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### Title

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### Permalink

<https://escholarship.org/uc/item/1sh9x59w>

### Journal

Nature, 488(7411)

### ISSN

0028-0836

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### Publication Date

2012-08-01

### DOI

10.1038/nature11263

Peer reviewed



Published in final edited form as:

Nature. 2012 August 16; 488(7411): 394–398. doi:10.1038/nature11263.

## APJ ACTS AS A DUAL RECEPTOR IN CARDIAC HYPERTROPHY

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### Abstract

Cardiac hypertrophy is initiated as an adaptive response to sustained overload but progresses pathologically as heart failure ensues<sup>1</sup>. Here we report that genetic loss of APJ confers resistance to chronic pressure overload by dramatically reducing myocardial hypertrophy and heart failure. In contrast, mice lacking apelin (the endogenous APJ ligand) remain sensitive, suggesting an apelin independent function of APJ. Freshly isolated APJ-null cardiomyocytes exhibit an attenuated response to stretch, indicating that APJ is a mechano-sensor. Activation of APJ by stretch increases cardiomyocyte cell size and induces molecular markers of hypertrophy. Whereas apelin stimulates APJ to activate G $\alpha_i$  and elicits a protective response, stretch signals in an APJ-dependent G-protein-independent fashion to induce hypertrophy. Stretch-mediated hypertrophy is prevented by knockdown of  $\beta$ -arrestins or by pharmacological doses of apelin acting through G $\alpha_i$ . Taken together, our data indicate that APJ is a bifunctional receptor for both mechanical stretch

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**Competing interests.** Authors don't have any competing interest regarding this publication.

**Respective author contribution.** MSC and CH designed, performed experiment, analyzed data and prepared manuscript. CEW, SR, SAM, KW, SM, JW, NHP, TA, and PK designed and performed experiment, analyzed data. DC, GPV, RB, OFB, LHS, EA, JHB designed experiment. MM designed experiments and prepared manuscript. PRL designed, supervised experiments, analyzed data and prepared manuscript.

and for the endogenous peptide apelin. By sensing the balance between these stimuli, APJ occupies a pivotal point linking sustained overload to cardiomyocyte hypertrophy.

GPCRs have been widely implicated in the control of cardiac function. These receptors couple to heterotrimeric GTP-binding proteins of the  $G\alpha_s$ ,  $G\alpha_i$ ,  $G\alpha_{q/11}$  and  $G\alpha_{12/13}$  families, and transduce the GPCR signal to intracellular targets. Numerous studies have linked  $G\alpha_s$  to increased contractility,  $G\alpha_{q/11}$  to pathological hypertrophy<sup>2,3</sup>, and  $G\alpha_i$  to cardioprotection<sup>4</sup>. APJ is a GPCR identified as the receptor for the adipokine apelin<sup>5,6</sup>. Apelin-activated APJ signals through  $G\alpha_i$  exerting a positive effect on cardiac contractility<sup>7-9</sup> and a vasodilator activity that counteracts angiotensin-II-induced atheroma<sup>10,11</sup>. Apelin administration blunts progression to hypertrophy (Suppl. Fig. 1 and Suppl. Tables 2-3) and apelin-KO mice show susceptibility to heart failure<sup>12</sup> (also see Suppl. Fig. 1 and Suppl. Table 1). Thus, apelin and its receptor APJ are emerging as potential therapeutic targets.

