1. Publishable summary (expected length: max. 500 words)

Arterial calcification and valvular calcifications are associated with significant morbidity and mortality. Studies have now conclusively showed that an osteochondrogenic differentiation of vascular smooth muscle cells (VSMC) is a key mechanism in the development of vascular calcification, opening a direction for therapeutic interventions. We used VSMCs, ex-vivo mouse aortic rings and human calcified aortic valves to show that HDAC4 is upregulated in vascular and valve calcification. Despite being exclusively cytoplasmic in VSMCs we show using both gain- and loss- of function approaches that HDAC4 is a positive regulator of the process. The cytoplasmic location of HDAC4 is controlled by the activity of salt inducible kinase (SIK) and the inhibition of SIK sends HDAC4 to the nucleus and inhibits the ossification process. In the cytoplasm HDAC4 binds to the LIM domains of the adaptor protein ENIGMA (Pdlim7) to promote vascular ossification of VSMCs. We identified a new role and a new mechanistic paradigm for HDAC4.We provide multiple lines of evidence that the cytoplasmic activity of HDAC4 promotes vascular calcification, and identify HDAC4, SIK and ENIGMA as mediators of this pathological process

2. Project objectives for the period (expected length: max. 500 words)

The specific aims of the proposal were:

- **Aim 1: Map the interactions between class IIa HDACs and ENIGMA in detail**
- **Aim 2: Determine the endogenous class I HDACs-ENIGMA-Class IIa HDACs ternary complex**
- **Aim 3: Determine the role of the ENIGMA complex in hypertrophy in vivo**

We discovered an interaction between the class IIa HDAC4 and ENIGMA and mapped it in dtail, as outlined in Aim1. We also discovered that HDAC4 is upregulated in vascular and valve calcification. We therefore shifted the focus of the research to understand the role of HDAC4 and HDAC4-ENIGMA in vascular calcification.

3. Work progress and achievements during the period (expected length: max. 2000 words):

Aim 1: Map the interactions between class IIa HDACs and ENIGMA in detail

High resolution mapping of the interaction domains and analysis of the specificity of the interaction among the various family members.

1.1 *High resolution mapping of the ENIGMA interacting domains on Class IIa HDACs*:

We have analysed the structure of HDAC4, analysed reported motifs and generated truncation mutants:

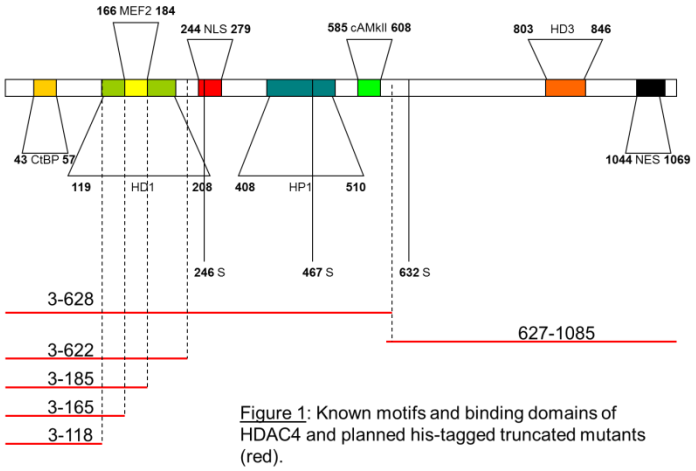
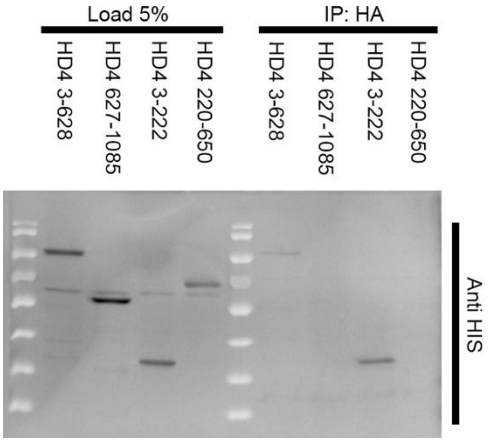
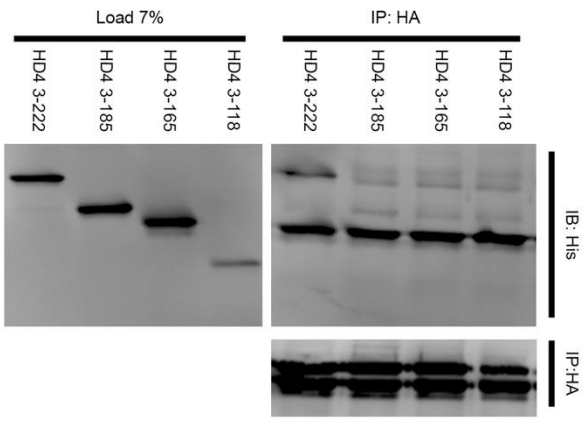


Figure 1: Known motifs and binding domains of HDAC4 and planned his-tagged truncated mutants (red).

