Supplementary Material

Detailed Methods

Antibodies. Antibodies to GTP bound Gα13 and Gα13 (Kit# 80401, Neweast Biosciences), phospho-HDAC4 and phospho-HDAC5 (3443, 3424, Cell Signaling), HDAC4 (2072, Cell Signaling), HDAC5 (2082, Cell Signaling), connexin 37 (cx37A11-A, Alpha Diagnostic International), connexin 40 (20466, Santa Cruz), APJ (LS-A64, MBL), GAPDH (2118s, Cell Signaling) were used for western blotting. Antibodies to CD31 (557355, BD Biosciences), SMA conjugated with cy3 (c-6198, Sigma), PCNA (2014-03, DAKO), connexin 37 (40-4200, Invitrogen), connexin 40 (20466, Santa Cruz), HDAC4 (2072, Cell Signaling) and HDAC5 (2082, Cell Signaling) were used for immunostaining on the cryosections. Alexa 488 (A11008 and A11001, Invitrogen), Alexa 568 (A11011 and A10037, Invitrogen) conjugated secondary antibodies and rat detection kit for anti-mouse CD31 (Biocare Medical) were used.

Embryo harvest and Immunohistochemistry. Embryos were collected at various stages of development from pregnant females, genotyped and photographed as per standard methodology. Embryos were fixed in 4% PFA at 4 °C overnight and cryoprotected in 30% sucrose (in 1× PBS) at 4 °C overnight. Embryos were then embedded in optimal cutting temperature medium (OCT) (Sakura Tissue-Tek), frozen solid in cryomolds, sectioned on a Leica CM1950 at 10 µm and stored at −20 °C. Cryosections were then air dried for 10 min and washed in 1× PBS for 15 min at room temperature. Sections were blocked in 5% heat-inactivated goat serum in 1× PBS for 1 h at room temperature and then probed with primary antibodies overnight at 4 °C. After incubation, slides were washed with 1× PBS, blocked for 1 h at room temperature and probed with secondary antibodies for 1 h at room temperature. After washing, slides were mounted in mounting medium with DAPI (Invitrogen). H&E staining was performed using standard methods.

***In situ* hybridization.** Mouse Klf2 probe was amplified from mouse lung cDNA by PCR and cloned into the pCR2.1 TOPO vector (Invitrogen) using the following primers: Forward primer-5'-CTTGCACATGAAGCGACACA-3'; Reverse primer-5'-CCGTGATTCCTCCAAAGATC-3' (463 base pairs). Sense and antisense digoxigenin (DIG)-labeled riboprobes were generated by PCR in the presence of DIG-dUTP M13 and T7 primers (Invitrogen). Incubation was done at 65 °C overnight. DIG was detected by a digoxigenin-specific, horseradish peroxidase–labeled antibody (Roche-11093274910). For alkaline phosphatase reaction, AP substrate (NBT/BCIP-Roche-11681451001) was used and the slides were mounted in aqueous mounting medium.

G protein activity measurement. COS7 cells were transfected with either pcDNA3 or APJ expression construct with wildtype Gα13 expression construct. Membranes were harvested from transfected cells by sucrose gradient differential centrifugation. Isolated membrane fractions were segregated into paired samples upon resuspension with HEM buffer (20 mM HEPES, pH 7.4, 1.4 mM EGTA, and 12.5 mM MgCl2). Samples were incubated 10 minutes at room temperature with excess GDP (2mM), followed by addition of [35S] GTPγS along with either vehicle (HEM) or apelin 13 peptide (Sigma). Samples were incubated for 1 h at 4 ℃ on a rotator. Mouse anti-Gα13-GTP antibody or anti-mouse IgG was added, and samples were incubated overnight at 4℃. Protein A/G PLUS-agarose beads (Santa Cruz) were pre-incubated with cold GTPγS to reduce non-specific binding. Saturated beads were washed to remove unbound cold GTP before being added to each sample, and incubated for 1 hour. Beads were then washed three times, resuspended in HEM, and transferred to scintillation plate. Samples were counted three times to produce final CPM readings.

