], suggesting that at least some Runx2 expression is required for the process, but VSMCs specific overexpression of Runx2 in transgenic mice did not induce aortic calcification[10], showing that upregulation is not sufficient. Rats with chronic renal failure develop severe vascular calcification with marked upregulation of the transcription factor Sox9 but without significant changes in Runx2 aortic expression levels [11]. Lomashvili et al also did not find significant upregulation of Runx2 expression in cultured rat aortas under calcifying conditions[12].

Our data agree with these observations, and we a see prominent upregulation of Sox9 in VSMC culture with either HPM induced differentiation (Fig 1A) or HDAC4 over-expression (Fig 2A) as well as HDAC4 over-expression in aortic rings (Fig 2C). We now also show that this upregulation depends on ENIGMA, since knockdown of ENIGMA prevents the HDAC4 upregulation of Sox9 (Fig 8E). We also see similar effects on Runx2. We see a trend for upregulation of Runx2 with HPM or HDAC4 in VSMCs (that did not reach statistical significance) (Fig 1A, Fig 2A) and a statistically significant upregulation in Runx2 with HDAC4 in the aortic rings (Fig 2C).

Together our data and the published data suggests that Sox9 and Runx2 are involved in vascular calcification, and also suggest that like in bone the relationship between these factors is complex. It is possible that in vascular calcification some subset of cells differentiates to chondrogenic like fate, with upregulation of Sox9 and without Runx2 upregulation, while other adopt an osteoblast fate with downregulation of Sox9 and upregulation of Runx2. Since the cytoplasmic protein ENIGMA is required for HDAC4 function, we do not think, and did not find evidence for direct control of HDAC4 on the nuclear factor Sox9. We could not find established upstream factors of Sox9, to test their control by HDAC4.

2. What is the role and regulation of Osteocalcin in vascular calcification?

Osteocalcin is a secreted protein that is expressed by osteoblasts, odontoblasts and hypertrophic chondrocytes at the late mineralization state and accumulates in the bone extra-cellular matrix [13]. In bone development, osteocalcin appear only in the matrix mineralization phase and strongly binds calcium ion in the extracellular matrix, and therefore, osteocalcin is one of the most frequently used markers for osteoblast differentiation [14]. The regulation of Osteocalcin expression is not well understood. In rat and human osteoblasts, vitamin D increases the expression of Osteocalcin by interaction with a vitamin D responsive element (VDRE) element present in the promoter of the genes. The mouse promoter lacks this VDRE element, and in mouse osteoblasts, vitamin D inhibited the expression of Osteocalcin [15]. The osteocalcin promoter also contains potential Runx2 binding sites, termed previously Osf2 sites. Accumulating data suggests that most tissue specific gene expression is controlled by distal enhancers and not by proximal promoters [16], therefore these data, that is mostly derived from luciferase assays on the proximal Osteocalcin promoter, should be interpreted with caution.

Osteocalcin is upregulated during VSMCs calcification, and our data is in agreement with vascular calcification data in patients and rodents that also showed the presence of osteocalcin in calcified plaques [17]. We show that Osteocalcin is a major target gene for the cytoplasmic HDAC4-ENIGMA complex. HDAC4 induces the upregulation of Osteocalcin in cultured VSMCs (Fig 2A) and in aortic rings (Fig 2C), and knockdown of HDAC4 suppresses it (Fig 2E). This induction depends on the cytoplasmic protein ENIGMA as overexpression of HDAC4 with knockdown of ENIGMA does not result in Osteocalcin upregulation (Fig 8E). We also show that the nuclear 3SA mutant of HDAC4 does not induce the upregulation of Osteocalcin (Fig 3C), and that inhibition of SIK in VSMCs and in rings prevents its upregulation (Fig 5A and C). In agreement with our results, Osteocalcin gene expression in bone was decreased nearly 4-fold in mice with osteoblast specific knockout of HDAC4 compared with control mice [18]. This regulation of Osteocalcin expression by class II HDACs appeared to be specific to HDAC4, as mice lacking HDAC5, did not demonstrate a decrease in bone Osteocalcin expression [18].

Osteocalcin knockout mice develop bones normally, showing that osteocalcin can serve as a marker but is not required for normal bone formation [19]. Surprisingly, it was shown that when overexpressed, osteocalcin functions as a stimulator of VSMCs calcification, upregulating Sox9, Runx2, ALP, proteoglycans, and calcium mineral accumulation. Moreover, in vivo administration of Osteocalcin siRNA, prevented vitamin D induced vascular calcification development [20]. This study shows that in contrast with bone, Osteocalcin is both necessary and sufficient for the induction of vascular calcification, and that the upregulation of Osteocalcin predates, and induces the upregulation of Sox9 and Runx2, rather than result from it. Together the data suggest that Osteocalcin may be the primary target gene of the cytoplasmic HDAC4-ENIGMA complex in VSMCs, and it is possible that the up-regulation of Osteocalcin, as reported before, results in the upregulation of Sox9 and promotes calcification.