We performed co-immunoprecipitation studies with HA-enigma and the above HIS-HDAC4 fragments:



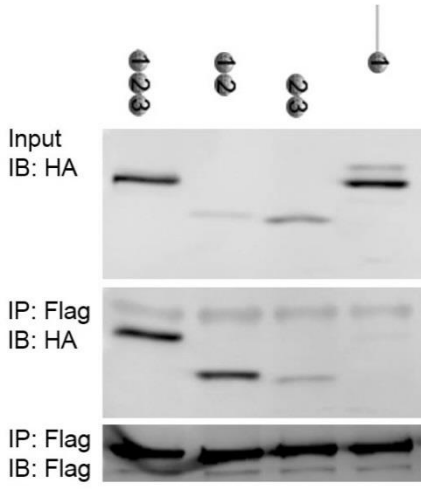
This analysis showed that fragments of HDAC4 containing amino acids 3-628, or amino acids 3-222 interacted with Enigma. Therefore amino acids 3-222 on HDAC4 are sufficient for the interaction. We further investigated the interaction domain on HDAC4:



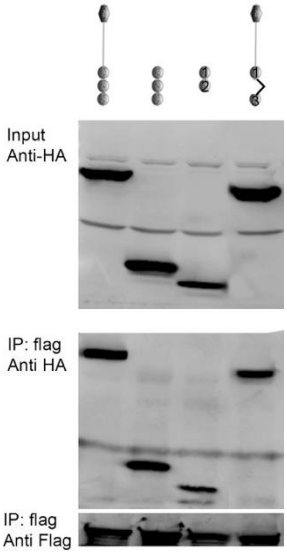
This analysis showed that fragments HDAC4 amino acids 3-222 interacted with Enigma, but smaller fragments did not. This suggests that the interaction domain is between amino acids 185-222 of HDAC4.

1.2 *High resolution mapping of the Class IIa HDACs interacting domains on ENIGMA*:

Enigma has 3 Lim domains that are similar in structure, but not identical. In accordance with our plan we mapped the interaction domain on Enigma using co-immunoprecipitation with flag-HDAC4 and HA-tagged truncation mutants of Enigma containing parts of the 3X LIM domain:



This analysis showed that mutants that contained either Lim domain 1&2 or 2&3 were sufficient to the interaction, but the one containing Lim domain 1 alone did not. These studies initially suggested that Lim domain 2 may be necessary for the interaction. To further test this we created a mutant with Lim domain 1 and 3 (but with deletion of lime domain 2). Our co-immunoprecipitation studies showed that this mutant can also bind HDAC4:



This experiment showed that any combination of two of the three Lim domain (1&2, 2&3, 1&3) was sufficient for the interaction.

Next, we assessed the expression of HDAC4 in a vascular smooth muscle model and in human calcified and control valves.