We examined the response of APJ knockout mice to sustained pressure overload by transaortic constriction (TAC). Although deletion of APJ resulted in some prenatal lethality<sup>13,14</sup>, all viable APJ-KO mice displayed normal adult appearance and cardiovascular parameters at baseline (Suppl. Table 4). However, APJ-null animals were resistant to the pathological hypertrophic response to TAC (Fig. 1a-d) observed both in WT and in apelin-KO mice (Suppl. Fig. 1g-i). APJ-KO mice responded to TAC by initially increasing cardiac mass but the maladaptive progression to dilated ventricular hypertrophy was blunted shortly after injury (Suppl. Table 4). The protective effect persisted long-term (Fig. 1a, b and g,h) in all parameters measured, including diminished cardiomyocyte size (Fig. 1c, d), reduced fibrosis (Fig. 1e, f), sustained cardiac contractility (Fig. 1g) relative to WT and apelin-KO mice (Suppl. Tables 1, 4), and reduced heart weight/body weight ratio (Fig. 1h). Baseline cardiac contractility measured as percent fractional shortening (%FS), was approximately 38% across genotypes. After 90 days of TAC, % FS decreased to  $22 \pm 2\%$  in WT,  $23 \pm 1\%$  in apelin KO mice, but remained at  $34 \pm 2\%$  in APJ-KO mice ( $p=0.01$  between APJ-KO and WT) (Fig. 1g and Suppl. Tables 1, 4). In summary, both WT and apelin-KO mice presented clear signs of heart failure after 90 days of TAC, while APJ-KO mice were almost unaffected. The maintenance of cardiac function in the APJ-KO demonstrates that the expression of APJ is necessary to elicit heart failure in response to pressure overload.

The different responses of apelin-KO and APJ-KO mice to TAC imply that either apelin can act independently of APJ, or that APJ transduces a signal independently of apelin. We tested the first hypothesis by infusing APJ-KO mice with apelin (285  $\mu\text{g}/\text{kg}/24\text{h}$ ) and examining two readouts: contractility under TAC, and vascular tone. Notably, apelin infusion did not increase cardiac contractility (%FS) in TAC-APJ-KO mice, in contrast to the characteristic improvement seen in TAC-WT animals (Suppl. Fig. 2a). In the absence of apelin infusion, endogenous levels of apelin in blood increased after TAC from 1ng/ml to 2ng/ml and that rise was not-different in WT and APJ-KO mice, making it unlikely that the protection achieved in the APJ-KO is due to hyper-activation of apelin signaling (Suppl. Fig. 2b). To test vascular tone, systolic and diastolic blood pressures were increased by infusion of Ang-II (1,000 ng/kg/min). Apelin infusion significantly decreased systolic blood pressure in WT

animals but not in APJ-KO mice (Suppl. Fig. 2c–f), further suggesting that apelin activity requires APJ.

Since the mechanical properties of the heart change dramatically during pressure overload<sup>15</sup>, and the structurally related angiotensin receptor (AT-1) can act as a mechanosensor<sup>16</sup>, we asked whether APJ responds to mechanical stretch. Initially these experiments were challenging as cultured cardiomyocytes consistently down-regulate the expression of endogenous APJ (Suppl. Fig. 3a), and studies had to be restricted to freshly isolated adult cardiomyocytes. We mimicked the effect of pressure overload by using a carbon fiber (CF) technique<sup>17</sup> to stretch cardiomyocytes and evaluated their Frank-Starling Gain (FSG). FSG is a dimensionless metric of the force that can be recruited by stretch<sup>18</sup>. Freshly isolated adult cardiomyocytes from WT mice displayed a significantly higher FSG than cardiomyocytes from APJ-KO mice (Fig. 2a–c). Treatment with apelin decreased the FSG in WT cardiomyocytes but showed no effect in APJ-KO cells, (Fig. 2d). Therefore, apelin modulated the response to stretch only in cardiomyocytes with APJ receptors.

Engineered cells stably expressing human APJ (APJ-HEK) responded to apelin by increasing the content of pERK (Fig. 3a, b) whereas parental (HEK) cells showed no significant change. Stretch also increased pERK content in cells expressing APJ (Fig. 3a,b). pERK levels, therefore, reflect the cellular response to APJ activation by either stretch or apelin. pERK was therefore used as a simple readout of APJ activation. The G $\alpha_i$  inhibitor PTX blocked the ability of apelin, but not stretch, to induce APJ-dependent phosphorylation of ERK (Fig. 3a,b), first suggesting that separate mechanisms link stretch and apelin to APJ intracellular signaling.