2. Why does osteocalcin expression increase with increased HDAC4 expression, given that HDAC4 is generally a transcriptional inhibitor? (Figure 2A and Supplemental Figure 1A)

The referee raise and important question concerning the mechanism by which the cytoplasmic HDAC4-ENIGMA complex controls gene expression, and specifically the expression of Osteocalcin.

As the referee justly pointed out, nuclear class IIa HDACs can act as transcriptional repressors. Indeed, reporter assays in cultured cells showed that HDAC4 can repress the transcriptional activity of Runx2. In these studies, the constitutively nuclear mutant HDAC4-3SA was a more potent inhibitor of Runx2 activity than the wild type one [21]. Despite the ability of HDAC4 to repress Runx2 activity in promoter assays, the role of HDAC4 as suppressor of Runx2 in vivo was called into question. Using an osteoblast specific knockout of HDAC4 it was also shown that HDAC4 does not inhibit Runx2 function in osteoblasts in vivo [3]. Our data strongly shows that HDAC4 acts in the cytoplasm. Together with the inability of the nuclear 3SA-HDAC4 to control Osteocalcin expression we can conclude the HDAC4 does not control Osteocalcin expression through a transcriptional repression mechanism.

Cytoplasmic complexes can modulate gene expression by many mechanisms. A complex can induce the passage of cytoplasmic components such as transcription factors, or molecules such as calcium into the nucleus, can induce posttranscriptional modification such as phosphorylation, can control the mRNA stability and translation rate, or can induce or prevent the degradation of proteins. Specifically, it was shown that ENIGMA elicited p53 degradation by inhibiting the E3 ubiquitin ligase MDM2 self-ubiquitination and increasing its ubiquitin ligase activity toward p53 in cancer cells [22]. Mouse p53-null osteoprogenitor cells have increased proliferation, increased expression of Runx2, increased osteoblast maturation, and develop osteosarcomas [23]. While an ENIGMA mediated p53 degradation induced induction of vascular calcification is an attractive hypothesis, we have so far been unable to show how HDAC4 can mediate this process. We have also not been able to document an HDAC4-ENIGMA mediated protein degradation process, but of course cannot rule this type of mechanism.

Multiple lines of evidence show that VSMCs respond to mechanical signals [24]. In aortic aneurysms, calcifications are usually localized in the outer part of the media and delimit the external

side of the aneurysmal dilatation, where stress is high. Histological examination of aortic valves has revealed distinct areas of calcification [24]. These areas correspond to sites where the greatest flexion stress (and hence strain) occurs, suggesting that biomechanical factors are involved in valve calcification. More directly, it was shown that the differentiation of cells to osteoblasts in response to biochemical cues can be modulated by matrix stiffness, with stiffer matrices promoting differentiation to an osteoblast fate [25,26].

Data suggests that mechanical stress can be channeled along cytoskeletal filaments and act directly on the nucleus to regulate gene expression (reviewed in[27]). In support of this, it was shown that tension applied to the cell surface is transmitted directly to the nucleus and can result in its physical distortion. The nucleus is physically connected to the cell surface through the cytoskeleton and the linker of nucleoskeleton and cytoskeleton (LINC) complex[27]. This complex is composed of SUN and KASH family members, which are membrane proteins of the inner nuclear membrane and the outer nuclear membrane respectively. KASH proteins interact with cytoskeletal elements through their C-terminal extremity, including intermediate filaments, actin filaments and microtubules, whereas SUN proteins are connected to lamins by their tails. SUN and KASH proteins interact within the perinuclear space, forming a bridge that connects the cytoskeleton with the nucleoskeleton. Importantly, the LINC complex was shown to transfer mechanical stresses from the cytoskeleton to the genome and control gene expression [28]. This mechanism may contribute to vascular calcification as the overexpression of the nuclear lamina lamin A/C protein was shown to promotes osteogenic differentiation of mesenchymal stem cells [29].