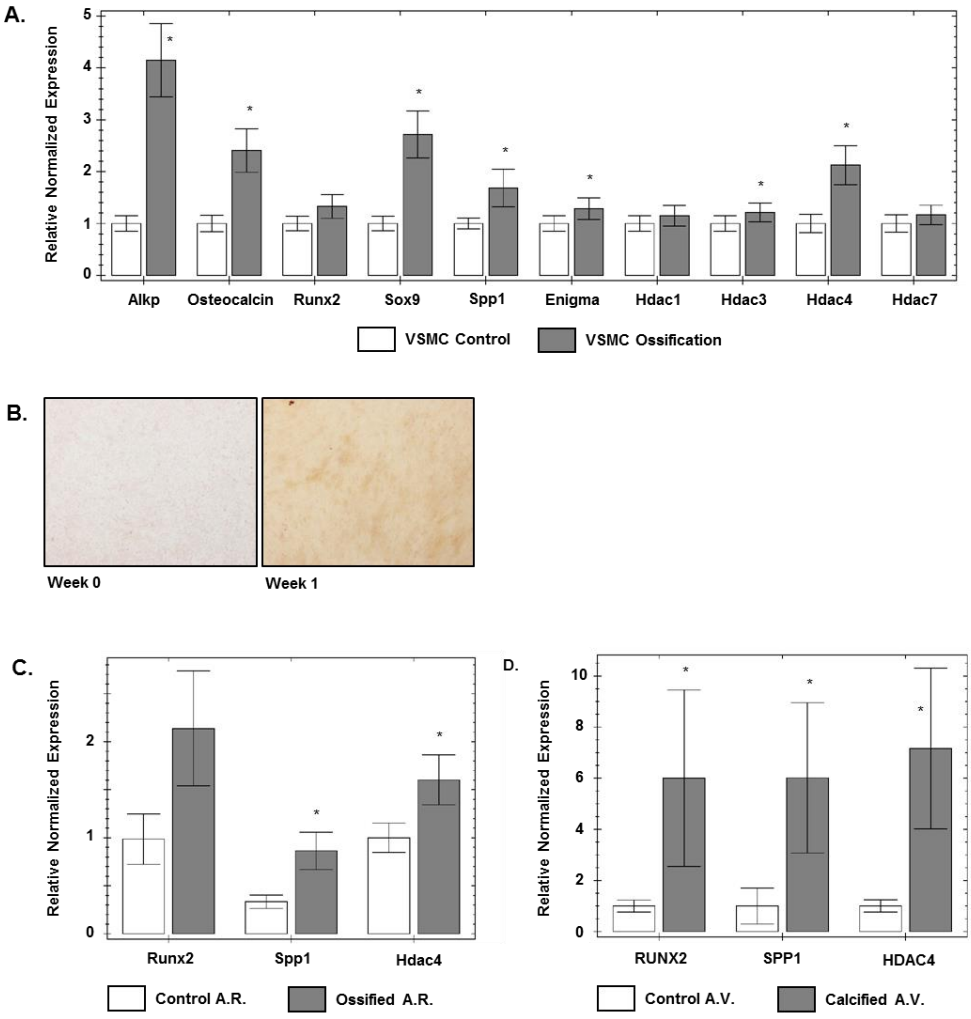


Figure 1. Histone deacetylase 4 (HDAC4) is upregulated during vascular and valve ossification. A, Gene expression qRT-PCR analysis of osteochondrogenic markers and HDACs in VSMC following treatment with osteoinductive media (VSMC ossification, grey) or control (VSMC control, white) showing upregulation of bone and cartilage markers as well as HDAC4. **B,** Alizarin red calcium staining of VSMC after 1 week of osteoinductive media (right) or control (left) showing increased matrix calcification levels. **C,** Gene expression qRT-PCR analysis of ossification markers Runx2 and Spp1 and of HDAC4 in the mouse aortic rings assay treated with (Ossified AR) or without (Control AR) osteoinductive media. **D,** Gene expression qRT-PCR analysis of ossification markers Runx2 and Spp1 and of HDAC4 in calcified Human aortic valves compared to controls. **A, C**, Mean±SE of 4 to 6 biological replicates for each group in 2-3 separate experiments (*P<0.05). **D,** Mean±SE, n=3 in each group (*P<0.05).

This analysis showed that HDAC4 is upregulated in the vascular and valve calcification. We then showed that overexpression of HDAC4 enhances the ossification process, while knockdown of HDAC4 blunts it

