Interference with ERK-dimerization at the nucleocytosolic interface targets pathological ERK1/2 signaling without cardiotoxic side-effects

Dysregulation of extracellular signal-regulated kinases (ERK1/2) is linked to several diseases including heart failure, genetic syndromes and cancer. Inhibition of ERK1/2, however, can cause severe cardiac side-effects, precluding its wide therapeutic application. ERK$^{T188}$-autophosphorylation was identified to cause pathological cardiac hypertrophy. Here we report that interference with ERK-dimerization, a prerequisite for ERK$^{T188}$-phosphorylation, minimizes cardiac hypertrophy without inducing cardiac adverse effects: an ERK-dimerization inhibitory peptide (EDI) prevents ERK$^{T188}$-phosphorylation, nuclear ERK1/2-signaling and cardiomyocyte hypertrophy, protecting from pressure-overload-induced heart failure in mice whilst preserving ERK1/2-activity and cytosolic survival signaling. We also examine this alternative ERK1/2-targeting strategy in cancer: indeed, ERK$^{T188}$-phosphorylation is strongly upregulated in cancer and EDI efficiently suppresses cancer cell proliferation without causing cardiotoxicity. This powerful cardio-safe strategy of interfering with ERK-dimerization thus combats pathological ERK1/2-signaling in heart and cancer, and may potentially expand therapeutic options for ERK1/2-related diseases, such as heart failure and genetic syndromes.
As the Raf1/MEK/ERK1/2 signaling cascade is causatively involved in frequent diseases such as heart failure and cancer, but also in rare developmental syndromes (so called RASopathies), it is an important therapeutic target. Several pharmacological antibodies blocking the extracellular domain of the epidermal growth factor receptor tyrosine kinase or small molecules targeting kinases of this signaling cascade with differential selectivity are available. However, the wide therapeutic application of these compounds is limited by side effects, inducing the development of severe cardiomyopathy with decreased ejection fraction and the development of resistances to these compounds.

In the heart, the Raf/MEK/ERK1/2 cascade is involved in adverse remodeling, but is also responsible for cardiomyocyte survival and protection from stress-induced cardiomyocyte death. Inhibition of Raf/MEK/ERK1/2 signaling by above-mentioned inhibitors renders the heart more vulnerable to injury, but conversely, activation of the cascade can also be a trigger for adverse cardiac remodeling and subsequent cardiac dysfunction. Thus, suppression of Raf/MEK/ERK1/2-mediated pro-hypertrophic and adverse remodeling processes and preservation of its pro-survival events are clearly warranted, but still remain an unmet clinical need.

Previously, we showed that autophosphorylation of ERK1/2 at threonine 188 in mouse ERK2 (resp. T208 in mouse ERK1; subsequently referred to as ERKT188-phosphorylation) is central for nuclear ERK1/2 target activation and for inducing ERK1/2-mediated pathological cardiac hypertrophy in response to several pathological stimuli. These include several Gq- and Gs-coupled G protein-coupled receptors (GPCR) that activate ERK1/2 by the phosphorylation of the so-called highly conserved TET motif (resp. T183/Y185 in mouse ERK2 or often referred to as T202/Y204 in human ERK1) triggering homo- or hetero-dimerization of ERK1/2 and subsequently facilitating intermolecular ERK autophosphorylation at threonine 188. Experimental data using a phosphorylation-deficient mutant (ERK2ΔT188A), that is dominant-negative for ERK1 and ERK2 autophosphorylation at threonine 188 suggested that interference with ERKT188-phosphorylation may offer a possibility to selectively target pathological cardiac hypertrophy without affecting anti-apoptotic signaling of ERK1/2.

Interestingly, ERK2ΔT188A only inhibited nuclear ERK1/2 target phosphorylation whilst cytosolic signaling remained unaffected.

Even though many mechanistic details of the Raf/MEK/ERK1/2 signaling cascade have been discovered, no therapeutic tools are currently available to differentially target this cascade in the nucleus and cytosol. In order to design a cardio-safe ERK1/2 targeting strategy, we show that (i) the ERK-ERK interaction is a prerequisite for ERKT188 phosphosylation which in turn is a central molecular event for nuclear ERK localization and signaling, (ii) ERKT188 phosphosylation is also upregulated in human colon and lung cancer, and (iii) interference with nuclear ERK signaling is a cardio-safe targeting strategy to prevent pathological ERK1/2 signaling in the heart and in tumor cells. This strategy holds therapeutic potential for heart failure and possibly cancer therapy and other ERK1/2-related diseases.

Expression of ERK2Δ4 attenuates cardiac remodeling. To assess the function and potential side-effects of monomeric ERK2 in the heart, we generated mice with cardiac-specific ERK2Δ4 expression (ERK2Δ4-tg; Supplementary Fig. 2A) and subjected ERK2Δ4-tg and non-transgenic control mice (Wt) to transverse aortic constriction (TAC), a stimulus for chronic pressure overload resulting in cardiac remodeling.

Echocardiographic fractional shortening and ventricular diameters of ERK2Δ4-tg were indistinguishable from Wt mice, and velocities of contraction and relaxation, even in response to dobutamine stress, were similar (Fig. 1c and Supplementary Table 1). The hypertrophic response to TAC of ERK2Δ4-tg in comparison to Wt mice, however, was significantly attenuated, as monitored by heart weight-to-tibia length and heart weight-to-body weight ratios, echocardiographic left ventricular wall thickness and cardiomyocyte size assessed by histology (Supplementary Table 1 and Fig. 1d). Intertitial fibrosis and mRNA expression levels of brain natriuretic peptide (Nppb) and collagen (Col3a1) that are common markers for pathological remodeling were also reduced in ERK2Δ4-tg mice after TAC compared to Wt animals (Fig. 1e, Supplementary Fig. 2B, C and Supplementary Table 1). Cardiomyocyte death, however, was comparable in ERK2Δ4-tg and Wt mice as shown by TUNEL and caspase-3 activity analyses (Fig. 1f and Supplementary Fig. 2D).

Of note, physiological growth of the heart that is needed to adapt the heart to altered work load as during exercise or pregnancy and that does not impair cardiac function and integrity, was not affected by ERK2Δ4. Physiological growth of mouse hearts at least until the age of 9 months, or in response to exercise, i.e. a period of voluntary running in a wheel for three weeks, remained normal in ERK2Δ4 overexpressing mice. In addition, ERK2Δ4 expression did not affect cardiac function, degree of interstitial fibrosis or Nppb and Col3a1 transcription levels (Supplementary Tables 2, 3, Supplementary Fig. 2E–H). Also, survival of ERK2Δ4-tg was comparable to non-transgenic Wt mice (Supplementary Fig. 2I). Of note, all experimental parameters assessed in Wt and ERK2-wt overexpressing mice have previously been shown not to differ, i.e. under basal conditions.
conditions, after TAC or with regard to physiological hypertrophy, therefore, we used Wt littermates as controls within this study.10,13

Hence, monomeric ERK2 expression has no adverse effects on cell survival, cardiac function, or remodeling and does not interfere with normal postnatal or exercise-induced cardiac growth even though it attenuates cardiomyocyte hypertrophy in response to pathophysiologically hypertrophic stimuli such as phenylephrine or chronic pressure overload.