ENIGMA is a cytoskeletal associated protein. Members of the PDLIM family of proteins were shown to be important for mechanosensing at focal adhesions [30]. We discovered ENIGMA as a HDAC4 binding partner using the RRS modified yeast-two-hybrid system. As shown in our manuscript HDAC4 bind the 3-LIM domain of Enigma (Fig 7). To further understand this cytoplasmic complex, and to understand how this cytoplasmic complex drives vascular calcification, we performed a second RRS screen with Enigma PDZ domain. This screen (Fig EV6), identified the cytoskeletal proteins alpha-actinin, and palladin as Enigma PDZ- domain binding partners. The α-actinins are a family of spectrin-like actin-binding proteins with critical roles in cytoskeleton maintenance. Stiff substrates were shown to increase expression of focal adhesion components, including α-actinin [25], and α-Actinin-3 deficiency is associated with reduced bone mass in human and mice [31]. Palladin is an actin filament (F-actin) binding protein that directly crosslinks actin filaments [32]. Mutations in the Ig3 domain of palladin that interrupt actin binding result in disruption of the actin cytoskeleton [33]. Palladin was shown to be upregulated in response to both cyclic tensile strain and osteogenic differentiation [34]. These data show that ENIGMA binds cytoskeletal proteins with its PDZ domain, and HDAC4 with its 3-LIM domain, and suggest that this complex is involved in the cytoskeleton response and sensing of the extracellular matrix stiffness.

To investigate the role of direct transmission of stress from the cytoskeleton to the nucleus, we examined the members of the LINC complex. Sun2, a member of the LINC complex, is an integral membrane proteins of the inner nuclear membrane connecting the nuclear envelope to the cytoskeleton [35]. We show that knockdown of Sun2 resulted in blunting of HPM induced upregulation of Osteocalcin (Fig EV6D), demonstrating that the integrity of the LINC complex is required for osteogenic differentiation of VSMCs, and that Osteocalcin gene expression is controlled by the nuclear mechanosensing apparatus. Although immunostaining of endogenous HDAC4 is difficult, due to the low abundance of this protein and the quality of available antibodies, we also now show that we can detect accumulation of HDAC4 in focal adhesion structures in VSMCs (Fig EV6E). Focal adhesions are structure that are known to transmit mechanical forces from the ECM to the cell. We propose that during vascular calcification HDAC4 is upregulated and accumulates in the cytoplasm in response to phosphorylation by SIK. Cytoplasmic HDAC4 is recruited to the cytoskeleton through association with the protein Enigma. We speculate that the ENIGMA-HDAC4 is part of the cytoskeletal mechanosensing apparatus. ENIGMA mediated recruitment of HDAC4 to the cytoskeleton, may modify the response of the cytoskeleton to the extra-cellular matrix stiffness, and this modified stress can be transmitted directly to the nucleus via the LINC complex to change gene expression.

We do not yet know what other proteins participate in the complex, and did not find ways to directly prove that this complex can sense the stiffness of the extra-cellular matrix, aside from the

experiments shown. We hope that future studies will be able to address these points. We have shown that HDAC4 requires the cytoskeletal protein ENIGMA, which is attached to other key cytoskeletal regulatory proteins, proving a cytoskeletal mechanism for the HDAC4-ENIGMA complex. In addition to the new data and figures that were added to the revised manuscript, we also added parts to the discussion.

3. In Figure 3B/C it is difficult to determine if the cytosolic GFP is just background in the 3SA-infected VSMCs. Perhaps a western of nuclear vs cytosolic HDAC4 and HDAC4-3SA should be performed on the infected VSMCs. Also, representative pics of HeLa experiments (similar to Figure 3B) should be provided in the supplement.

We thank the referee for the suggestion. We performed a biochemical nuclear/cytoplasmic fractionation of flag tagged wildtype and 3SA HDAC4, and analyzed it using western blot. This analysis is shown in Fig EV3C and documents the cytoplasmic localization of wildtype HDAC4 and the nuclear localization of 3SA HDAC4. The revised manuscript now reads: "We further confirmed the cytoplasmic localization of wild-type HDAC4 and the nuclear localization of 3SA HDAC4 using biochemical fractionation (Fig EV3C)."

We also include representative pictures of the HeLa experiments in Fig EV3B.

4. The findings in Supplemental Figure 1 are downplayed by placing them in the supplemental data. These experiments seem to be critical as they demonstrate that cytosolic HDAC4 is required for the calcification process, and it is suggested that they be moved to Figure 3, and maybe Figure 3A can be moved to the supplement.

We moved the cartoon (formerly Fig 3A) to Fig EV3A, and now display the experiments in Fig 3C and D.