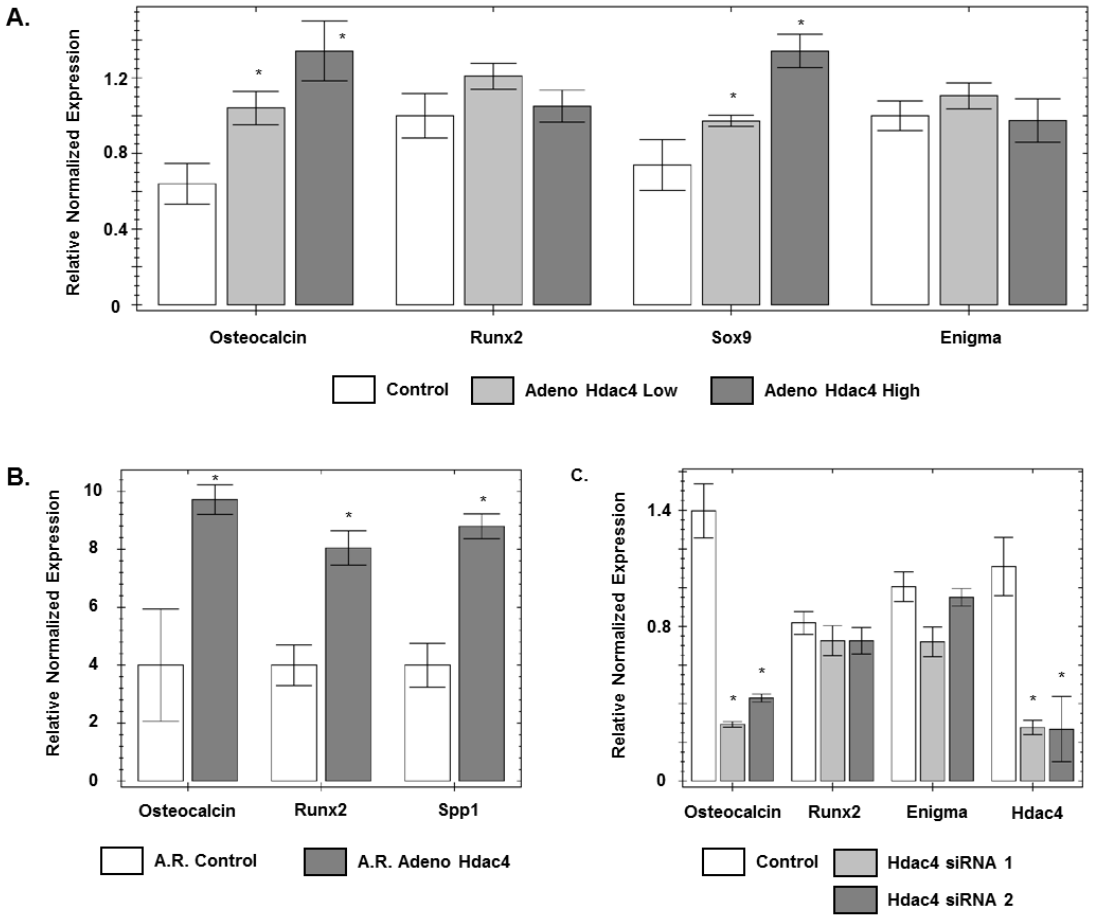
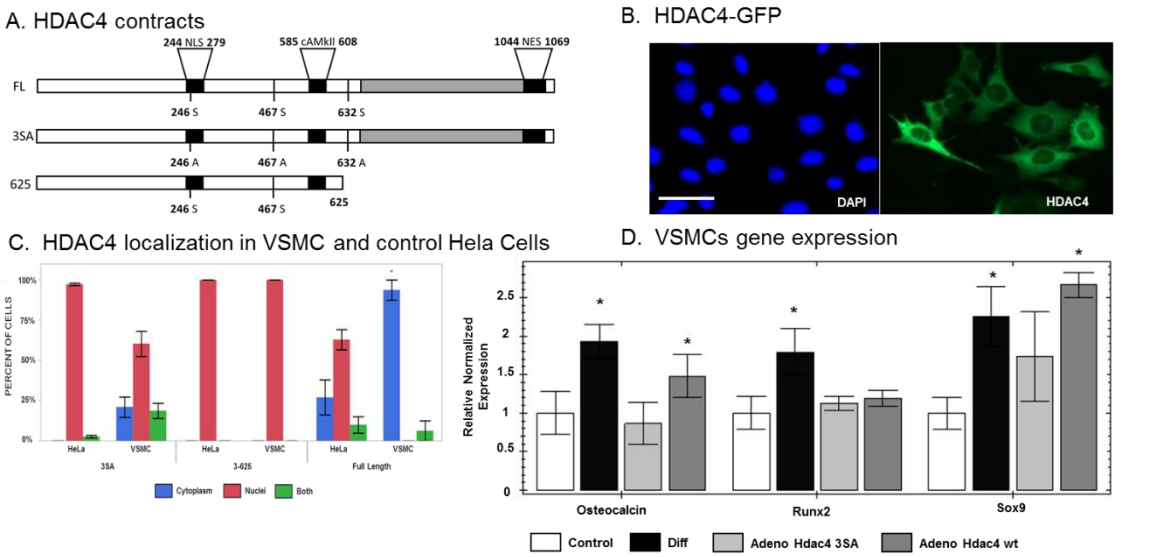