We next examined whether activation of APJ by stretch or apelin differentially modulate G-protein-generated second messengers. Activation of G $\alpha_i$  is associated with inhibition of adenylyl-cyclase and reduced cAMP, whereas activation of G $\alpha_s$  increases cAMP<sup>19</sup>. cAMP did not increase after application of stretch or apelin, arguing against activation of adenylyl-cyclase (and G $\alpha_s$ ) (Fig. 3c–d, no isoproterenol conditions). In contrast, when isoproterenol was used to elevate the intracellular concentration of cAMP<sup>20</sup>, apelin addition decreased cAMP levels in the APJ-HEK cells (Fig. 3c). This effect of apelin was partially inhibited by PTX, consistent with the involvement of G $\alpha_i$  (Fig. 3c). Apelin did not modify isoproterenol-stimulated cAMP formation in untransfected HEK controls, showing that the decrease is mediated by APJ. These data agree with previous reports<sup>21</sup> and demonstrate that G $\alpha_i$  transduces the signal initiated by apelin binding to APJ. In contrast, stretch reduced cAMP in parental HEK as well as in APJ-HEK cells (Fig. 3d), indicating that, although stretch can activate G $\alpha_i$  signaling, this response is not mediated through APJ.

The G $\alpha_{16}$  subunit couples any activated GPCR to phospholipase C, which results in accumulation of inositol phosphates (IP1), and thus provides a general readout of G-protein activation<sup>22</sup>. Stretch did not increase IP1 production whereas apelin did so in a dose-responsive manner (Fig. 3e). Importantly, for any given concentration of apelin, stretch consistently reduced G-protein activation (Figure 3e). There was a decrease in maximal levels and a shift in EC<sub>50</sub> from  $5.1 \times 10^{-9}$  to  $5.5 \times 10^{-8}$  when both stimuli were applied simultaneously (Fig. 3e). These experiments demonstrate that APJ activation by stretch is

largely G-protein independent and that stretch interferes with apelin-mediated G-protein activation by APJ.

Using a  $\beta$ -arrestin/APJ complementation assay, apelin was found to induce a dose-dependent increase in  $\beta$ -arrestin signaling, as expected (Fig. 3f). Stretch in the absence of apelin boosted  $\beta$ -arrestin signaling 2-fold over baseline (Fig. 3g). Notably, stretch increased  $\beta$ -arrestin recruitment in response to apelin at all doses tested (Figure 3g). Taken together, stretch causes APJ to signal less effectively/potently via G-proteins, but to more effectively recruit  $\beta$ -arrestin.

In order to investigate whether these distinct mechanisms of APJ action differentially affect cardiac hypertrophy, we examined cardiomyocyte cell growth and the expression of molecular markers of pathological hypertrophy in neonatal rat ventricular cardiomyocytes (NRVC)<sup>23,24</sup>. NRVC in culture respond to mechanical stretch<sup>25</sup>, mainly through the angiotensin II receptor AT-1<sup>26</sup> and the endothelin1 receptor ET-A<sup>27</sup>, which are also GPCRs. To test the specific effect of APJ in cardiac stretch/hypertrophy, we used pharmacological inhibitors of AT-1 (100 nM candesartan) and ET-A (300 nM BQ123), hereafter labeled as “inhibitors”. To overcome APJ down-regulation during cardiomyocyte culture, we reestablished APJ expression by adenoviral transduction (about 90% efficiency) with either control GFP (Ad-GFP) or an APJ-GFP fusion protein (Ad-APJ-GFP). Hypertrophy was assessed by the characteristic increase in perinuclear immunolocalization of atrial natriuretic factor (ANF) (Fig. 4a–m). Remarkably, apelin treatment did not increase ANF immunostaining, in Ad-APJ-GFP nor in Ad-GFP-infected cells (Fig. 4d–f and m). In contrast, stretch applied in the presence of inhibitors significantly increased the number of perinuclear ANF<sup>+</sup> cells in APJ-restored cardiomyocytes ( $27 \pm 1\%$ ), but not in control cardiomyocytes infected with Ad-GFP ( $5.8 \pm 2.1\%$ ) (Fig. 4g–I, m). Co-stimulation with apelin and stretch reduced the number of ANF<sup>+</sup> cells (Fig. 4j–l, m). Stretch also induced specific changes in the expression of other molecular markers of hypertrophy, including an increased ratio of  $\beta$ MHC/ $\alpha$ MHC (Fig. 4n) and increased cell size (Fig. 4o). Similar to its effects on ANF, the addition of apelin also attenuated effects of stretch on other parameters of hypertrophy (Fig. 4m–o). The induction of ANF by stretch was PTX-insensitive, but the ability of apelin to antagonize stretch-induced hypertrophy was prevented by treatment with PTX (Fig. 4w).