Immunoprecipitation and immunoblotting. For the active Gα13 immunoprecipitation, HUVEC cells were either incubated with 1 μM apelin 13 for 30 min after serum starvation or transfected with an APJ expression construct. Cells were harvested in lysis buffer using the Gα13 activation assay kit (NewEast Biosciences). Active Gα13 proteins were immunoprecipitated by using anti-Gα13-GTP antibody and washed five times. Precipitated proteins were resolved by SDS/PAGE, transferred to PVDF membranes, and immunoblotted with anti-Gα13 (total) antibody. Proteins were visualized by using a chemiluminescence system (Thermo Scientific). For HDAC4/5 phosphorylation, HUVECs were transfected with either expression vectors or knockdown siRNAs along with the appropriate controls for 48 hours. A subset was stimulated with apelin 13. RIPA lysis buffer (Thermo Scientific) containing Halt Protease and Phosphatase Inhibitor cocktail (Thermo Scientific) was used for cell lysis. Protein contents were measured using a Bio-Rad DC assay kit. Western blots were carried out as previously described.[1] Each western blot is a representative of three independent experiments of triplicate samples.

Realtime PCR analysis. RNA was extracted using the miRNasy® Mini kit (Qiagen) according to the manufacturer's instructions. DNase treated RNA was reverse-transcribed with iScript reverse transcriptase kit (Bio-Rad). For quantitative PCR analyses, mouse or human Taqman probes (Applied Biosystems) or designed primers (Online Table III) were used. 18S probe (Applied Biosystems) was used for an internal normalization control. Quantitative PCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad) and Power SYBR Green (Applied Biosystems) according to the manufacturer's protocol. The RT-PCR assays were performed on Bio-Rad CFX96 thermal cycler. Each condition was repeated three times in triplicates.

DNA constructs. The sense and antisense strands of the 41 basepair KLF2 promoter (Online Figure V) (Keck Oligo Lab, Yale University) were cloned into pGL3 vector using NheI and XhoI sites. 3X-MEF2-luciferase reporter, HDAC4-GFP, and HDAC5-GFP plasmids were kindly provided by Dr. Eric Olson. KLF2-221 bp and KLF2-221 bp-MEF2 mt constructs were kindly provided by Dr. Mukesh Jain.[2] Expression constructs for apelin, APJ, APJ-GFP, MEF2A and MEF2C (Origene), wildtype Gα13, constitutively active Gα13, Gαi and Gαq (www.cdna.org), HDAC4-FLAG and HDAC5-FLAG (Addgene) were used.

Luciferase assays. Either COS7 cells or HUVECs were transfected and lysed in lysis buffer (Promega). Dual Luciferase Reporter System (Promega) was used according to the manufacturer's protocol. All experiments were performed three times in triplicates.

Chromatin immunoprecipitation assay. HUVECs were transfected with control siRNA or apelin-APJ siRNAs (Invitrogen) for 48 hours and native protein-DNA complexes were cross-linked by treatment with 1% formaldehyde for 15 minutes. Simple ChIP Plus Enzymatic Chromatin IP kit (Cell Signaling) was used per the manufacturer's protocol. Briefly, equal aliquots of isolated chromatin were subjected to immunoprecipitation with anti-MEF2 antibody (Santa Cruz; sc-313X), or rabbit IgG control. PCR reactions of immunoprecipitated DNA were performed to validate MEF2 binding on the *KLF2* promoter. PCR primers used: FWD: TGTCAGCGCAAGGCCCAG and REV: GATGGGGACGAGCTCCGG. PCR products were separated by gel electrophoresis and visualized by SYBRsafe (Invitrogen).

Immunocytochemistry. For the apelin 13 effect on HDAC4/5 translocation, HUVEC cells plated on glass bottom culture dish (Mat Tek) were transfected with either FLAG or GFP-tagged HDAC4 and HDAC5 expression vectors for 24 hours. Cells were imaged using a Nikon Eclipse Ti confocal microscopy before and after treatment with apelin 13 (1 μM for 1 h at 37℃). For the effect of APJ on HDAC4/5 translocation, HUVECs were transfected with FLAG-tagged HDAC4

or HDAC5 with either GFP vector (pEGFP-N1) or APJ-GFP. 24 hours after transfection, cells were fixed with 3% paraformaldehyde, washed, and permeabilized with 0.2% Triton X-100/PBS for 10 min, and blocked with 10% fetal bovine serum in 0.1%/PBS for 10 min. Cells were incubated with Cy3 conjugated anti-FLAG antibody in blocking solution for 2 h at room temperature. Dishes were mounted using mounting medium with DAPI (Invitrogen). Analysis of fluorescent staining was performed using confocal microscopy. Isolated heart ECs from mice were treated as above and stained for Hdac4 or Hdac5. In HUVECs, the HDAC4/5 data is presented as percent cells with cytoplasmic HDAC4/5, as in basal condition the majority of cells had the transfected HDACs being localized to the nucleus. In isolated heart endothelial cells and in endocardium, this data is presented as percent cells without nuclear Hdac4/5, as the majority of cells had endogenous Hdac4/5 in both the cytoplasm and the nucleus, and responded to apelin by translocation of the nuclear fraction to the cytoplasm.