5. In Figure 6, the reversal of aortic calcification by HG-9-91-01 in vivo seems like a promising therapeutic approach. How does the dosage and treatment time compare to what might be used in humans? Perhaps off-target and side effects of the pan-SIK inhibitor should be mentioned in the discussion?

We thank the referee for his comment. Vascular and valve calcifications are years long processes, although their course is not linear. That is, once significant calcification occur the process appear to accelerate. Some insights could be gained from the design of the few clinical trials that aimed to combat this process. The SEAS randomized clinical trial recruited patients with mild-to moderate, asymptomatic calcific aortic stenosis [36]. The patients received either 40 mg of simvastatin plus 10 mg of ezetimibe or placebo daily and followed to a median of 52.2 months. Although the study failed to reach its clinical outcome goals, one could envision that a human clinical trial of drugs targeting vascular or valve calcification would have similar design and time-frame.

We used HG-9-91-01 as a proof of concept, but we do not believe this specific molecule is anywhere near clinical application. Higher affinity SIK inhibitors will have to be developed, and their pharmacodynamic and pharmacokinetic properties will have to be studied in detail. Of course, as noted by the referee, the potential toxicity/off-target/side effects of the approach must be studied. In line with the referee suggestion we have added a section in the discussion about off-target effects:

"The SIKs are expressed in several tissues. The inhibition of SIK was shown to reprograms macrophages to an anti-inflammatory phenotype [37]. HG-9-91-01 was shown to enhanced gluconeogenic gene expression and glucose production in hepatocytes [38]. Once daily treatment with the small molecule SIK inhibitor YKL-05-099 was shown to mimic skeletal effects of PTH and increase bone mass [1]. Therefore, any potential use of SIK inhibitor must include a thorough analysis of off-target effects. "

6. In Figure 8, Enigma protein levels in Enigma siRNA experiments should be determined. Can HDAC4 cytosolic localization be forced by overexpression of Enigma in VSMCs? Unaltered HDAC4 levels and localization after knockdown of Enigma suggests other proteins are involved in HDAC4-regulated calcification. Perhaps the role of osteocalcin should be investigated?

We now added protein level analysis of ENIGMA in the siRNA experiments. These experiments are now showed in Fig EV5A. We also show that over-expression of ENIGMA cannot force the cytoplasmic localization of 3SA HDAC4 (Fig EV5B). We also now show that overexpression of ENIGMA cannot drive osteochondral differentiation (Fig 8D).

We agree with the referee, and have no doubt that other proteins are part of the cytoplasmic HDAC4-ENIGMA complex. To identify these proteins, we performed a second RRS screen with Enigma PDZ domain. This screen (Fig EV6A-C), identified the cytoskeletal proteins alpha-actinin, and Palladin as ENIGMA PDZ- domain binding partners. These binding partners show the association of ENIGMA to cytoskeletal regulatory proteins, and suggest a role for the complex in mechanosensing. We also agree with the reviewer that Osteocalcin is a major target gene for the ENIGMA-HDAC4 complex, that can modulate Osteocalcin expression. As discussed above the overexpression of osteocalcin was shown to be sufficient to induce differentiation and mineralization of vascular smooth muscle cells [20]. Yet, once expressed Osteocalcin is secreted outside the cell, and therefore we do not believe that Osteocalcin binds the ENIGMA-HDAC4 complex.

7. The authors might also include in the discussion the relative importance of cytosolic HDAC4 in regulating calcification of VSMCs versus decreased gene regulatory function. For example, does cytosolic HDAC4 allow for increased expression of calcification genes that enhance calcification (other than the markers reported herein), or is HDAC4 involved in another cytosolic mechanism that promotes calcification, such as by forming a matrix for calcium deposition?

We thank the referee for his comment. We added a paragraph in the discussion section detailing the cytoplasmic control of Osteocalcin and the proposed mechanosensing mechanism, that was discussed in more details in response to comments 1 and 2.

Minor points:

1. In Figure 2, controls for Ad exps and siRNA exps should be included: protein levels and localization of HDAC4 in VSMCs and aortic rings need to be shown (FLAG immunostaining would be great for Ad exps).

We added the necessary control experiments:

For Fig 2A and B (adeno-HDAC4 expression in VSMCs) we now added protein level analysis of HDAC4 using a western blot, displayed in Fig EV2A and showing about 1.6-fold increase HDAC4 protein levels after over-expression for the low dose Adeno and 2.5-fold for the high dose.