Several control experiments confirmed that APJ is directly involved in the response to stretch: NRVC treated with forskolin responded to apelin only upon reconstitution of APJ expression, as monitored by their ability to decrease intracellular cAMP levels (Fig. 4p). The possibility that stretch induces the secretion of factors that might indirectly activate hypertrophy through APJ was considered but appears unlikely since conditioned medium from APJ-transfected cardiomyocytes did not induce ANF expression (Fig. 4q). Moreover, the concentration of apelin in the media remained unchanged (approximately 5ng/ml) with or without stretch (Fig. 4r), and conditioned media from stretched cells could not activate  $\beta$ -arrestin recruitment (Fig. 4s). Single cell analysis of low multiplicity APJ-GFP transduced, cells showed that cardiomyocytes require APJ to induce ANF upon stretch (Fig. 4t–v, note perinuclear ANF immunostaining only in GFP<sup>+</sup> cells in panel v), further demonstrating that APJ activation through stretch is sufficient to elicit cardiac hypertrophy. Prior studies

showed that APJ interacts with AT-1 and apelin antagonizes AT-1 function<sup>11</sup>. As shown in Fig 4x, in the presence of AT-1 inhibitors, only those cells expressing APJ responded to stretch by significantly increasing perinuclear ANF expression. In the absence of AT-1 inhibitors, APJ-transduced and non-transduced cells reached the same maximal level of ANF expression (Fig. 4y). Thus, blocking AT-1 does not impair the ability of APJ to respond to stretch, indicating that APJ alone is sufficient to transduce a stretch-induced hypertrophy signal.

Importantly, siRNA specific for  $\beta$ -arrestin1 or  $\beta$ -arrestin2 (Fig. 4z) blocked the stretch induction of hypertrophic markers ( $\beta$ MHC/ $\alpha$ MHC Fig. 4z, and ANF not shown) with an additive effect when both siRNAs were used together. These data substantiate the model that APJ signaling through  $\beta$ -arrestin mediates stretch-induced myocardial hypertrophy.

**In summary**, the mechano-response of APJ is necessary (blunted hypertrophic response to TAC of APJ-KO mice, Fig. 1) and sufficient (stretch induction of ANF expression occurs in cells expressing APJ, Fig. 4) to trigger myocardial hypertrophy in a  $\beta$ -arrestin-dependent manner (Fig. 4z). Apelin does not induce hypertrophy, but instead blunts stretch-induced hypertrophic induction (Fig. 4j-l-o,t-y), suggesting the ability of apelin to override pathological signaling from stretch. At a mechanistic level, APJ transduces apelin and stretch signaling differently. The response to apelin appears to be G-protein-PTX-sensitive, whereas that induced by stretch is PTX-insensitive and G-protein independent in the absence of exogenous apelin (Fig 3). Stretch profoundly affects apelin signaling, diminishing G-protein activation while augmenting  $\beta$ -arrestin recruitment (Fig. 3e-g). These data indicate that APJ integrates apelin and stretch stimuli, biasing the levels of G-protein signaling versus  $\beta$ -arrestin recruitment accordingly.

These results have implications for the consideration of APJ as a drug target, since APJ/stretch can be pathological. Therefore, a beneficial effect will be obtained not by general apelin receptor agonism, but rather by selectively inhibiting the ability of APJ to respond to mechanical stretch or by blocking its interaction with molecules that initiate pathological signaling cascades.

## Summary of Methods

All experiments were performed in accordance with relevant guidelines and regulations. The Sanford-Burnham Medical Research Institute's Animal Care & Use Program is accredited by AAALAC International and a Multiple Project Assurance A3053-1 is on file in the OLAW, DHHS.