Whole-mount immunostaining. For whole-mount immunostaining, embryos were fixed in 4% paraformaldehyde at 4 °C overnight and dehydrated with methanol, then rehydrate through descending graded alcohols to PBS. The samples were then blocked in 1% BSA/PBS-Tween at 4 °C overnight and incubated overnight at 4°C with anti-CD31 antibody. The samples were washed 4 times for 45 min each in PBS-Tween and incubated with goat anti-rat Alexa488 antibody at 4 °C overnight.

Microarray. HUVECs were transfected with apelin and APJ siRNA, MEF2A and MEF2C siRNA, Gα13 siRNA or negative control siRNA (Invitrogen). After 48 hours, RNA was extracted using miRNasy® Mini Kit (Qiagen). The RNA was quantified and the RNA quality was verified. The HumanHT-12 v4 Expression BeadChip Kit (Illumina) was used according to the manufacturer's protocol by the Yale Center for Genome Analysis. Microarray results were analyzed using the bead array and limma packages in R/Bioconductor (v 2.14/2.09). Differential gene expression was defined as a two-fold difference in log intensity between samples.

Supplemental References

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Online Table I. Gene expression analyses of HUVECs subjected to 1) apelin/APJ, 2) Gα13, or 3) MEF2A/C knockdown.

	List of Upregulated Genes (>2 fold in all three conditions)	List of Downregulated Genes (< 0.5 fold in all three conditions)
Name of genes	ANKRD9 AP1M1 ARMCX6 BCAT2 CENPB EIF4EBP2 FBXL18 FEM1A KHSRP LLGL1 LOC100134073 LOC401115 LOC653381 LYPD1 LYPD1 MED16 MICB MPRIP NOX4 PAK4 ZDHHC8 PIP5K1C PTOV1 RAB11FIP3 SH3GL1 SPOCD1 STC2	ADAMTS9 ALOX5AP ANGPTL4 APLN ARSD C10orf54 C20orf160 CAV2 C22orf29 CCL7 CCL20 CETP CSF2 CX3CL1 CXCR7 CXXC5 CYTL1 DUSP6 DYSF EBI3 FAM116B FLJ10986 GALNT1 GJA4 GJA5 GPR126 IL1A IL27RA IL8 IQCK KRCC1 LIPG LOC285943 LTB MGAT4A MSMP MYL12A MYL5 PALM PLVAP PPAP2B

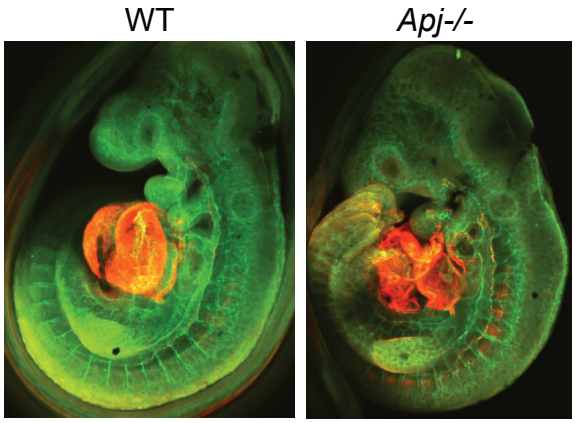
		S100A3 SDPR SELE SERPINB1 SH2D3C SLC16A3 SLCO2A1 ST3GAL5 TANK TNFRSF4 TNFRSF6B VCAM1 VIP

Online Table II. Putative endothelial apelin/APJ, Gα13, MEF2 targets identified by microarray and qPCR analyses.