For Fig 2 C and D (adeno-HDAC4 expression in aortic rings), we tried extracting proteins from calcified aortic rings using several approaches and protein extraction buffers, however we got very poor yields, most probably because of the highly fibrotic nature and high elastin content of these tissues. We also failed to reliably perform immunostaining on the rings because of very high background staining. We instead verified the over-expression of HDAC4 by qRT-PCR. The human HDAC4 used in this study is almost completely identical to the mouse HDAC4 at the amino acid level (95% identity), but has a lower identity (86%) at the RNA level. Therefore qRT-PCR primers that recognize the over-expressed human HDAC4 do not recognize the endogenous mouse HDAC4. The analysis is now shown in Fig EV2B, and confirms the efficient transduction of the rings. This analysis however cannot be used to quantify the degree (fold-ratio) of HDAC4 overexpression over endogenous mouse HDAC4, since the level of human HDAC4 in control rings is 0 (the fold expression level in HDAC4 transduced rings is infinitesimally high since [human HDAC4 concentration in transduced rings]/0 = ∞) . Fig EV2B therefore may somehow suggest that the overexpression is about 1900 fold, and that is of course not the case. We tried to explain this in the legend as best as we can. Unfortunately, we were unable to find primer pairs that would quantify both the endogenous mouse and human HDAC4 with the exact efficiency to allow head to head comparison. As shown in Figs 1C and 1D there is a comparable 2-fold increase in HDAC4 in VSMCs at both mRNA and protein levels, showing that mRNA levels can be used as surrogate for protein levels in these cells for HDAC4. We used the same virus concentrations for both rings and VSMCs, documented an efficient viral transduction in the rings, and since we saw about a 2.5-fold

increase in protein levels in the VSMCs, it is very likely that the overexpression in the rings was similar.

As requested by the reviewer in comment 3, we performed a biochemical nuclear/cytoplasmic fractionation of VSMCs and assessed the localization of overexpressed wt HDAC4 and 3SA HDAC4. This analysis is displayed in Fig EV3C and confirms the cytoplasmic localization of wt HDAC4 and the nuclear localization of 3SA HDAC4 using Flag immunoblot.

For fig 2E (siRNA knockdown of HDAC4), we now added a protein level analysis in Fig EV2D, that demonstrates the significant knockdown of HDAC4 protein. Again, this analysis show a high correlation between mRNA levels (Fig 2E) and protein levels (Fig EV2D) for HDAC4.

2. What happens to calcification of aortic rings with the addition of HDAC4 in the presence of HPM?

We added the requested analysis in Fig EV2C. HDAC4 overexpression in HPM treated aortic rings did not result in additional increase in Osteocalcin or Spp1. There was a non-significant trend for an additional increase in Runx2 levels. The revised manuscript now reads: "Overexpression of HDAC4 in HPM treated aortic rings did not result in additional markers upregulation (Fig EV2C)"

3. Error bars are missing for Figure 3C, 4B, supplemental Figure 2A. There seems to be large variability in HDAC4 localization in response to 10uM HG-9-91-01 (Figure 4B versus Supplemental Figure2A), please comment.

Error bars were added to the figures that are now showed in Fig 3B, Fig 4B, Fig EV4A. The 10uM dose HG-9-91-0 appear to be somewhat toxic for the cells, and that likely caused the high variability. This dose was not used in the rest of the study.

Comments from external advisor:

The paper is very interesting and might provide new insights. But further investigations need to be conducted. I agree with your concern that it is not clear how HDAC4 regulates calcification in concert with ENIGMA. It is not surprising that HDAC4 regulates calcification in view of pervious reports about the interaction with Runx2. But here, the authors suggest a cytoplasmic role, which somehow contradicts the previous data and potentially provides a new conceptual thinking. It is indeed important to describe the cytoplasmic functions (because HDAC4 lives mostly in the cytoplasm and it seems to bind to acetylated proteins without deacetylating them) but so far this paper only gives a rough idea but not very deep insights. I think the authors need to substantiate their claims and in particular they need to show how HDAC4 regulates ENIGMA and - most importantly - they need to demonstrate that HDAC4 and ENIGMA not only act in parallel but that they indeed depend on each other.