**pERK**T188 has strong impact on hypertrophic gene expression.

The introduction of an aspartic acid (d) in ERK2 mimics ERKT188-autophosphorylation13. Interestingly, NRCM transduced with ERK2-D4 that additionally stimulates a constitutive ERKT188-phosphorylation (ERK2-D4D) showed - in contrast to ERK2-D4 - comparable hypertrophic responses to PE as NRCM transduced with ERK2-wt (Fig. 2a and Supplementary Fig. 3). Nuclear localization studies of ERK2-wt, ERK2-D4, and ERK2-D4D, N-terminally tagged with yellow fluorescent protein (YFP; YFP-ERK2-wt, YFP-ERK2-D4, and YFP-ERK2-D4D) in COS7 cells and in NRCM further revealed that ERKT188-phosphorylation is essential for the nuclear ERK2 accumulation, but not sufficient (also refer to Supplementary Fig. 6H): PE stimulation led to a significant nuclear localization of YFP-ERK2-wt and ERK2-D4D but not of monomeric ERK2-D4 (Fig. 2b and Supplementary Fig. 4A–C). These experiments identify ERK(T188)-phosphorylation as the decisive trigger for nuclear ERK localization and cardiomyocyte hypertrophy and endorse ERKT188-autophosphorylation as a potential target to specifically interfere with nuclear ERK signaling. To further evaluate the different outcomes of detrimental ERK-activating stimuli involving ERK(T188)-phosphorylation and more physiological ERK-activating stimuli, we used ERK2T188D + PE as a pathological stimulus involving ERK(T188)-signaling and MEK155218/222-DD (MEK-DD) and IGF as adaptive/physiological ERK1/2 stimuli23,24. MEK-DD is a constitutively active MEK1 mutant that can activate ERK1/2 independently from extracellular signals such as GPCR/G-protein activation. The insulin-like growth factor IGF has been associated with a physiological type of cardiac hypertrophy25. Expression levels or concentrations of ERK2T188D + PE, MEK-DD and IGF were adjusted for comparable effects on cardiomyocyte hypertrophy (Fig. 2c). Under these conditions, we evaluated YFP-ERK2 localization, ERK(T188) and ERK(TEY) phosphorylation, and expression of genes known to be involved in pro-hypertrophic signaling. While comparable pERK(TEY) levels were detected in response to all three hypertrophic stimuli, only ERK2T188D + PE resulted in increased ERK(T188)-phosphorylation levels (Fig. 2d). In line with the induction of pERK(T188), YFP-ERK2-wt showed significant nuclear localization in cells treated with ERK2T188D + PE but not in the presence of
Fig. 2 Nuclear ERK2 accumulation requires ERK\textsuperscript{T188D} phosphorylation and impacts on hypertrophic gene regulation. a Images and quantification of cell size of phalloidin-stained NRCM transduced with Flag-tagged ERK2-wt or Flag-ERK2\textsuperscript{Δ174–177,T188D} (ERK2-Δ4D) in response to PE (4 μM, 24 h) (n = 5 independent experiments and 25 cells per group and experiment; scale bar 20 μm). b Nuclear-to-cytosolic ratios of YFP-tagged ERK2-wt (ERK2-wt), YFP-ERK2\textsuperscript{Δ174–177} (ERK2-Δ4), and YFP-ERK2\textsuperscript{Δ174–177,T188D} (ERK2-Δ4D) in NRCM in response to PE (4 μM, 10 min). Shown are confocal images displaying distribution of YFP-tagged ERK2 constructs (yellow) and DAPI-stained cell nuclei (blue) (scale bar 10 μm). Quantification: Supplementary Fig. 4A. c, d NRCM transduced with Flag-tagged ERK2\textsuperscript{T188D} (ERK2\textsuperscript{T188D}) or HA-tagged MEK1\textsuperscript{Δ218/222DD} (MEK-DD) treated with PE (4 μM, 24 h) or IGF (30pM, 24 h). c Representative phalloidin-stained NRCM (scale bar 25 μm) and cell size analysis (n = 3 independent experiments and 70–100 cells per group and experiment). d Representative western blots of pERK(TEY) and pERK(T188) were reproduced three times with similar results. e Nuclear-to-cytosolic ratios of NRCM transduced with YFP-tagged ERK2-wt and MEK-DD if indicated and stimulated with PE (4 μM, 10 min). Shown are confocal images of YFP-ERK2, DAPI-stained cell nuclei (scale bar 10 μm), and quantitative analyses (n = 30–70 cells per group of four independent experiments). f NRCM transduced with Flag-tagged ERK2\textsuperscript{T188D} or the HA-tagged MEK\textsuperscript{Δ218/222DD} mutant treated with PE (4 μM, 24 h) or IGF (30pM, 24 h). mRNA expression of connective tissue growth factor (Ctgf), transforming growth factorβ1 (Tgfb1), transforming growth factorβ2 (Tgfb2), transforming growth factorβ3 (Tgfb3), Toll-like receptor2 (Tlr2), ETS domain-containing protein Elk-1 (Elk1), insulin-like growth factor (Igf1), sarcoplasmic reticulum calcium ATPase 2A (Serca2a), peroxisome proliferator-activated receptor gamma coactivator 1-α (Ppargc1a), nuclear factor erythroid 2-related factor 2 (Nrf2), heart- and neural crest derivatives-expressed protein 2 (Hand2), ATP synthase, H\textsuperscript{+}-transporting, mitochondrial F1F0 complex, subunit E (Atp5k), myocyte-specific enhancer factor 2A (Mef2a), E3 ubiquitin-protein ligase TRIM63 (Murf), and cytochrome C (CytoC) normalized to glycerinaldehyde-3-phosphate dehydrogenase (Gapdh). n represents number of samples of biologically independent experiments measured in triplicates and are indicated in bar graph. Error bars are mean ±s.e.m.; ordinary one-way ANOVA with Tukey as post hoc test was used except for a where Bonferroni was used as post hoc test. Source data are provided as a Source Data file.
using cells overexpressing Flag- and HA-tagged ERK2-wt and HA-tagged ERK1-wt, respectively, revealed that ERK1/2 stimulation using carbachol or PE induces a stable interaction between Flag- and HA-tagged ERK hetero- or homodimers, a phenomenon that was prevented in the presence of EDI suggesting that EDI may affect both, ERK1 and ERK2, signaling (Fig. 3b and Supplementary Fig. 5A–C). EDI was N-terminally tagged with myc for visualization of successful transfection using anti-myc antibodies. Similarly, a fragment of EDI (JOLU22) presenting peptide penetratin and fluoresceinylated, also abolished the PE-induced interaction of HA- and Flag-tagged ERK2 in a proximity ligation assay (Supplementary Fig. 5C). A cross-linking experiment in cells overexpressing Flag-ERK2 further revealed that EDI specifically binds to activated but not inactive, unstimulated ERK2, as suggested by the molecular weight/mobility shift of ERK2 after stimulation with carbachol (Supplementary Fig. 5D). And, as hypothesized from the experiments using monomeric ERK2-A4, EDI efficiently prevented ERK(T188)-phosphorylation without inhibitory impact on ERK activation (pERK[TEY]) (Supplementary Fig. 5E, F).

To evaluate whether EDI’s effects on ERK-phosphorylation translate into inhibition of cardiomyocyte hypertrophy, we virally transduced NRCM with EDI (Supplementary Fig. 6A). This led to
a significant prevention of ERK\textsuperscript{T188}-phosphorylation (Fig. 3c and Supplementary Fig. 6B) and cardiomyocyte hypertrophy in response to PE (Fig. 3d). ERK activation and cell survival, however, were comparable to control conditions as observed with monomeric ERK2 (Fig. 3c, e, Supplementary Fig. 6B). Further, PE-induced cytosolic substrate phosphorylation was unaffected by EDI as shown by B-cell lymphoma 2-interacting mediator of cell death (BIM) phosphorylation\textsuperscript{29}, contrary to nuclear substrate phosphorylation as shown by significantly reduced ELK1 phosphorylation (Fig. 3c and Supplementary Fig. 6B). These selective effects of EDI on nuclear ERK effects were further validated by EDI’s inhibitory effect on nuclear ERK2 accumulation (Fig. 3f and Supplementary Figs. 4A and 6C).