### APJ and apelin KO mice

APJ-KO mice were obtained from Deltagen. Apelin-KO mice are described elsewhere<sup>13</sup>. Both APJ and apelin lines (male and female) were in C57Bl/6 genetic background in a 99%–100% purity, as demonstrated by microsatellite analysis (Radil). For detailed methods, see supplementary information.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

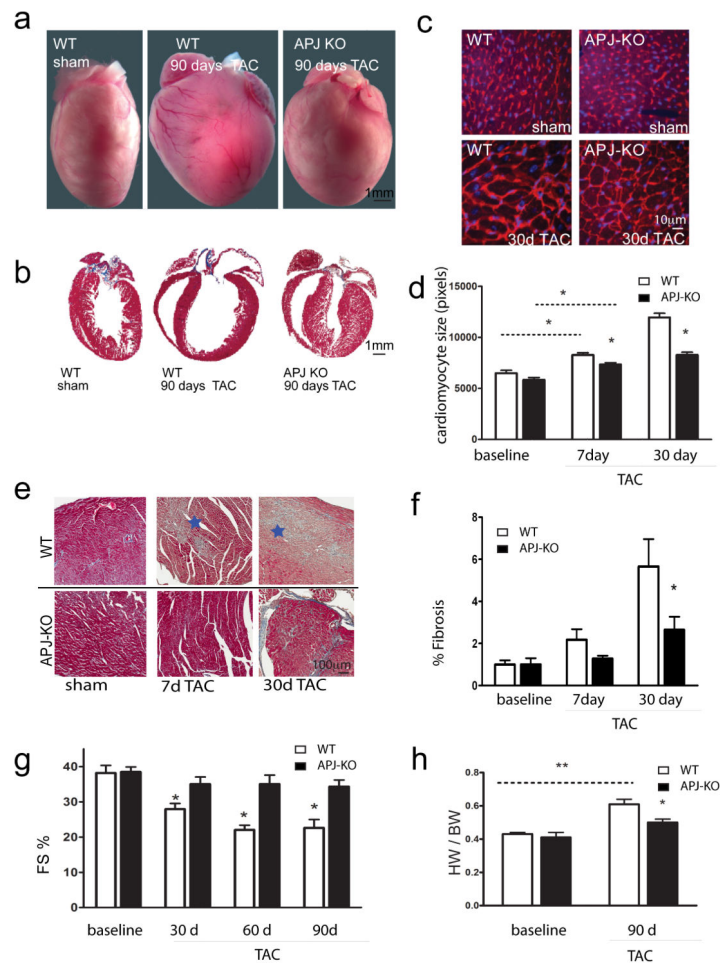
We are thankful to Nicole Ling, Sherry Zhao and Farid Abdel-Wahhab for excellent technical assistance. Dr. Eduard Sergienko for help optimizing arrestin assay. Dr. Eileen Adamson for critical reading of the manuscript. Marga Querol for graphics design. This work was supported by Wyeth Sponsored Research Agreement and NIH grant R01HL086879 to PRL; NIH grants R37HL059502 and R01HL083463 and the Sanford Children's Center to MM; NIH grant R01HL054732, grants from the Ellison Medical Foundation and the Muscular Dystrophy association to RB, NIH grant (NS05422) and Florida Department of Health grant 06-NIR-09 to LHS, and NIH grants RO1HL28143, P01 HL085577 to JHB. MCS has received support from CIRM (clinical fellow), Italian Ministry of Research and Education, and the Italian Society of Cardiology (SIC and Sanofi-Aventis Foundation). CH holds an AHA Postdoctoral Award, SR is a Sanford Children's Health Research Center fellow. PK is supported by SAF2010-15050 MICINN, Spain.