We thank the advisor for his comment. We agree that a major point that was not fully explored in the original manuscript, was the interdependence of HDAC4 on ENIGMA for function. To assess if HDAC4 and ENIGMA not only act in parallel but that they indeed depend on each other, we initially over-expressed ENIGMA and assessed the effects on osteochondral genes. The overexpression of ENIGMA did not result in upregulation of osteochondral markers, and in fact resulted in a very modest, yet significant downregulation of Osteocalcin expression (Fig 8D). This downregulation likely resulted from perturbation of the cytoskeleton-ENIGMA-HDAC4 cytoplasmic complex stoichiometry by ENIGMA overexpression. This phenomena, often termed 'squelching' is repression of activity with high overexpression of a complex member that sequesters other limiting components required for the activation**.** The requirement of ENIGMA together with the inability of ENIGMA overexpression to promote osteochondral differentiation on its own, shows that ENIGMA depends on other proteins for this function. Importantly, we next overexpressed HDAC4 together with control or ENIGMA siRNA. While HDAC4 overexpression was sufficient to induce osteochondral differentiation, the concomitant knockdown of ENIGMA significantly blunted this ability (Fig 8E). These data show that HDAC4 and ENIGMA depend on each other to drive vascular calcification.

We further investigated the mechanisms by which the HDAC4-ENIGMA cytoplasmic complex can control gene expression, and specifically the expression of Osteocalcin. As pointed by the advisor, nuclear class IIa HDACs can act as transcriptional repressors. Indeed, reporter assays in cultured cells showed that HDAC4 can repress the transcriptional activity of Runx2. In these studies, the constitutively nuclear mutant HDAC4-3SA was a more potent inhibitor of Runx2 activity than the wild type one [21]. Despite the ability of HDAC4 to repress Runx2 activity in promoter assays, the role of HDAC4 as suppressor of Runx2 in vivo was called into question. Using an osteoblast specific knockout of HDAC4 it was also shown that HDAC4 does not inhibit Runx2 function in osteoblasts in vivo [3]. Our data strongly shows that HDAC4 acts in the cytoplasm. Together with the inability of the nuclear 3SA-HDAC4 to promote calcification or control Osteocalcin expression we can conclude the HDAC4 does not promote vascular calcification expression through a transcriptional repression mechanism.

Multiple lines of evidence show that VSMCs respond to mechanical signals [24]. In particular, it was shown that the differentiation of cells to osteoblasts in response to biochemical cues can be modulated by matrix stiffness [25,26]. We performed a second RRS screen with Enigma PDZ domain. This screen (Fig EV6), identified the cytoskeletal proteins alpha-actinin, and palladin as ENIGMA PDZ- domain binding partners. The α-actinins are a family of spectrin-like actin-binding proteins with critical roles in cytoskeleton maintenance. Stiff substrates were shown increased expression of focal adhesion components, including α-actinin [25], and α-Actinin-3 deficiency is associated with reduced bone mass in human and mice [31]. Palladin is an actin filament binding protein that directly crosslinks actin filaments [32]. Mutations in the Ig3 domain of palladin that interrupt actin binding result in disruption of the actin cytoskeleton [33]. Palladin was shown to be upregulated in response to both cyclic tensile strain and osteogenic differentiation [34]. These data show that ENIGMA binds cytoskeletal proteins with its PDZ domain, and HDAC4 with its 3-LIM domain, and suggest that this complex in involved in the cytoskeleton response to mechanical stress. We also added immunostaining analysis of endogenous HDAC4 in VSMCs that show that HDAC4 can localize to focal adhesion structures (Fig EV6E), structure that are known to sense transmit mechanical forces from the extra-cellular matrix to the cell.

Data suggests that mechanical stress can be channeled along cytoskeletal filaments and act directly on the nucleus to regulate gene expression (reviewed in[27]). In support of this, it was shown that tension applied to the cell surface is transmitted directly to the nucleus and can result in its physical distortion. The nucleus is physically connected to the cell surface through the cytoskeleton and the linker of nucleoskeleton and cytoskeleton (LINC) complex[27].

To investigate the role of direct transmission of stress from the cytoskeleton to the nucleus, we examined the members of the LINC complex. Sun2, a member of the LINC complex, is an integral membrane proteins of the inner nuclear membrane connecting the nuclear envelope to the cytoskeleton [35]. We show that knockdown of Sun2 resulted in blunting of HPM induced upregulation of Osteocalcin (Fig EV6D), demonstrating that the integrity of the LINC complex is required for osteogenic differentiation of VSMCs, and that Osteocalcin gene expression is controlled by the nuclear mechanosensing apparatus. We propose that during vascular calcification HDAC4 is upregulated and accumulates in the cytoplasm in response to phosphorylation by SIK. Cytoplasmic HDAC4 is recruited to the cytoskeleton through association with the protein ENIGMA. Mechanistically we have now established that HDAC4 requires the cytoskeleton associated protein ENIGMA for its cytoplasmic function. We speculate that the ENIGMA-HDAC4 is part of the cytoskeletal mechanosensing apparatus. ENIGMA mediated recruitment of HDAC4 to the cytoskeleton, may modify the response of the cytoskeleton to the extra-cellular matrix stiffness, and this modified stress can be transmitted directly to the nucleus via the LINC complex to change gene expression.