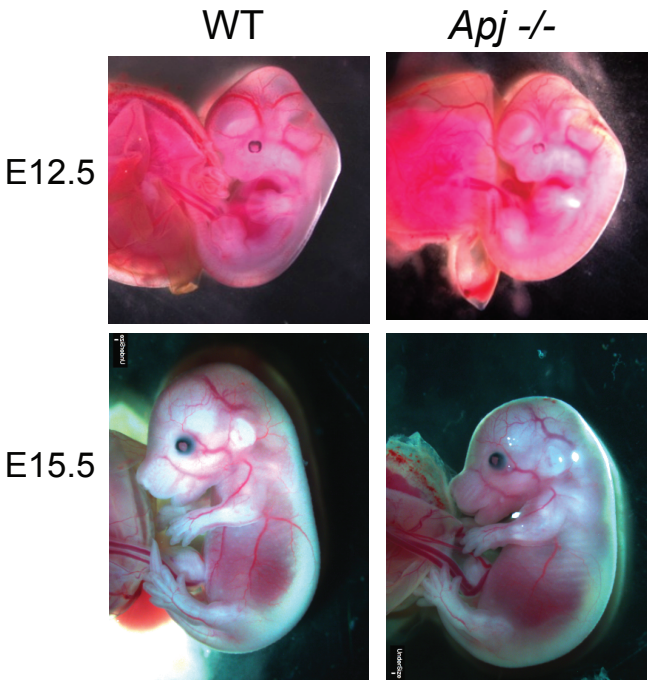
Gene	Role in development/gene function	Reference
KLF2	Global -/- is embryonic lethal due to impaired blood vessel maturation.	3, 4
VCAM1	Global -/- is embryonic lethal due to defective chorio-allantoic fusion and myocardial defects.	5, 6
Connexins 37 and 40	Double -/- mice have vascular developmental defects and hemorrhages and die between E12.5 and birth.	7
Krcc1	Secreted FGF binding proteins. Knockdown results in embryonic lethality within 5 days. Predicted to be a MEF2 transcriptional target.	8
Myl12a	Non-muscle regulatory light chain. Predicted to be a MEF2 transcriptional target.	9
Plvap	Global -/- is embryonic lethal due to vascular permeability. Predicted to be a MEF2 transcriptional target.	10
SerpinB1	Inhibitor of neutrophil serine proteases. Predicted to be a MEF2 transcriptional target.	11
St3gal5	Catalyzes the formation of ganglioside GM3 which is important in cell differentiation, cell proliferation and integrin-mediated cell adhesion. Predicted to be a MEF2 transcriptional target.	12

Online Table III. Primer sequences for quantitative PCR analyses for gene expression.

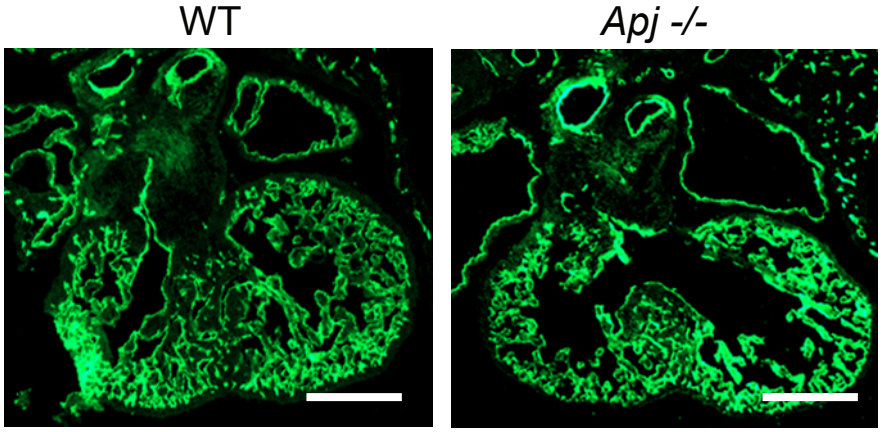
NAME of GENE	PRIMER SEQUENCE	
FOR MOUSE RNA		
KRCC1 (NM_145568)	Forward Sequence	GAGACTCCCATCTGGAACCAAC
	Reverse Sequence	TCTGAGAAGCCATCCCTGGTGA
MYL12A (NM_026064)	Forward Sequence	GTTCGCCATGTTTGACCAGTCC
	Reverse Sequence	CGTCCAGGTATTCGTCAGTTGG
PLVAP (NM_032398)	Forward Sequence	GTTGACTACGCGACGTGAGATG
	Reverse Sequence	AGCTGTTCCTGGCACTGCTTCT
SERPINB1 (NM_025429)	Forward Sequence	GGATGCTCCATTCCGACTGAGT
	Reverse Sequence	AGTTCTCCACCCTGGTAAGGCA
ST3GAL5 (NM_001035228)	Forward Sequence	ACTCCAGCCAAAGCACTTCAGG
	Reverse Sequence	GATGTGTAGCCAAGACAACGGC
VCAM1 (NM_011693)	Forward Sequence	GCTATGAGGATGGAAGACTCTGG
	Reverse Sequence	ACTTGTGCAGCCACCTGAGATC
FOR HUMAN RNA		
KRCC1 (NM_016618)	Forward Sequence	GAAACCATCCAGACCTACCCAAG
	Reverse Sequence	CCTCGTGAACTGACAGTAGCTC
MYL12A (NM_006471)	Forward Sequence	GTTTGACCAGTCGCAGATTCAGG
	Reverse Sequence	TAGATACTCATCAGTTGGATTCTTC
PLVAP (NM_031310)	Forward Sequence	CAATCAGAGGTACATGGCTGCC
	Reverse Sequence	CTATCTCCACCTCCAGCGTCTT
SERPINB1 (NM_030666)	Forward Sequence	AGCTCAGCATGGTCATCCTGCT
	Reverse Sequence	CGAGATTCTCAGGTTTAGTCCAC
ST3GAL5 (NM_003896)	Forward Sequence	AGAGCCTCAGTCAAGGTTCTGG
	Reverse Sequence	GAGGTCATATCCAAAACCCGCC
VCAM1 (NM_080682)	Forward Sequence	GATTCTGTGCCCACAGTAAGGC
	Reverse Sequence	TGGTCACAGAGCCACCTTCTTG