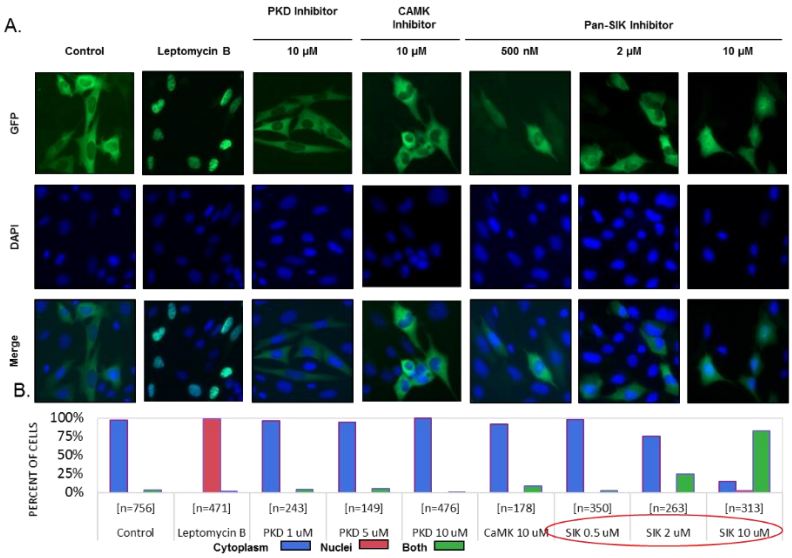
Figure 2. Effects of overexpression and knockdown of HDAC4 on ossification. A, VSMCs were transduced with adenoviral vector encoding for flag tagged human HDAC4 or control betal-gal. Gene expression qRT-PCR analysis shows dose dependent up-regulation of ossification markers. **B,** Aortic rings were transduced with adenoviral vector encoding for flag tagged human HDAC4 or control betal-gal. Gene expression qRT-PCR analysis shows up-regulation of ossification markers. **C,** VSMCs were transfected with two different siRNAs for HDAC4 or control siRNA. Gene expression qRT-PCR analysis shows similar level of knockdown of HDAC4 for the two siRNAs and downregulation of the ossification marker Osteocalcin. **A-C**, Mean±SE of 4 to 6 biological replicates for each group in 2-3 separate experiments (*P<0.05).

HDAC4 can shuttle between the nucleus and cytoplasm. Surprisingly we found that HDAC4 is exclusively cytoplasmic in VSMCs. We also show that while overexpression of HDAC4 induced calcification, the nuclear mutant HDAC4-3SA did not increase calcification.



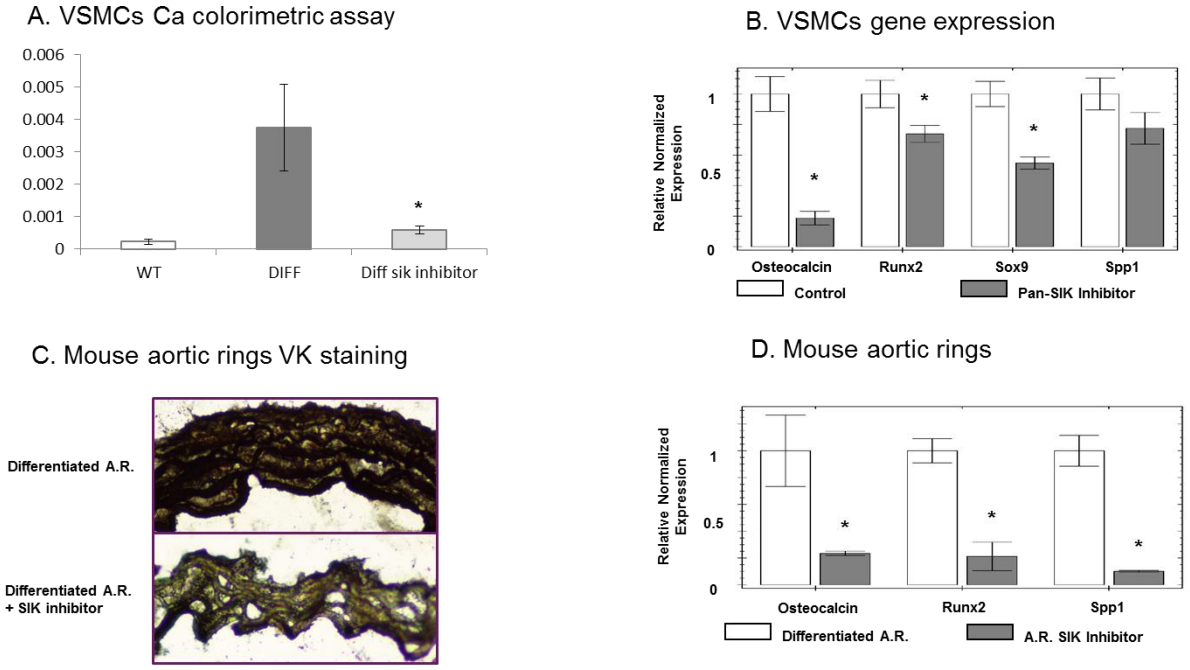
VSMCs were transfected with full-length HDAC4, with a 3SA mutant in which 3 Serine residues 246/467/632 were mutated to Alanine, or with truncated amino-acid 3-625 HDAC4 GFP constructs (A). Surprisingly, full-length HDAC4 was exclusively cytoplasmic in VSMCs, but not in control HeLa cells (B,C). The HDAC4-3SA mutant was predominantly nuclear in both VSMCs and in HeLa cells (C). Unlike the wildtype HDAC4, this mutant did not induce the upregulation of osteogenic markers (D).