To test the specificity of EDI for ERK, i.e. the ERK-ERK interface\textsuperscript{26}, we performed several additional experiments: first, to ensure that EDI behaves as a structural counterpart of ERK2, we analyzed whether the isolated peptide has the potential to form a tertiary structure, i.e. an α-helix containing structure, as postulated from the crystal structure of ERK2. For these analyses, infrared spectroscopy of purified His\textsubscript{6}-EDI was performed, which revealed an amide I band maximum at ~1656 cm\textsuperscript{-1} that is characteristic for an α-helical protein\textsuperscript{29} (Fig. 3g). Secondly, we analyzed whether the peptide is specific for the leucine zipper-like structure containing the ERK-ERK interface or if it also interferes with other leucine zipper-like structures. This was achieved by the analysis of the interaction of the transcription factors Myc and Max\textsuperscript{30} in a proximity ligation assay. While EDI interfered with the ERK-ERK interaction, it had no impact on the PE-induced Myc-Max interaction (Supplementary Fig. 6D). Thirdly, we analyzed the impact of the EDI on cardiomyocyte hypertrophy in the absence of ERK1 and ERK2. siRNA-mediated knock-down of ERK1/2 (Supplementary Fig. 6E) abolished the anti-hypertrophic effect of EDI in PE-treated NRCM (Fig. 3h), which was not the case in the presence of endogenous ERK1/2 (Fig. 3d and Supplementary Fig. 6F). Of note, compensatory/non-ERK1/2-mediated hypertrophic signaling pathway may be responsible for cardiomyocyte hypertrophy in this experimental setting similarly as discussed for ERK1/2 knockout mouse models\textsuperscript{1,32}. Fourthly, EDI did not prevent ERK-mediated PE-induced cardiomyocyte hypertrophy or nuclear ERK localization in the presence of an ERK\textsuperscript{T188}-phosphorylation simulating ERK2 mutant (ERK2\textsuperscript{T188D}), suggesting that EDI affects ERK1/2 upstream of ERK\textsuperscript{T188}-phosphorylation (Supplementary Fig. 6G, H).

These experiments support the specificity of EDI for the ERK-ERK interface, and reveal its function as a valuable tool to interfere with endogenous ERK\textsuperscript{T188}-phosphorylation, ERK1/2 signaling, and ultimately ERK1/2 function.

AAV9-EDI gene therapy rescues TAC-induced heart failure. To test EDI in a heart failure model, we subjected C57BL/6 mice to TAC surgery to induce chronic pressure overload and applied an AAV9 vector encoding EDI or enhanced green fluorescent protein (AAV9-eGFP) under the control of the CMV-enhanced myosin light chain promotor for efficient cardiac expression (Supplementary Fig. 7A)\textsuperscript{21,33}. AAV9-EDI treatment of WT mice resulted in a significant protection from pathological cardiac remodeling after TAC. Left ventricular wall thickness, heart weight-to-tibia length and heart weight-to-body weight ratios, and histological cardiomyocyte cross-sectional area were significantly reduced. In addition, parameters associated with cardiac function and fibrosis were improved compared to AAV9-eGFP-treated control mice, i.e. fractional shortening, left ventricular dilation, interstitial fibrosis, marker gene expression, and pulmonary congestion, and even survival of AAV9-EDI-treated mice was slightly (not significantly) improved compared to control mice. Furthermore, apoptosis was significantly reduced in EDI-treated mice (Fig. 4a–f, Supplementary Fig. 7A and Supplementary Table 4). Of note, baseline parameters after 4 weeks of AAV9-eGFP or AAV9-EDI application were comparable with age- and gender-matched untreated control mice (Supplementary Table 5). The prominent impact of EDI on chronic pressure overload-induced changes in the heart was also reflected by gene array analysis: the top 100 regulated genes related primarily to extracellular matrix organization and structure, based on GO functional enrichment analysis (Fig. 4g and Supplementary Table 6). In addition, TAC-induced changes in gene expression categorized into functional profiles, i.e. cardiac hypertrophy, extracellular matrix, cell survival, and heart failure, were largely prevented by treatment with EDI (Supplementary Fig. 7B and Supplementary Tables 7–10).

Further, EDI-treated mice revealed a significant inhibition of ERK\textsuperscript{T188}-phosphorylation and phosphorylation of nuclear but not cytosolic ERK targets (Fig. 4h and Supplementary Fig. 7C). Interestingly, the heatmap visualization of \textit{Nfat} and \textit{Myc}-related genes shows that the presence of EDI prevented the activation of the related gene networks in response to TAC (Supplementary Fig. 7D and Supplementary Tables 11, 12). Both, \textit{Nfat} and \textit{Myc} signaling are strong triggers for cardiac remodeling processes and are enhanced by nuclear ERK\textsuperscript{T188},34,35. These findings highlight EDI’s impact on cardiac function and remodeling as well as its gatekeeper role at the nucleocytoplasmic interface.

**EDI reduces tumor proliferation and cardiomyocyte toxicity.**

The Raf/MEK/ERK1/2 pathway is one of the most frequently dysregulated signaling pathways in cancer, in particular in melanoma, pancreatic, oral squamous cell, and colorectal cancers\textsuperscript{36–38}. Cardiotoxicity as well as drug resistance are severe limitations of prolonged treatment with FDA approved drugs that target Raf/MEK/ERK1/2 signaling in cancer\textsuperscript{3,39,40}. To test, whether the apparently cardio-safe EDI might also be effective in cancer and may thus represent a possible treatment strategy circumventing cardiotoxic side effects, we studied the role of ERK\textsuperscript{T188}-phosphorylation in cancer.

We found ERK\textsuperscript{T188}-phosphorylation to be strongly upregulated in colorectal and lung cancer as compared to healthy colon or lung tissue (Fig. 5a, b). EDI displayed an inhibitory effect on colon cancer cell proliferation (LS174T and HT29 cells) at least as strong as the effect of the MEK inhibitor PD98059 at concentrations previously shown to effectively reduce cancer cell proliferation\textsuperscript{7} (Fig. 5c and Supplementary Fig. 8). Both EDI and PD98059 reduced ERK\textsuperscript{T188}-phosphorylation to a similar extent, whereas phosphorylation of the TEF motif of ERK1/2 was only inhibited by PD98059 corresponding to their effects in cardiomyocytes (Fig. 5d). These results suggest that specific interference with ERK\textsuperscript{T188}-phosphorylation may be as efficient as global kinase inhibition to attenuate cancer cell proliferation. Interestingly, Akt activation, one well-known compensatory mechanism in response to ERK1/2 inhibition in HT29 cells, was only induced by PD98059 but not by EDI, which may suggest that specific vs. global ERK1/2 inhibition may help to circumvent compensatory mechanisms to some extent (Fig. 5e)\textsuperscript{41,42}. A gene array further revealed that the peptide strongly repressed gene expression associated with cell cycle and cell proliferation (Fig. 5f and Supplementary Tables 13–15). Expression levels of several genes were further analyzed in the colon cancer cell lines LS174T and HT29 by quantitative real-time PCR analysis: EDI was at least as efficient as PD98059 with regard to the suppression of the expression of the selected genes (Fig. 5g).
These findings demonstrate that ERK\textsuperscript{T188} phosphorylation is strongly upregulated in colorectal and lung cancer and functions as a growth promoting trigger within the Raf/MEK/ERK1/2-signaling cascade in colon cancer cells. In line with these findings, our data suggest that selective inhibition of ERK\textsuperscript{T188} phosphorylation may be advantageous in cancer therapy: this strategy efficiently attenuates cancer cell proliferation, may even circumvent compensatory mechanisms to some extent, but most importantly is cardio-safe in vitro and in mice, in contrast to PD98059 or other inhibitors of the Raf/MEK/ERK1/2 signaling cascade such as cetuximab and the clinically used MEK inhibitors trametinib, selumetinib, cobimetinib, and binimetinib. Like PD98059, all newer generation MEK inhibitors prevented ERK\textsuperscript{T188} phosphorylation, nuclear ERK target phosphorylations and cardiomyocyte hypertrophy but also inhibited – in contrast to EDI – the phosphorylation of ERK(TEY) and of cytosolic ERK target proteins of which the Bcl-2-associated agonist of cell death (Bad) is of particular importance for cell survival\textsuperscript{43} (Fig. 6a, b and Supplementary Fig. 9A–C). The non-cardiotoxicity of EDI compared to the MEK inhibitors was validated by TUNEL assays and evaluation of mitochondrial membrane potential in response to oxidative stress; while the membrane potential was depolarized in the presence of all MEK inhibitors, EDI protected the latter from collapsing (Fig. 6c, d vs. Fig. 3e and Supplementary Fig. 9D–F). These experiments further substantiate the essential cytosolic role of ERK1/2 signaling and the subsequent need for more specific or differential ERK1/2 targeting strategies.