We do not yet know what other proteins participate in the complex, and did not find ways to directly prove that this complex can sense the stiffness of the extra-cellular matrix, aside from the experiments shown. We hope that future studies will be able to address these points. We have shown that HDAC4 requires the cytoskeletal protein ENIGMA, which is attached to other key cytoskeletal regulatory proteins, proving a cytoskeletal mechanism for the HDAC4-ENIGMA complex. In addition to the new data and figures that were added to the revised manuscript, we also added parts to the discussion.

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2nd Editorial Decision 12 April 2017

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees. Referee 3 still has a few more comments that I would like you to address before we can proceed with the official acceptance of your manuscript.

- Please note that no statistics can be calculated when n<3, eg in figures EV2 and EV5. If n=2 you can show the single data points of both experiments along with their mean but please delete the error bars.
- Some of the references need to be completed: 8, 47, and 58.
- The blot in Fig7B is overexposed, can you please provide a better image?

- The images in Figs 2D and 5D appear to be identical. Please explain. Control experiments must be included for each experiment.
- Please label the gel bands in the source data with names and/or frame the bands that were used for the figure.
- The source data for EV Figs 2, 3, 5 need to be labeled source data for EV figures 2, 3, 5 and need to be provided in a single ziped file. The source data for the main figures are fine.
- Fig EV6C needs to be explained better, what is B1, B2, etc? The clones in the lanes B1 and B2 seem to be identical. Please explain and provide source data.
- EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1: The authors have adequately addressed my concerns.

Referee #3: The authors have dealt adequately with most concerns, however some of the new data need minor adjustments.

Minor points:

1. In the figure legends throughout the manuscript the authors should denote how data is normalized as it seems inconsistent.

2. The authors should display the location of the GFP tag on the designated constructs (Fig 3 and EV3A).

3. Controls are missing in EV5B and EV6E. Also, the legend for EV6E needs to describe the experiment as it is unclear. Why does HDAC4 have a focal adhesion staining here but not throughout the manuscript?

4. Phosphorylation of HDAC4 by SIK or another kinase was not reported and therefore should not be used as a mechanistic explanation of the results. These suggestions should be downplayed or HDAC4 phosphorylation should be investigated.

2nd Revision - authors' response 18 April 2017

POINT BY POINT RESPONSE TO EDITORIAL COMMENTS

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees. Referee 3 still has a few more comments that I would like you to address before we can proceed with the official acceptance of your manuscript.

Please note that no statistics can be calculated when n<3, eg in figures EV2 and EV5. If n=2 you can show the single data points of both experiments along with their mean but please delete the error bars.

Figures were replaced with single data points of both experiments along with their means.

Some of the references need to be completed: 8, 47, and 58.

Corrected

The blot in Fig7B is overexposed, can you please provide a better image?

A better image is provided

The images in Figs 2D and 5D appear to be identical. Please explain. Control experiments must be included for each experiment.

We thank you for the remark. The experiments in 2D and 5D were performed simultaneously: Aortas were dissected from Apo E-/- mice, and cut into rings that were embedded in collagen. The rings were divided to the different experimental groups. Rings from all groups were harvested, sectioned, stained and imaged together. In figure 5D we showed the effects of SIK inhibition, and rings treated with HPM without SIK inhibitor served as control. We also added the image from figure 2D: rings treated without HPM and without SIK inhibitor for side-by-side comparison, but we apologize that we did not explained it clearly. We now supply an image from a different control ring (from the same experiment) to figure 5D. If you think this is still confusing, we can combine figures 2D and 5D to one figure.

Please label the gel bands in the source data with names and/or frame the bands that were used for the figure.

Corrected

The source data for EV Figs 2, 3, 5 need to be labeled source data for EV figures 2, 3, 5 and need to be provided in a single ziped file. The source data for the main figures are fine.
Provided

Fig EV6C needs to be explained better, what is B1, B2, etc? The clones in the lanes B1 and B2 seem to be identical. Please explain and provide source data.