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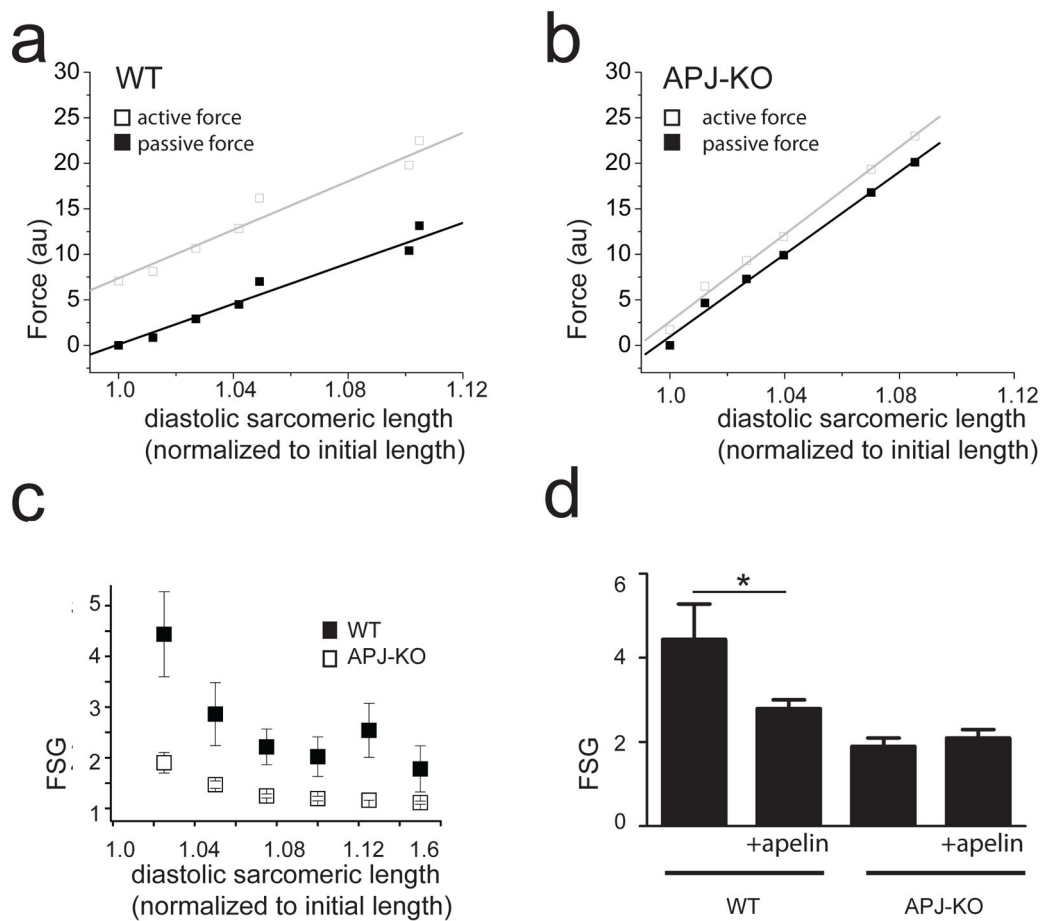
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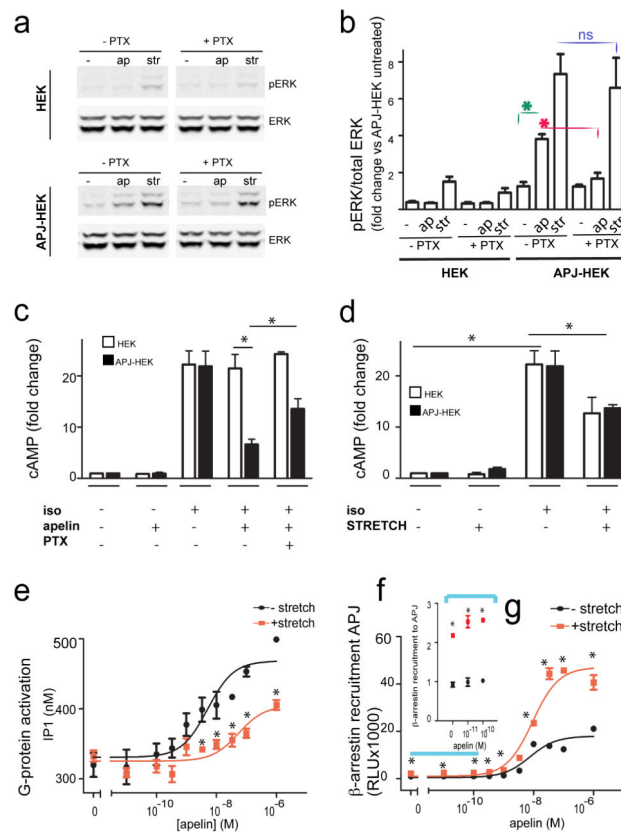
### Figure 1. APJ-KO mice are protected from hypertrophy after TAC