**Discussion**

This study reveals that interfering with ERK dimerization is highly effective in targeting pathological ERK1/2 signaling in the heart, without causing cardiotoxic side-effects. This principle may also apply more generally to unfavorable activation states characterized by increased ERK\textsuperscript{T188} autophosphorylation, as suggested here by similar effects in cancer cells (Fig. 5). Targeting the ERK-ERK interface offers an elegant way to specifically interfere with nuclear ERK1/2 signaling. Using this peptide-based strategy, we showed (i) that ERK-ERK interaction is a prerequisite for ERK\textsuperscript{T188} autophosphorylation of endogenous ERK1/2; (ii) that ERK\textsuperscript{T188} phosphorylation is a central molecular event for nuclear ERK localization and signaling (Fig. 2) since simulation of ERK\textsuperscript{T188} phosphorylation in monomeric ERK2 (ERK2-Δ4D) enabled monomeric ERK2 to accumulate in the nucleus and to

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**Fig. 4 AAV9-EDI gene therapy protects from pressure overload-induced heart failure.** a–h Cardiac characterization of wild-type mice before (Con; 8-week-old C57BL/6 mice) and 4 weeks after transverse aortic constriction (TAC) and treatment with an adeno-associated virus serotype 9 (AAV9) vector encoding the peptide (EDI) or eGFP as a control (GFP) under the control of the CMV-enhanced myosin light chain promotor fragment (CMV-MLC0.26 kb). a Histological analyses: representative H&E (left panel; scale bar 50 μm) and Sirius Red (right panel; scale bar 200 μm)-stained pictures of left ventricular myocardial sections, quantifications are shown in Supplementary Table 4. b Representative M-mode echocardiograms. c Echocardiographic fractional shortening (Con, n = 18; GFP + TAC, n = 11; EDI+ TAC, n = 14 mice per group). d Lung weight-to-tibia length (LW/TL) ratios (Con, n = 8; GFP + TAC, n = 11; EDI + TAC, n = 11 mice per group). e Caspase-3 activity in heart lysates (Con, n = 7; GFP + TAC, n = 9; EDI + TAC, n = 10). f Kaplan–Meier survival curves of AAV9-eGFP (n = 24) and AAV9-EDI (n = 19) treated mice after TAC. g Microarray gene expression analysis of left ventricular myocardial tissue (Con, n = 3; GFP + TAC and EDI + TAC, n = 4 mice per group). Heatmap visualization of the top 100 genes with the highest variance across the samples. For gene list and changes refer to Supplementary Table 6. h Representative immunoblots of heart lysates. Analysis of ERK phosphorylations (pERK(TEY), n = 6; pERK(T188), n = 4) and ERK1/2 target phosphorylation (nuclear pELK[S383], n = 6; cytosolic pp90RSK[S380], n = 8); n numbers represent biologically independent experiments. For quantification refer to Supplementary Fig. 7C. Error bars are mean ± s.e.m. For statistical analyses ordinary one-way ANOVA and Bonferroni as post hoc test was applied except for where a survival curve comparison was applied. Source data are provided as a Source Data file.
Fig. 5 EDI reduces tumor cell proliferation. a, b Analysis of human colon adenocarcinoma and lung cancer. a Immunohistochemistry (IHC) of pERK(T188) (left: colon, scale bar 50 μm, n = 9; right: lung, 200 μm, n = 5). b Representative western blots and quantifications of pERK(TEY) and pERK(T188)) (colon, n = 9 patients; lung, n = 4 patients; normal (N) and tumor (T) tissue). c–g LS174T and HT29 colon cancer cells transduced with LacZ (Con) or EDI and pretreated with PD98059 (30 μM, 48 h) (c, d). c [3H]Thymidine incorporation (Con, PD98059, 50 kDa (x-fold of N)). d Representative western blots and western blot analyses of pAKT1(S473) in HT29 cells treated as described above (n = 28). Microarray gene expression analysis of LS174T transduced with Con or EDI (n = 3 per condition). Heatmaps of top 100 genes with highest variance across samples and of transcriptional changes of genes related to proliferation and cell cycle. g mRNA expression of cyclin B1 (Ccnb1), cyclin A2 (Ccn2), TTK Protein Kinase (Ttk), deoxythymidylate kinase (Dyrmk), cyclin-dependent kinase 1 (Cdk1), kinetochore protein NDC80 homolog (Ndc80), nuclear transcription factor Y subunit alpha (Nfyα), and E2F transcription factor 8 (E2F8) normalized to glycerinaldehyde-3-phosphate dehydrogenase (Gapdh). Error bars are mean ± s.e.m.; n numbers represent biologically independent experiments; n numbers of g are displayed in the graph; ordinary one-way ANOVA (c–e, g) and Bonferroni as post hoc test was applied except for b where an unpaired and two-sided Student’s t-test was applied. Source data are provided as a Source Data file.

normally respond to hypertrophic stimuli; (iii) that ERK<sup>T188</sup>-phosphorylation is not restricted to cardiomyocytes but is also strongly upregulated in human colon and lung cancer (Fig. 5a, b) and may thus represent a potential target and marker in cancer therapy; and (iv) that interference with nuclear ERK signaling is efficient and sufficient to interfere with maladaptive ERK1/2 signaling in the heart and in proliferating tumor cells and – most importantly – that it promises to be a cardio-safe approach to correct maladaptive, dysregulated ERK1/2 signaling (Fig. 7).