The cytoplasmic location of HDAC4, and the nuclear location of the 3SA mutant in which 3 Serine residues 246/467/632 were mutated to Alanine, suggested that phosphorylation was responsible for the cytoplasmic location. To identify the responsible Kinase we used inhibitors of known HDAC4 kinases. We found that inhibition of salt inducible kinase, resulted in nuclear translocation of HDAC4.



VSMCs transfected with full length GFP-HDAC4 and treated with the indicated inhibitor and concentration. The pan-SIK inhibitor induced dose dependent nuclear accumulation of HDAC4 (A). HDAC4 localization was scored as being exclusively cytoplasmic (blue), exclusively nuclear (red) or as a cytoplasmic and nuclear localization (green) (B).

We show that inhibition of SIK not only sends HDAC4 to the nucleus, but also inhibits the ossification process.



SIK inhibitor blunted the ossification of VSMCs (A,B), aortic rings (C,D), and in mice aortas (E).

Finally, we show that HDAC4 binds to ENIGMA in the cytoplasm. Enigma does not control the cytoplasmic localization of HDAC4, since knockdown of ENIGMA does not cause shuttling of HDAC4 to the nucleus. However, ENIGMA is required for the pro-calcification, because knockdown of ENIGMA blunts the calcification process.

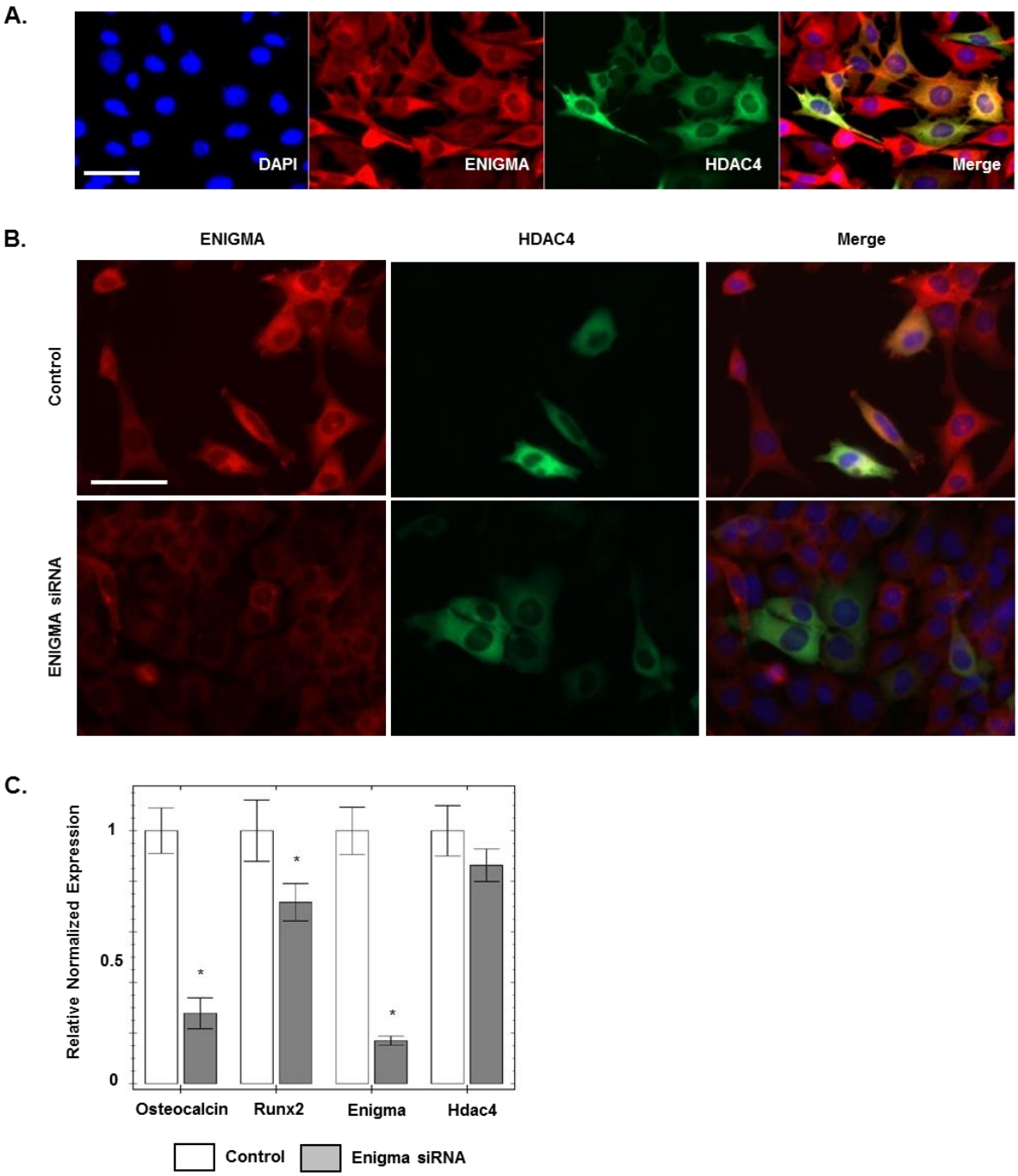


Figure 7. ENIGMA co-localize with HDAC4 in the cytoplasm and promotes ossification in VSMCs. A, VSMC were transfected with GFP-HDAC4, cells were fixed with formaldehyde, immune-stained with anti-ENIGMA antibodies, and nuclei were counterstained with Dapi. Images show cytoplasmic co-localization of ENIGMA and HDA4C. Size bar = 25 µm **B,** VSMCs were transfected with GFP-HDAC4 and Enigma siRNA or control, fixed with formaldehyde, immune-stained with anti-ENIGMA antibodies and nuclei were counterstained with Dapi. Knockdown of ENGIMA did not change the cytoplasmic localization of HDAC4. Size bar = 25 µm **C,** VSMCs were transfected with Enigma siRNA or control, and osteochondrogenic markers were analyzed by qRT-PCR. Mean±SE of 4 biological replicates for each group in 2 separate experiments (*P<0.05).

Advancement beyond the state of the art in the field.