**a**, Anatomical view and **b**, Histological sections of WT and APJ-KO mice 90 days after surgery. **c**, Cell membrane staining (wheat germ agglutinin). **d**, Quantification from (c). **e**, Trichrome staining (fibrosis in blue, stars). **f**, quantification of (e). **g**, Fractional shortening (%FS) decreased in WT mice after TAC, but did not change significantly in the APJ-KO mice. APJ-KO mice fail to develop heart failure upon sustained TAC as shown by echocardiographic analysis. **h**, Heart weight-to-body weight ratio (HW/BW) at baseline and in TAC operated mice, 90 days after surgery (see Suppl. Table 4 for details). Error bars are SEM. \* $p < 0.05$  between indicated groups, ANOVA.

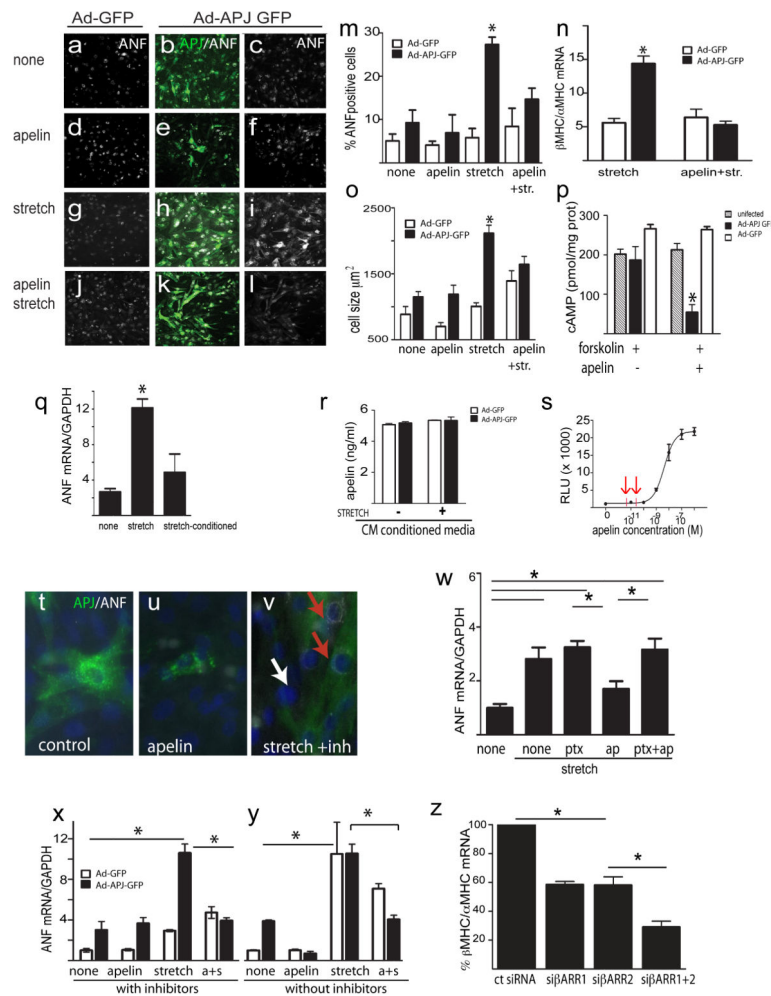


**Figure 2. APJ mediates a stretch response that can be modulated by apelin**

**a–b**, Representative force measurements (arbitrary units) for end-diastolic and end-systolic length-tension relationships in adult cardiomyocytes from WT (a) and APJ-KO (b) mice plotted against diastolic length (normalized to unstretched length). Cells were paced at 1Hz. **c**, Frank-Starling Gain (FSG) attained by dividing the active force by the passive force from experiments in (a) and (b) plotted as a function of diastolic length ( $n= 8$  WT and 7 APJ-KO cardiomyocytes). **d**, Average FSG (at 1.02 sarcomeric length from c) is shown for APJ-KO and WT both with (+) and without (–) 10 nM apelin administration ( $n=6$  for WT+apelin and  $n=7$  for APJ-KO+apelin). Error bars are SEM.