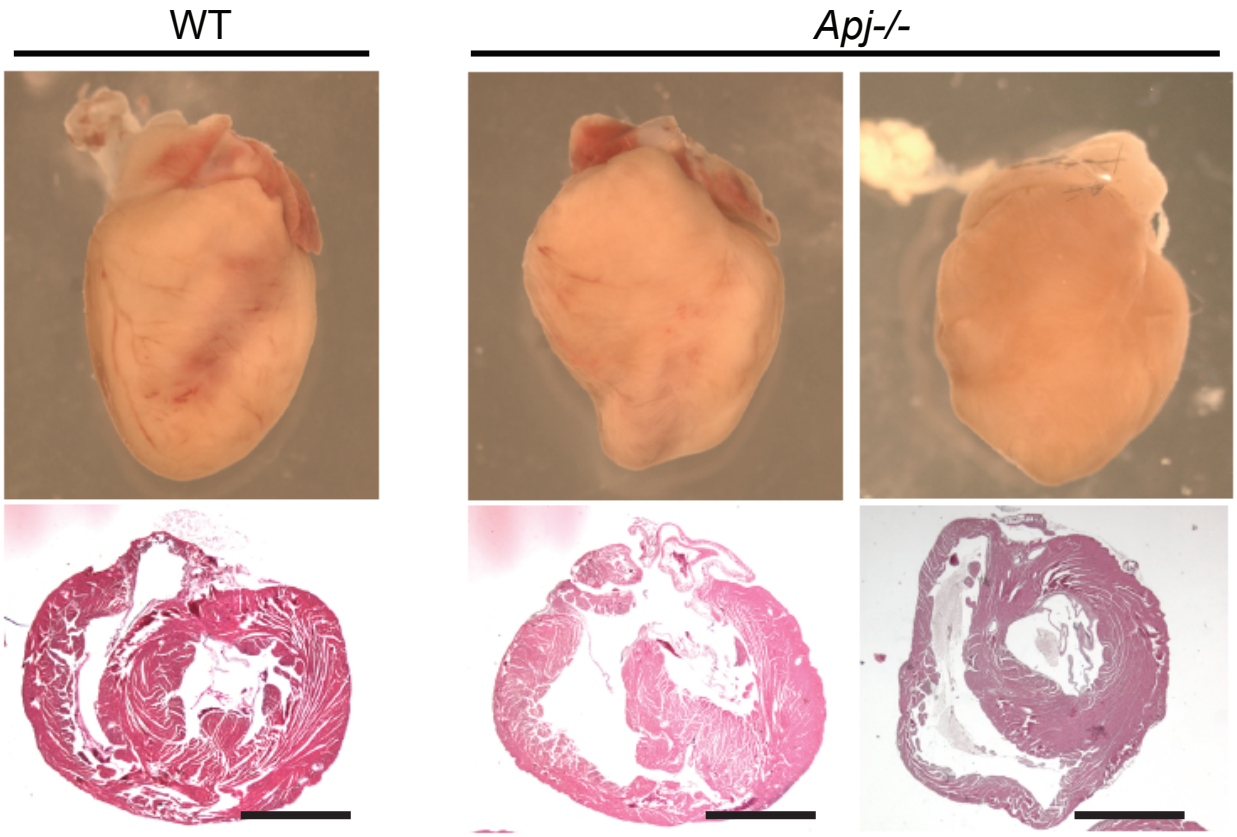
Online Figure I. CD31 staining of wildtype and *Apj-/-* E10.5 embryos show comparable CD31 staining (n=3 per group). CD31 staining is shown in green, SMA staining is shown in red.