Several in part contradictory studies have shown that ERK1/2 can trigger maladaptive cardiac hypertrophy, but also physiological hypertrophy<sup>23,44</sup> or confer no impact on cardiac hypertrophy<sup>11–13,44–50</sup>. While it is generally accepted that ERK1/2 activation is essential for cardiomyocyte survival<sup>48</sup>, the outcome of these mouse studies on ERK1/2-mediated cardiac hypertrophy may depend on the model, circumstances, and upstream signals. The identification of ERK<sup>T188</sup>-autophosphorylation and molecular prerequisites for ERK<sup>T188</sup>-phosphorylation provide additional insights into ERK1/2 signaling and in the distinct outcomes of the mouse studies. Interestingly, only certain instances or signals upstream of ERK1/2 lead to ERK<sup>T188</sup>-phosphorylation, and those have been shown to be rather maladaptive than physiological as physiological hypertrophy does not trigger ERK<sup>T188</sup>-phosphorylation and its inhibition has no impact on physiological hypertrophy (ref. 10; Supplementary Fig. 2 and Supplementary Tables 2 and 3). However, ERK<sup>T188</sup>-phosphorylation has been identified to affect ERK localization and functional outcome. Mice with cardiac overexpression of ERK2<sup>T188D</sup> showed that simulation of ERK<sup>T188</sup>-phosphorylation aggravates the pathological outcome of hypertrophic disease triggers and that over-expression of a phosphorylation-deficient mutant reduces the hypertrophic response<sup>12</sup>. In this study here, we made use of the peptide EDI in vitro and in vivo (AAV9-EDI gene therapy) that prevented ERK<sup>T188</sup>-phosphorylation of endogenous ERK1/2. This “tool-peptide” modulated endogenous ERK1/2 signaling without artificial overexpression of a catalytically active protein kinase mutant and therefore provided a valuable tool to gain insights into endogenous ERK1/2 signaling. In the current study,
we convincingly show that interference with ERK$^{T188}$-phosphorylation indeed prevents cardiac hypertrophy and maladaptive remodeling, interferes with major hypertrophic signaling pathways$^{19,22,44,45}$ such as Myc- and NFAT-associated gene regulation in mice after TAC, and that ERK$^{T188}$-phosphorylation is a central molecular event for nuclear ERK localization and signaling. As Crepaldi and co-workers state in their current review on ERK as a key player in the pathophysiology of cardiac hypertrophy$^{24}$, cardiac hypertrophy is a complex response to various physiological and pathological stimuli, and ERK activation seems to be involved in both adaptive and maladaptive hypertrophy, depending on the pathophysiological context. Our current and previous experiments$^{10,13,14}$ clearly suggest that pERK$^{T188}$ is a key driver of ERK1/2-mediated pathological cardiac hypertrophy. The comparison of ERK1/2 stimuli involving (ERK2$^{T188D} +$ PE) or circumventing ERK$^{T188}$, phosphorylation (e.g. MEK-DD) revealed divergent patterns of gene regulation. In particular, ERK$^{T188D} +$ PE modulated gene expression, i.e. suppressed genes involved in adaptive cardiac hypertrophy and induced genes involved in pathological processes (Fig. 2f). Thus, ERK$^{T188}$-phosphorylation and subsequent nuclear ERK localization/accumulation are key determinants of gene expression and ERK-mediated pathological cardiomyocyte hypertrophy.

Selective interference with ERK$^{T188}$-phosphorylation or ERK dimerization, in contrast to global ERK1/2 kinase inhibition and presumably also ERK kinase inhibitors$^{31}$, offers the possibility to preserve beneficial cytosolic, and to specifically interfere with maladaptive nuclear ERK1/2 signaling in cardiomyocytes. The strong effect of EDI on gene regulation and thus cardiac remodeling as well as cancer cell proliferation may be due to direct or indirect activation of nuclear transcription factors by ERK1/2, but also kinase-independent ERK1/2 effects such as a direct binding of ERK to oligonucleotides, as shown for ERK2, activated ERK2, and ERK2$^{T188D}$ (ref. 52). In line with the protection of cardiomyocyte cell death, EDI further preserved the functional integrity of cardiomyocyte mitochondria, i.e. depolarization of the mitochondrial membrane potential, in response to oxidative stress which is in clear contrast to MEK inhibition by PD98059 or new...
generation MEK inhibitors (Fig. 6d and Supplementary Fig. 9F)33. These preserved cytosolic functions of ERK1/2 in cells transduced with EDI (vs. MEK inhibition) may be particularly relevant in patients with a second source of cardiac injury (e.g. ischemia, other toxic drugs, or hypertension). Of note, ERK1/2 knockout mice were particularly susceptible to cardiac dysfunction in response to chronic pressure overload2,11.

A small molecule compound, DEL22379, has been described previously as a compound that binds within the ERK dimer interface similar to the peptides EDI and JOLU22 used in our study. The outcome of this compound on cancer cells, however, was different: DEL22379 efficiently induced cancer cell apoptosis, whereas our peptide displayed rather strong anti-proliferative effects as substantiated by the gene array analyses—a difference in the mode of action that needs further evaluation (Fig. 5f, g)34,45. Thus, slight differences in the binding mode within the ERK dimer interface result in diverse functional outcomes. These different targeting modes within the dimer interface (DEL vs. EDI) may also translate into different outcomes in neonatal cardiomyocytes with regards to cardiotoxic side-effects. In addition, long-term side-effects of EDI and the MEK inhibitors will require careful attention, as for example potential physiological functions of ERKT188 phosphorylation are thus far unknown. Altogether, our data suggest that the ERK dimer interface is a tractable therapeutic target that may represent a “hot spot” for efficient ERK targeting. Subsequent work with in-depth analyses to better understand the potential of this hot-spot is required to further refine the herein presented anti-nuclear ERK strategy.

New targeting strategies of ERK1/2 signaling are of major therapeutic interest, since permanent activation and inactivation of this cascade both appear detrimental. Novel ERK1/2 targeting strategies should increase the therapeutic efficacy by circumventing drug-resistance and reduce cardiac side-effects that currently limit their application2,4,56–58. In this regard, interference with ERK dimerization holds promise, since AKT activation as one compensatory mechanism that contributes to drug-resistance was prevented in HT29 cells, and most importantly no side-effects were detectable in the AAV9-EDI model nor for the overexpression of monomeric ERK2 (Figs. 4 and 5e, Supplementary Fig. 2 and Supplementary Table 5). Thus, our targeting approach may also facilitate the application of the Raf/MEK/ERK1/2 cascade inhibitors for long-term therapy, which would be needed for the therapy of heart failure and rare genetic syndromes like RASopathies, but also with respect to chronic side effects of cancer treatment13,37,47,59–63. This targeting strategy exemplified by EDI thus may fulfill all key aspects of a potentially perfect Raf/MEK/ERK1/2 inhibitor. It will be interesting to assess whether this targeting strategy also overrides the MEK inhibitor resistance due to the recently described ERK2 mutations in human cancer, ERK2(E322K) and ERK2(D321N) located within the highly conserved common docking (CD) region close to the proposed binding site of EDI/JOLU2254,65.

Moreover, ERKT188-phosphorylation has important characteristics of a biomarker for certain diseases: in our study, we show for the first time that ERKT188-phosphorylation is not restricted to pathological remodeling in the heart, but is also upregulated in cancer tissue, and thus may be central for maladaptive processes (Fig. 5)10,45. ERKT188-phosphorylation was increased in 7/9 colon tumor and in 4/4 lung tumor samples, whereas pERK1/2[TEY] was rather decreased in colon cancer and unchanged in lung tumors. This is in line with the well-known variable time course of pERK1/2[TEY] due to concomitant activation of different phosphatases (Fig. 5a, b)66,67. Preliminary experiments suggest that ERKT188-phosphorylation is protected from dephosphorylation within the nucleus (data not shown). ERKT188-phosphorylation may thus become a valuable marker for the identification of Raf/MEK/ERK1/2 participation in pathological conditions/tumors.