we showed that the expression of HDAC4 is upregulated during vascular ossification in different models and in patients, while the localization of HDAC4 remains exclusively cytoplasmic. Despite this cytoplasmic location we show using both loss- and gain of function approaches that HDAC4 is functional and promotes the process. The cytoplasmic location of HDAC4 depends on the activity of the protein kinase SIK and the inhibition of SIK sends HDAC4 to the nucleus and impedes the ossification process. In the cytoplasm HDAC4 binds the cytoskeleton associated proteins ENIGMA, which is required for vascular ossification.

Impact

The understanding that vascular ossification is an active process suggests that therapeutic agents may be able to modify its development. However, to date, no such therapies are available. We identified here three novel modulators - the class IIa HDAC4, the adaptor protein ENIGMA and the protein kinase SIK that together positively regulate this pathological process. Inhibition of this pathway may be able to blunt vascular ossification and prevent disease progression.

Transfer of knowledge

I teach the course physiology 1 (cardiovascular and pulmonary physiology) to third year medical students at the faculty of medicine at the Technion-Israel Institute of Technology

I have also initiated and in charge of the course titled “Things you should know – introduction to graduate biomedical students”. This course is an overview of the main approaches in biomedical studies, and is now an obligatory course for all first year graduate students at the faculty of medicine.

I mentor four PhD students in my lab, and in addition two residents from the hospital have an extended research project in my lab.

6. Dissemination activities (expected length: max. 800 words)

Publication: a manuscript describing our results is in review.

Mutlak M, Kehat I. Extracellular signal-regulated kinases 1/2 as regulators of cardiac hypertrophy. Front Pharmacol. 2015 Jul 24;6:149.

Koren L, Alishekevitz D, Elhanani O, Nevelsky A, Hai T, Kehat I, Shaked Y, Aronheim A. ATF3-dependent cross-talk between cardiomyocytes and macrophages promotes cardiac maladaptive remodeling. Int J Cardiol. 2015 Nov 1;198:232-40.

Abend A, Kehat I. Histone deacetylases as therapeutic targets--from cancer to cardiac disease. Pharmacol Ther. 2015 Mar;147:55-62.