Online Figure II. Representative embryos from E12.5 (n=5 per group) and E15.5 (n=4 per group) showing grossly normal appearance of *Apj-/-* embryos.

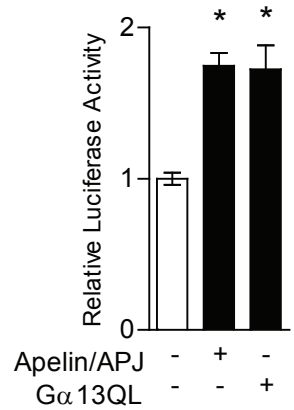


Online Figure III. An E12.5 heart with CD31 staining showing the presence of VSD in ~20% of *Apj-/-* embryos (1 out of 5). Bars indicate 300 μm.

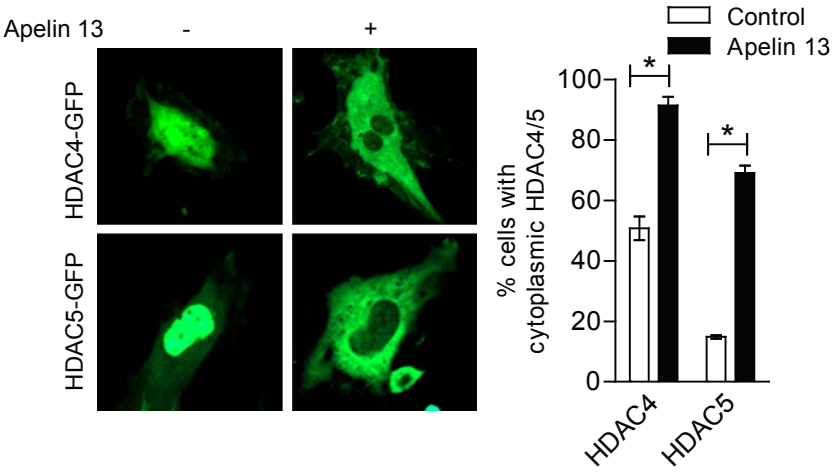


Online Figure IV. Hearts of adult *Apj-/-* mice demonstrated presence of ventricular septal defect (2 out of 10) as well as enlarged or deformed right ventricle (4 out of 10). Bars indicate 5 mm.

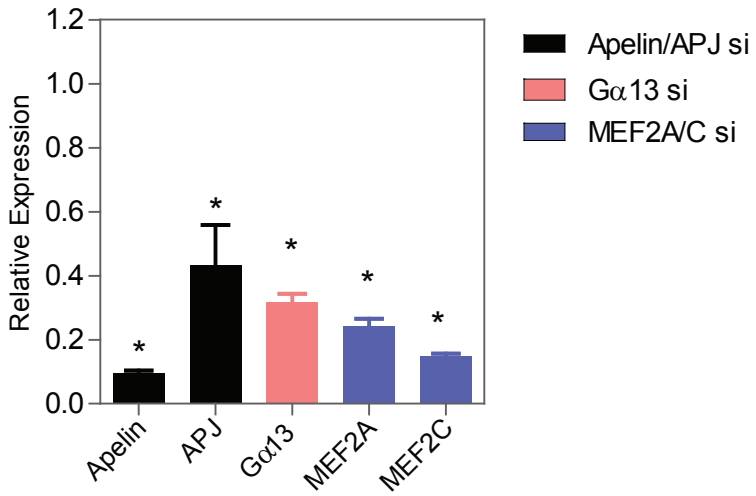
Online Figure V. Sequence of the 41 bp KLF2 promoter containing MEF2 binding sites.



Online Figure VI. Overexpression of apelin and APJ or Ga13-QL lead to significant induction of luciferase activity driven by 3X MEF2 binding site in HUVECs. **P*<0.01 vs. control.



Online Figure VII. Stimulation of HUVECs with apelin 13 leads to cytoplasmic translocation of GFP tagged HDAC4 and HDAC5. *P<0.01.



Online Figure VII. Relative knockdown levels achieved for apelin, APJ, Gα13, MEF2A and MEF2C in HUVECs wit the three knockdown conditions. *P<0.01 vs. control.