We deeply apologize for mislabelling the clones on the image. In the RRS yeast-two-hybrid screen, positive clones were picked and library (prey) plasmids were isolated. These 'positive hits' library plasmids were then transformed to yeast together with the bait plasmid for a second round of validation. Two yeast colonies were picked from each such transformation and streaked on a plate. Clones were numbered by row, such that in the first row we streaked two colonies from clone#1, two colonies from clone#2, and two colonies from clone #3, in the second row – clones #4, #5, #6, etc. The plate was grown in 24^{o}C , and then replica plated on four differential media plates: GAL-LU (galactose without uracil and leucine), GAL-MUL (galactose without methionine, uracil and leucine), -MUL (glucose without methionine, uracil and leucine), and YPD. These four plates were grown at 36^{o}C. Images from these four plates, showing positive clones on all four media types are shown in figure EV6C. The source image is now also provided. Clones #2,5,8, and 11 were independent clones of actinin (different library plasmids of the same protein). The cartoons of these clones are shown in figure EV6A. Clones #3, and 7 were identical clones of paladin (since they were the exact same library clone we consider them a duplicate, and not independent clones, and only one of them is shown). The cartoon of this clone in shown in figure EV6B. The designation 'B' was an internal code for the bait used, and we now removed it. Instead we use clone#2, #5, etc.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We added a synopsis text and image

I look forward to seeing a final version of your manuscript as soon as possible.

POINT BY POINT RESPONSE TO REFEREE COMMENTS

Referee #1: The authors have adequately addressed my concerns.

Referee #3: The authors have dealt adequately with most concerns, however some of the new data needs minor adjustments.

Minor points:

1. In the figure legends throughout the manuscript the authors should denote how data is normalized as it seems inconsistent.

We apologize. Normalization is now denoted in all legends.

2. The authors should display the location of the GFP tag on the designated constructs (Fig 3 and EV3A).

GFP location is now shown

3. Controls are missing in EV5B and EV6E. Also, the legend for EV6E needs to describe the experiment as it is unclear. Why does HDAC4 have a focal adhesion staining here but not throughout the manuscript?

Negative controls are now shown for figures EV5B and EV6E. In Figure EV6E we stained wild type VSMCs with anti HDAC4 antibody. The legend now states: "Wildtype VSMCs were fixed with formaldehyde, immune-stained with an anti- HDAC4 antibody (green), phalloidin (red) and nuclei were counterstained with Dapi (blue). Representative images are shown. Scale bar = 10 µm." In contrast to the very bright GFP staining, the anti HDAC4 antibody staining is weak. We think that the very bright cytoplasmic GFP likely masks the weak signal of accumulation of HDAC4 in focal adhesions.

4. Phosphorylation of HDAC4 by SIK or another kinase was not reported and therefore should not be used as a mechanistic explanation of the results. These suggestions should be downplayed or HDAC4 phosphorylation should be investigated.

We apologize for not emphasizing it clearly, but two papers showed direct phosphorylation of HDAC4 by SIK. The Introduction reads: "The N-terminal domain also contains three conserved Serine residues that can undergo phosphorylation by several kinases including calcium/calmodulin-dependent kinase II (CamK II) [12], Protein Kinase D (PKD) [13], and salt inducible kinase 1,2 and 3 (SIK1, SIK2, and SIK3) [14,15]." References 14 and 15 describe the phosphorylation of HDAC4 by SIK.

4th Editorial Decision 26 May 2017

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.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Izhak Kehat
Journal Submitted to:	EMBO reports
Manuscript Number:	EMBOR-2016-43686V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was choses base on the size of similar studies in the field, P.17
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was choses base on the size of similar studies in the field, no statistical methods were used, P. 17
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusions were made, no statistical methods were used
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Littermate ApoE-/- mice were arbitrarily divided to receive treatment or control, P.15
For animal studies, include a statement about randomization even if no randomization was used.	Littermate ApoE-/- mice were arbitrarily divided to receive treatment or control, P.14
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	All data and sections were processed simultanously, P.14
4.b. For animal studies, include a statement about blinding even if no blinding was done	There was no blinding and all sections were processed simultanouly
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All the data meet the assumptions of the testes and the statistical methods are specified in each panel of the figure legends
Is there an estimate of variation within each group of data?	yes (SE or SD)
Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Provided in the method. P.16-17
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	A new batch of cell line was provide for this study from ATCC, P.14

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	ApoE-/- mice were purchased from The Jackson Laboratory. all animals were housed in a temperature-controlled environment with 12 hours light/dark cycles with food and water available ad libitum. Mice were aged 8 weeks when entering the study. P. 14
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All mice procedures were performed in accordance with institutional guidelines. P.14
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirm

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The study was approved by an institutional review committee of the Rambam Health Care Campus and subjects gave informed consent. P.15
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	The study was approved by an institutional review committee of the Rambam Health Care Campus and subjects gave informed consent. P. 15
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No