The identification of ERKT188-phosphorylation as a maladaptive trigger of cardiac hypertrophy and heart failure initiated the search for a differential targeting strategy for these central kinases ERK1/2 that are involved in many physiological and pathological processes. Harnessing the molecular prerequisites for ERKT188-phosphorylation, we here discovered a peptide/EDI-based targeting strategy of the ERK-ERK interface to interfere with ERKT188-phosphorylation. This strategy promises to be a powerful cardiac-safe treatment option to combat pathological ERK1/2-signaling in heart and cancer, and possibly other ERK1/2-related diseases requiring long-term treatments, such as heart failure and genetic syndromes.

Methods

Mice and rats. Transgenic mice overexpressing ERK2Δ174–177 (ERK2-Δ4-tg) under the control of the mouse α-myosin heavy chain (Myh6) promoter were generated by pronuclear injection of fertilized oocytes derived from FVB/N mice15,16. For the gene therapy approach, mice with C57BL/6J background were used. In all experiments, isogenic, age- and gender-littermates were used as controls. Pregnant Sprague Dawley rats (embryonic day 11) were purchased from Janvier. Animal care was performed corresponding to the Committee on Animal Research of the regional government (Regierung von Unterfranken and Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen LANUV-NRW) which reviewed and approved all experimental protocols (Az. 54–2531.01–62/06, Az. 55.2–2531.01–46/09, –20/10, –52/10, –38/11, –60/13 and –42/14 and Az. 81–02.04.2018.A082) according to the national legislation. All animals were maintained in accordance with federal guidelines: Standard chow diet and water were offered, 10% ad libitum; for housed sterilized plastic cages under specific pathogen-free conditions were used; as housing conditions 22 ± 2 °C, 12/12 light/dark cycle, 55 ± 10% humidity and <400 lux were maintained. Hygiene monitoring was done on quarterly basis.

Human colon and lung tissue. The use of the colon cancer samples was approved by the ethic committee of the University and University Hospital of Würzburg (20180829 01) and all patients gave written consent. Additional colon cancer and lung cancer samples were obtained from RWTH Aachen centralized Biomaterial Institute (bMB, RWTH CBMB). The bCBMB was reviewed and approved by the Ethics Committee of the Medical Faculty of the RWTH Aachen University. A mandatory prerequisite for incorporation of a biomaterial sample into RWTH CBMB is the written consent of the donor. Before signing, the donor is informed by a medical doctor about the research project and the intended storage of donated samples and associated data. The important contribution of the donor to biomedical research is addressed (quoted from https://www.cbmb.rwth-aachen.de/en/data-privacy). All procedures performed involving human tissue were in accordance with the ethical standards of the institutional research committee which are comparable with the 1964 Helsinki declaration and its later amendments.

Preparation and handling of NRCM. Neonatal rat cardiomyocytes (NRCM) were isolated from 1–2-day-old Sprague Dawley rats10,15,16. NRCM were cultured in Medium Eagle (MEM) containing 5% (V/V) fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 µg/mL amphotericin B, 5% (V/V) FCS, 100 µM 5-bromo-2′-deoxyuridine (5-BrdU), and 2 µg/mL vitamin B12 at 37 °C and 1% CO2. FCS concentration was reduced to 1% (V/V) after 24 h by followed an adenoviral transduction after 30–48 h. Cells were transduced with indicated adenoviruses encoding mouse Flag-ERK2 (ERK2-wt), HA-ERK1 (HA-ERK1), Flag-ERK2Δ174–177 (ERK2-Δ4-A4), Flag-ERK2T188D (ERK2-D), HA-ERK2T188D (ERK2-D), HA-MEK1S1212S222D (MEK-ΔD), YFP-ERK2-wt (YFP-ERK2-wt), YFP-ERK2Δ174–177 (YFP-ERK2-Δ4-A4), YFP-ERK2Δ174–177, TmT (YFP-ERK2-Δ4-D), myc-ERK2Δ177, 35′-peptide, “EDIT” (β-galactosidase (LacZ), or enhanced green fluorescent protein (GFP), respectively10,13,14. 48 h after transduction, NRCM were treated with either phenylephrine (PE; 4 µM, 10 min, 24 h) or for the isolucine inclusion assays for 30 h or for TUNEL assays (30 µM, 15 min) or hydrogen peroxide (H2O2; 100 µM, 1 h or 15 min)10. Before stimulation, cells were cultured in serum-starved medium. When indicated, cells were pre-incubated with PD98059 (30 µM, 1 h) trametinib (15 µM), selumetinib (1 µM), cobimetinib (5 µM), bimetinib (5 µM) or cotreatment (0.2 µg/ml, 24 h). NRCM used in Fig. 3h and Supplementary Fig. 6E, F were cardiomyocytes purchased from Lonza (rat cardiac myocytes, RCM-CM-561). They were transfected with siRNAs directed against ERK1 and ERK2 or respective scramble siRNA from Dharmacon using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions (ERK1: On-Target plus Smart Pool, Rat MAPK3 (50689); ERK2: sense 5′-CAAUGGUGCAGGAGACCUUGU-3′ and antisense 5′-AAUGUGCCAGCUCCAGUUU-3′; scramble: On-Target plus Nontargeting
siRNA#1) 24 h after plating and transduced with indicated adenoviruses 5 h later. ERK1/2 expression levels were analyzed 48 h after transfection.

Cell culture of HEK293, COS7, HT29, LS1T74, and H9c2 cells. Human embryonic kidney 293 (HEK293) and COS7 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) at 37 °C and 7% CO2 as previously described and maintained in foetal calf serum (FCS, Sigma). For HA- and myc-tagged constructs, i.e. LacZ as control or the myc-EDI, 48 h after infection, experiments were performed. When indicated, cells were treated with 10% DMSO (DMSO; Sigma).

ERK1/2 expression levels were analyzed 48 h after transfection. LS1T74 cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 and HT29 cells in McCoy’s 5A medium and H9c2 in DMEM at 37 °C and 5% CO2 and transduced with adenoviruses 48 h (LS1T74, HT29) and 24 h (H9c2) after seeding with indicated constructs. Lactac as control or the myc-EDI. 48 h after infection, experiments were performed. Antagonist CTCF and to present semiquantitative results, immunoblot signals were quantified by analysis of scanned immunoblots scanning with densitometry software. All measurements and analyses were performed in a blinded manner.

Quantification of nuclear-to-cytosolic ratios of YFP-ERK2. Quantification of nuclear-to-cytosolic ratios of YFP-ERK2 and YFP-ERK2-D (YFP-ERK2-D) was performed in COS7 cells and NRMC as previously described with or without prior stimulation using phenylephrine (PE, 10 μM for COS7 and 4 μM for NRMC; 10 min) and thereafter fixed using 4% paraformaldehyde for 10 min at room temperature. Nuclei were stained with DAPI (700 ng/mL). For examination of nuclear-to-cytosolic ratios of YFP-ERK2 localization, a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) was used. Yellow fluorescent protein (YFP) was excited at 488 nm and DAPI at 405 nm. Fluorescence images were taken at 530–600 and 430–463 nm, respectively. Confocal images were recorded with a Leica TCS SP5 laser scanning confocal microscope. All measurements and analyses were performed in a blinded manner.

\[^{3}H\]Isoleucine incorporation. Rates of protein synthesis in NRMC were determined by \[^{3}H\]isoleucine incorporation as previously described with or without prior stimulation using phenylephrine (PE, 4 μM, 24 h).

Voluntary running exercise. At the age of 8 weeks, male wild-type mice (FVB/N) and ERK2-tg transgenic mice (ERK2-tg, FVB/N) were subjected to cage with free access to a running wheel and daily running distances were recorded.