Koren L, Elhanani O, Kehat I, Hai T, Aronheim A. Adult cardiac expression of the activating transcription factor 3, ATF3, promotes ventricular hypertrophy. PLoS One. 2013 Jul 3;8(7

Kehat I. Novel strategies for the treatment of heart failure. Rambam Maimonides Med J. 2012 Apr 30;3(2)

List scientific conferences attended:

Heart Failure Association of the European Society of Cardiology -Translational Winter Research Meeting on Heart Failure. Les Diablerets, Switzerland. 18-21 January, 2012: Invited speaker

International Society for Heart Research, European Section, Israeli Subsection meeting. Ramat-Gan, Israel, 1 March 2012: Invited speaker

Emerging Faculty Teaching Skills Workshop. Washington DC, USA. Spetember 10, 2012: Selected by the Israeli heart society to participate in the American College of Cardiology skills workshop.

The Biochemistry, Biology and Pathology of MAP Kinases Meeting. Ma'ale Hachamisha, Israel. October 14-18, 2012: Speaker

Frontiers in Cardiovascular Regeneration International Symposium. San Diego, Ca, USA. November 7-8, 2012: Invited speaker

Biomedical Engineering & Internation society of heart research, European Section, Israeli Subsection meeting, Haifa, Israel. 19 February 2013: Invited speaker

The department of Cardiology and Angiology of the Hannover School of Medicine -advanced training seminar. Hannover, Germany. 17June, 2013: Invited speaker

University of Michigan – Israel Partnership for Research Seminar. Ann- Arbor, Mi, USA. October 18, 2013: Invited speaker

Israeli Society for Medical and Biological Engineering Annual meeting. Haifa, Israel. 27 February 2014: presentation by my PhD student

The 61st Annual Conference of the Israel Heart Society, Tel-Aviv, Israel, 30 April, 2014: abstract presentation by my PhD student

Hannover Medical School Fellow Seminar -Mechanisms of cardiac concentric and eccentric hypertrophy. Hannover, Germany. May 2014: Invited speaker

The 11th meeting of the myocardial and pericardial working group of the European society for cardiology. Cardiomyocyte remodeling and ventricular function. Tel-Aviv, Israel, February 4-6, 2015: Invited speaker

Scientific workshop about mechanotrasduction in cell biology, Biomechanics of cardiac cell hypertrophy, Paris, France. March 10-11, 2015: Invited speaker

Biomedical Engineering & International society of heart research, European Section, Israeli Subsection meeting, Genome organization in cardiac hypertrophy, Haifa, Israel. February 2015: Invited speaker

Outreach activities:

"Cardiac hypertrophy, the good, bad and the ugly". Frontiers in biomedical sciences: open talk series held at the Technion. 7 April 2013.

7. Project Management (expected length: max. 800 words)

Size and composition of the research group:

I have established my independent lab. I have four PhD students, two MD residents (one from internal medicine and one from cardiac surgery) who have a one year full time research project at the lab, and one full time technician.

Stable, permanent position:

I have attained a joint position: A tenured position at the Department of Cardiology and Rambam Research institute, Rambam Medical Center, and a tenure-track assistant professor position at the Department of Physiology, Faculty of Medicine, Technion-Israel Institute of Technology.

Independence and support from Host institute:

I have established my independent lab and I receive full support from both the Rambam Research institute, Rambam Medical Center and from the Department of Physiology, Faculty of Medicine, Technion. This support includes use of the interdisciplinary equipment unit, animal facility, animal imaging center, administrative and computer (IT) support.

Additional grants:

2014-2017 Ministry of Health Grant : Mechanism of Extra skeletal ossification 41,796 Eu

2014 University of Michigan-Israel partnership: small G proteins in hypertrophy. 20,000 USD

2012-2015 Niedersachsen - Israel Research Cooperation Program - Unraveling the mechanisms of asymmetrical growth and localized translation in cardiac myocytes: molecular imaging and identification of signaling responsive RNA-binding proteins- 105,000 Eu

2012-2016 Israel Science Foundation (ISF) 873/12 – Histone deacetylase complex in cardiac hypertrophy, 852,000 NIS (179,853 Eu)

2012-2015 Rappaport institute Grant – 60,000 USD

Collaborations:

Established collaboration with Prof. Dr. Jörg Heineke, Experimental Cardiology, Klinik für Kardiologie und Angiologie, Medizinische Hochschule Hannover – to study the cellular mechanisms of hypertrophy

Collaboration with Prof. Allan Saltiel, University of California at San Diego (UCSD) – to study the role of RhoJ proteins in hypertrophy.