**Figure 3. Stretch activation of APJ enhances  $\beta$ -arrestin while reducing G-protein signaling**  
**a**, Immunoblot and **b**, Quantification of ERK from APJ stably transfected (APJ-HEK) and parental (HEK) cells treated for 5 minutes with 100 nM apelin or stretch in absence or presence of PTX ( $G_{\alpha_i}$  inhibitor),  $n=3$ . **c**, Effect of 1  $\mu$ M apelin or **d**, stretch on cAMP levels. 1  $\mu$ M isoproterenol (iso) was used to artificially elevate cAMP to study  $G_{\alpha_i}$  activation,  $n=4$  (PTX). **e**, G-protein ( $G_{\alpha_{q,s,i}}$  and  $12/13$ ) activation by apelin and effect of stretch (red) in CHO cells expressing APJ and  $G_{\alpha_{16}}$ . Receptor stimulation activated the promiscuous  $G_{\alpha_{16}}$ , phospholipase-C $\beta$  and caused the accumulation of IP1 (representative of 3 experiments,  $n=4$  samples). **f-g**, Arrestin recruitment to the APJ receptor in response to apelin (black) and stretch (red) by an enzyme complementation assay in CHO cells expressing recombinant APJ and  $\beta$ -arrestin2 (representative of 3 experiments,  $n=3$  samples). (f) Represents full range of  $\beta$ -arrestin binding to APJ under either physiological or pharmacological doses of apelin. (g) Shows the data points for 0 – 10<sup>-10</sup> M apelin. RLU= Relative light units. Error bars represent SEM. \* $p<0.05$  between indicated groups, ANOVA.



**Figure 4. APJ activation through mechanical stretch elicits cardiac hypertrophy**  
**a–l**, ANF immunostaining (white) and nuclear DAPI staining (blue) of rat neonatal ventricular cardiomyocytes transduced with rat APJ (Ad-APJ-GFP) or control GFP (Ad-GFP, green). **m**, Quantification of from a–l (n=250–350 cells). **n**, qPCR analysis of the ratio between β- and α-MHCs, as an independent index of hypertrophy, n=3–5 samples. **o**, Mean cell sizes in a–l, n=24 cells. **p**, Responsiveness of transfected cells to apelin treatment, n=4–5. **q**, ANF expression in cardiomyocytes in the presence of conditioned-media from stretched cardiomyocytes (str. media), n=3. **r**, Apelin ELISA of conditioned media (12 h) from cardiomyocytes (CM) non-stretched and stretched. **s**, Apelin standard curve of CHO APJ-β-arrestin interaction assay; red arrows represent the response elicited by 2 samples from (r) **t–v**. Higher magnification image of NRVC showing ANF (white) and APJ (green) expression. White arrow indicates APJ<sup>+</sup> cells not expressing ANF. **w**, G<sub>αi</sub> inhibition with PTX blocked the ability of apelin to prevent ANF expression, n=5. **x–y**, qRT-PCR for ANF in cells treated with (x) or without (y) inhibitors, n=3. **z**, Diminished expression of hypertrophy markers in Ad-APJ-GFP cardiomyocytes transfected upon knockdown of βarrestin1 (siβARR1), βarrestin2 (siβARR2) or both (siβARR1+2), n=4. Except for (p) and (y) all experiments were performed in the presence of inhibitors of AT-1 (candesartan, 100

nM) and ET-A (BQ123, 300 nM) added one hour prior to stretch and/or apelin treatment until fixation. All are representative experiments performed at least three independent times. Error bars are SEM. \* $p < 0.05$  between indicated groups, ANOVA.

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