Transverse aortic constriction. Male wild-type mice at the age of 8 weeks (FVB/N) and ERK2-tg transgenic mice (ERK2-tg-tg, FVB/N) were subjected to transverse aortic constriction (TAC) using a 27-gauge needle to induce chronic left ventricular (LV) pressure overload. Before TAC and 6 weeks after TAC, echocardiography and cardiac catheterization was performed. Hearts were isolated for tissue sections were additionally counterstained with wheat germ agglutinin. Samples were analyzed using fluorescence microscopy (Leica; DM 4000B). All measurements and analyses were performed in a blinded manner.

**RESULTS**

**ERK1/2 expression levels were analyzed 48 h after transfection. LS1T74 cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 and HT29 cells in McCoy’s 5A medium and H9c2 in DMEM at 37 °C and 5% CO2 and transduced with adenoviruses 48 h (LS1T74, HT29) and 24 h (H9c2) after seeding with indicated constructs, i.e. LacZ as control or the myc-EDI. 48 h after infection, experiments were performed. When indicated, cells were treated with MEK inhibitors 1h, 24h or 48h as indicated. Cell culture media were supplemented with 10% (V/V) fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine.**
Echocardiography. Transthoracic echocardiograms were performed in a blinded manner using the Vevo700 or Vevo3100 high-resolution imaging systems.

Histological and morphometric analysis. Histological analysis, mouse hearts were fixed in 4% (V/V) paraformaldehyde and embedded in paraffin. Tissue sections (2 μm) were either stained with hematoxylin/eosin or with Sirius Red [11]. For determination of cross-sectional areas, 50–80 individual cells per mouse and 6–10 animals per group were analyzed on a computerized pixel counting. Only nucleated cardiac myocytes of transverse myocyte sections showing a centrally localized nucleus were included in the analysis. For quantification of fibrosis, Sirius Red stained sections of 6–10 animals per group and genotype were analyzed by semi automated image analysis. All measurements were performed in a blinded manner.

Left ventricular catheterization. To measure left ventricular pressure, a 1.4-F pressure catheter (Millar Instruments) was inserted into the right carotid artery and advanced to the left ventricle. Dobutamine was infused in increasing doses (75, 150, 375, 750, and 1500 mg/min) into the jugular vein. Data were analyzed using the Chart software (Chart5, AD Instruments) as previously described [12,13]. Eight-week-old male mice (FVB/N) or mice after 6 weeks of TAC were used for the analysis. Dobutamine concentration–response curves, a sample size of at least n = 8 for untreated mice and n = 9 (RUN or TAC surgery) was chosen based on prior knowledge of statistical power from previously published experiments [8,12,13,19].

Caspase-3 activity. Caspase-3 activity was determined using the Caspase-Glo 3/7 Assay kit (Promega). Frozen heart samples were homogenized in lysin buffer (see above) and then centrifuged for 10 min (25,200 g). Protein concentration was adjusted to 0.2 mg/ml. 50 μl of sample and 50 μl of reaction reagent were added in a white 96-well plate. After 1 h of incubation, luminescence of each sample was measured using the Perkin-Elmer Envision 2104 Multilabel Reader.

RNA preparation and real-time PCR. RNA from left ventricles, NRCM, H9c2, HT29, and Ls174T cells was isolated using the RNAasy Kit (Qiagen) or peqGOLD TriFast™ solution (Peqlab) and reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) for quantitative real-time polymerase chain reaction (RT-PCR). The C1000 Thermal Cycler (CFX96 (Bio-Rad) was used and data were analyzed according to the 2−ΔΔCT method [29]. For cDNA amplification of genes encoding brain nitric oxide synthetase (Npy), collagen type III alpha 1 (Col3a1), myc-EDI (mouse) forward primer 5′-ACTTCAGCTGATGAGTGA-3′; myc-EDI (mouse) reverse primer 5′-GAGCTCCTGCTGCTCGAAG-3′; Tgfb2 (mouse) forward primer 5′-GCTGCTCTGCTGCTCTGCTA-3′; Tgfb2 (mouse) reverse primer 5′-GAGAGCTGAGATGCTGAAGCAGGCA-3′; Tgfb1 (rat) reverse primer 5′-TCCCTGAGCTCCTGCTGCTA-3′; Tgfb1 (rat) forward primer 5′-GAGGCTACCTCCTCAAAGGAC-3′; Tgfb3 (mouse) forward primer 5′-ATGGAGCCGAAGCAGGCTGGA-3′; Tgfb3 (mouse) reverse primer 5′-GGCAAAAGAGTCTGGATGCCC-3′; Thr2 (rat) forward primer 5′-GACCTGGACAGCAGCAGGG-3′; Thr2 (rat) reverse primer 5′-TCCCTGCTGCTGCTGCTCTA-3′; Gapdh (mouse) forward primer 5′-TGGCGATGTGTGCGGTGTCT-3′; Gapdh (mouse) reverse primer 5′-CTGAGCTGACCATCTCAAGC-3′; Col3a1 (mouse) forward primer 5′-AAAACAGACGATTTCCATCAC-3′; Col3a1 (mouse) reverse primer 5′-ACCCCCAATGCTGATAGGG-3′; Nefya (mouse) forward primer 5′-ATGGAGCCGAAGCAGGCTGGA-3′; Nefya (mouse) reverse primer 5′-GGCAAAAGAGTCTGGATGCCC-3′; Nefya (human) forward primer 5′-GTTCGCTCCCTGCCATTCACA-3′; Nefya (human) reverse primer 5′-CAACAAGTTGGCCCAGAACA-3′; Cnb1 (human) forward primer 5′-CCAGAGAGCCAGACGAAATAG-3′; Cnb1 (human) reverse primer 5′-GGGCTAAGAGCAGCAAGAAAAG-3′; Cnna1 (human) forward primer 5′-CCCTCAAGAGAGCAGCAAGAAAAG-3′; Cnna1 (human) reverse primer 5′-CCCTCAAGAGAGCAGCAAGAAAAG-3′; Gapdh (human) forward primer 5′-CCGGTTCCCCACATTGCA-3′; Gapdh (human) reverse primer 5′-CTGAGCTGACCATCTCAAGC-3′.

RNA isolation and reverse transcriptase (RT)-PCR. Total RNA was isolated from indicated cells using peqGOLD TriFast™ solution (Peqlab) according to the manufacturer’s protocol. Isolated RNA was transcribed into cDNA using the Superscript® First-Strand Synthesis System (Invitrogen) and cDNA was amplified by PCR. PCR products were visualized in ethidium bromide containing agarose gel. The SYBR® (EKR2-309-357) was detected with the following primer pair: forward 5′-ATGGAGCAAAGAAGTCTCATCAGC-3′; reverse 5′-TCTGTATCTTCCCTGACAACT-3′. The PCR reaction mixture contained SsoFast EvaGreen Supermix (Bio-Rad), and following primers were used:

Proximity ligation assay. NRCM were seeded on coverslips and transduced with adenoviruses encoding Flag-tagged ERR2, HA-tagged ERR2, and myc-EDI or LacZ as indicated. COS7 cells were seeded on coverslips and transduced with Flag-tagged ERR2, HA-tagged ERR2, and myc-EDI (subcloned into pcDNA3 expression vector) or empty pcDNA3 as control (Con) as indicated. In total, 24 h after transfection or transduction, cells were serum starved and 48 h after transfection or transduction, cells were stimulated with PE (4 μM, 10 min, 37 °C). Cells were fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature and permeabilized with ice-cold methanol acetate, 1:1 (2 min). Cells were then incubated with 3% BSA (w/v) blocking solution (1 h, RT) and afterwards incubated with a reaction mixture containing SsoFast EvaGreen Supermix (Bio-Rad), and following primers were used:

Cross-linking experiments. For cross-linking experiments, HEK293 cells were transduced with Flag-tagged ERR2 and myc-EDI (myc-ERR2-309-357) that were subcloned into pcDNA3 expression vector or empty pcDNA3 as control (Con). In
IR measurements of the peptide EDI. Immobilized films of recombinant Hisc-tagged ERK230–357 (Hisc-EDI), purified from BL21 by His-tag affinity purification1, were prepared by immobilization of the Hisc-EDI from a 3.8 mg/mL solution in binding-buffer on gold substrates (Siens, Netherlands). After immobilization, the samples were rinsed with deionized water. IR Microscopy was performed using a Bruker Hyperion 3000 FTIR microscope with a Grazing Angle Objective at spectral resolutions of 4 cm⁻¹ in dry-air purged environment. A photovoltaic mercury cadmium telluride detector served for maximum linearity of the detected IR signals. AFM-IR was performed using a commercially available AFM-IR setup by Anasys Instruments (nanoIR2-FS) equipped with a tunable p-polarized NIR quantum cascade laser (QCL) by Daylight Solutions (MiRcat) with a spectral resolution of 1 cm⁻¹ at 20 cm⁻¹ per second sweep rate in ambient conditions. Grazing angle IR (GIR) spectrum is displayed reversed and baseline-corrected.

Assessment of mitochondrial membrane potential. After isolation, NRCM were seeded on poly-L-lysine-coated glass coverslips. In all, 48 h after adenoviral transduction (LacZ or EDI), cells were stimulated with H₂O₂ (15 min, 100 µM) and pretreated with trametinib (1 h, 15 µM) if indicated. H9c2 cells were seeded on poly-L-lysine-coated glass coverslips. In all, 48 h after adenoviral transduction (LacZ or EDI), cells were stimulated with H₂O₂ (1 h, 100 µM) and pretreated with trametinib (15 µM), selumetinib (1 µM), cobimetinib (5 µM), and binimetinib (10 µM) for 1 h if indicated. NRCM were stained with 1 nM MitoTrackerGreen for 1 h. Samples were analyzed using a Leica TCS SP5 confocal microscope. TMRM was excited at 561 nm and emission was measured between 580 and 700 nm. MitoTrackerGreen was excited at 488 nm and emission was measured between 500 and 530 nm. For quantification at least 85 NRCM and 15 H9c2 per condition and experiment were analyzed by computerized pixel counting. The investigators were blinded to experimental settings during data analysis.

Statistical analysis. For statistical analyses, we used GraphPad software (San Diego, USA), p < 0.05 was regarded as significant. We chose the sample sizes for all groups based on study feasibility and prior knowledge of statistical power from previously published experiments12,13,19,37,55. All data sets showed normal distribution. For adequate power, we generally chose a sample size of at least n = 6–8 for physiological experiments and at least n = 4 for biochemical experiments. Our results represent the mean±standard error (mean ± SEM). P-values are indicated within the graphs. We used Student’s t-test analysis for two-group comparisons (two-tailed) and one-way analysis of variance analyses (ANOVA; ordinary one-way ANOVA) if more than two groups were compared. We used Bonferroni test as post-hoc test if not stated otherwise. Parametric tests were chosen only when variances between the compared groups were not significantly different.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Gene array data are available from Array Express: accession numbers: E-MTAB-8110 and 8111. Full scans of the plots are available in Supplementary Fig 10. In addition, all source data are provided as a Source Data file. Figures and Supplementary Figures have associated raw data. Raw data for additional information required to interpret, replicate, or build upon the findings of this study are available from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file.

Code availability

The custom codes in R that generated the findings of this study are available by authors upon request.

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Microarray pre-processing and data analysis. Affymetrix gene expression data were pre-processed using ‘affyPLM’ packages of the Bioconductor Software29. Genes with the strongest evidence of differential expression were obtained using a linear model fit. Data obtained from eGFP-treated mice or LacZ transduced cells were used as reference. To annotate the microarrays, custom chip definition file version 22 from Brainarray based on Entrez ID’s was used. A false positive rate of α = 0.05 with false discovery rate (FDR) correction and a fold change greater 1.5 was taken as the level of significance. To unravel patterns in the gene expression data for different pathways heatmaps the ‘ComplexHeatmap’ package was used.
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Author contributions
A.T., T.B., C.S., S.K., M.W.H., P.G., W.S.-H., J.L., S.H., T.S., A.R., C.K., and K.L. performed experiments and analyzed data; P.N., A.W., R.K., H.-K.M.-H., A.R., N.F., J.E., D.D., A.E.-A., J.G.H., O.J.M., K.H., F.C., and A.Z. provided feedback and revised the manuscript; K.L. wrote the manuscript and designed the project.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to K.L.
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Angela Tomasovic1,2,19, Theresa Brand1,2,19, Constanze Schanbacher1,2,19, Sofia Kramer1, Martin W. Hümmer1,18, Patricio Godoy3, Wolfgang Schmidt-Heck4, Peter Nordbeck5, Jonas Ludwig6, Susanne Homann1, Armin Wiegering6,7, Timur Shaykhutdinov8, Christoph Kratz8,8, Ruth Knüchel9, Hans-Konrad Müller-Hermelink10, Andreas Rosenwald10, Norbert Frey11,12, Jutta Eichler6, Dobromir Dobrev13, Ali El-Armouche14, Jan G. Hengstler15,16, Alma Zernecke17 & Kristina Lorenz1,12,5,16

1Institute of Pharmacology and Toxicology, University of Würzburg, 97078 Würzburg, Germany. 2Leibniz-Institut für Analytische Wissenschaften – ISAS-e.V., 44139 Dortmund, Germany. 3IfADo-Leibniz Research Centre for Working Environment and Human Factors at the Technical University Dortmund, 44139 Dortmund, Germany. 4Leibniz Institute for Natural Product Research and Infection Biology - Hans Knoll Institute, 07745 Jena, Germany. 5Comprehensive Heart Failure Center, 97078 Würzburg, Germany. 6Department of Chemistry and Pharmacy, Friedrich-Alexander-Universität Erlangen-Nürnberg, 91058 Erlangen, Germany. 7Department of General, Visceral, Transplant, Vascular and Pediatric Surgery, University Hospital Würzburg, 97080 Würzburg, Germany. 8Leibniz-Institut für Analytische Wissenschaften – ISAS-e.V., 12489 Berlin, Germany. 9Institute of Pathology, University Hospital Aachen, RWTH Aachen, 52074 Aachen, Germany. 10Institute of Pathology, University of Würzburg, 97080 Würzburg, Germany. 11Department of Internal Medicine III, University of Kiel, 24105 Kiel, Germany. 12DZH (German Center for Cardiovascular Research), partner site Hamburg/Kiel/Lübeck, Kiel, Germany. 13Institute of Pharmacology, West German Heart and Vascular Center, University Duisburg-Essen, 45147 Essen, Germany. 14Department of Pharmacology and Toxicology, TU Dresden, 01307 Germany.
Dresden, Germany. Institute of Experimental Pharmacology and Toxicology, Cardiovascular Research Center, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany. DZHK (German Center for Cardiovascular Research), partner site Hamburg/Kiel/Lübeck, Hamburg, Germany. Institute of Experimental Biomedicine, University Hospital Würzburg, University of Würzburg, 97080 Würzburg, Germany. Present address: Department of Neurology, Hannover Medical School, 30625 Hannover, Germany. These authors contributed equally: Angela Tomasovic, Theresa Brand, Constanze Schanbacher. Email: lorenz@toxi.uni-wuerzburg.de