Hypoxia-inducible factor 2-alpha-dependent induction of amphiregulin dampens myocardial ischemia-reperfusion injury

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Myocardial ischemia–reperfusion injury (IRI) leads to the stabilization of the transcription factors hypoxia-inducible factor 1-alpha (HIF1-alpha) and hypoxia-inducible factor 2-alpha (HIF2-alpha). While previous studies implicate HIF1-alpha in cardioprotection, the role of HIF2-alpha remains elusive. Here we show that HIF2-alpha induces the epithelial growth factor amphiregulin (AREG) to elicit cardioprotection in myocardial IRI. Comparing mice with inducible deletion of Hif1a or Hif2a in cardiac myocytes, we show that loss of Hif2-alpha increases infarct sizes. Microarray studies in genetic models or cultured human cardiac myocytes implicate HIF2-alpha in the myocardial induction of AREG. Likewise, AREG increases in myocardial tissues from patients with ischemic heart disease. Areg deficiency increases myocardial IRI, as does pharmacologic inhibition of Areg signaling. In contrast, treatment with recombinant Areg provides cardioprotection and reconstitutes mice with Hif2a deletion. These studies indicate that HIF2-alpha induces myocardial AREG expression in cardiac myocytes, which increases myocardial ischemia tolerance.
Myocardial infarction is among the leading causes of death in the Western countries. It results from the occlusion of a coronary artery by an intracoronary thrombus, thereby preventing blood flow to the metabolically highly active myocardium. The mainstay therapy for acute myocardial ischemia currently focuses on timely reperfusion—for example by placement of an intracoronary stent. However, additional therapeutic approaches that render the myocardium more resistant to ischemia are an important area of investigation. Such approaches would contribute to the preservation of myocardium at risk and could potentially improve outcomes of patients suffering acute myocardial ischemia.

During myocardial ischemia, the supply and demand ratio for metabolites shifts dramatically—in particular for oxygen—thereby causing profound tissue hypoxia. Cellular responses to hypoxia lead to stabilization of hypoxia-dependent transcription factors. Indeed, previous studies have suggested the transcription factor hypoxia-inducible transcription factors (HIFs) in cardioadaptive responses. For example, HIFs mediate the cardioprotective response induced by ischemic preconditioning, where short time periods of ischemia treatment reduces myocardial infarct sizes. During ischemic preconditioning, HIFs are stabilized, however, mice with partial deletion of Hif1a are not protected by ischemic preconditioning. Similarly, HIFs have been implicated in mediating cardioprotection provided by remote ischemic preconditioning, a cardioprotective strategy where treatment of a limb for short time periods of ischemia results in attenuated myocardial infarct sizes.

Due to their high metabolic demand, the functional role of myocytes in cardioadaptive responses during ischemia has been the focus of many studies, and several have indirectly suggested myocardial-expressed HIFs in cardioprotection. However, a significant obstacle for the systematic investigation of HIFs in cardioprotection results from the fact that mice with homozygous deletions of HIFs die early during embryogenesis. To overcome this problem, we generated mice with inducible myocyte-specific deletion of Hif1a or Hif2a, the two predominant isoforms of HIFs that mediate alteration in genetic programs during inflammatory or ischemic conditions. For this purpose, we crossed transgenic mice with a floxed Hif1a or Hif2a gene with mice expressing Cre-recombinase under the control of a tamoxifen-inducible myocyte-specific promoter (Hif1aloxP/loxP Myosin-Cre+ and Hif2aloxP/loxP Myosin-Cre+ mice, respectively).

Exposure of these two mouse strains to IRI revealed a previously unappreciated role in cardioprotection for myocyte-dependent Hif2-alpha via induction of the growth factor amphiregulin (Areg).

**Results**

**Myocyte-specific Hif2a deletion enhances myocardial IRI.** Based on previous studies implicating HIFs in organ-protection during ischemia and reperfusion injury, we hypothesized that myocyte-specific HIFs dampen myocardial ischemia and reperfusion injury. To overcome the problem that mice with a global Hif1a or Hif2a deletion die during embryogenesis, we generated mice with induced deletion of Hif1a or Hif2a in cardiac myocytes. To achieve this, we crossed Hif1aloxP/loxP or Hif2aloxP/loxP mice with mice expressing tamoxifen-inducible Cre-recombinase under the Myosin-heavy chain promoter (Myosin-Cre+). To induce a Hif1a or Hif2a deletion, respectively, we treated these mice with daily tamoxifen injections for 5 days (1 mg i.p./day), followed by a 7 day recovery period (Fig. 1a). Subsequently, we exposed the mice to in situ myocardial ischemia-reperfusion. Western blot studies for Hif1-alpha or Hif2-alpha showed that protein levels increased following 60 min of ischemia and 120 min of reperfusion in Myosin-Cre+ mice. In contrast, the respective HIF-isoform in the post-ischemic myocardium decreased notably in Hif1a- or Hif2a-deficient animals (Fig. 1b–d; Supplementary Fig. 1). These findings demonstrate an increase of Hif1-alpha and Hif2-alpha levels following myocardial ischemia and reperfusion injury, while in genetic models for Hif1-alpha or Hif2a deletion this response is attenuated. These findings suggest that the mouse lines generated allow us to assess the individual function of myocyte-specific Hif1-alpha versus Hif2-alpha during myocardial ischemia and reperfusion injury.

To assess the functional role of cardiac myocyte-specific Hif1-alpha or Hif2-alpha in cardioprotection, we exposed Hif1aloxP/loxP Myosin-Cre+, Hif2aloxP/loxP Myosin-Cre+ mice to myocardial ischemia and reperfusion injury and measured myocardial injury by infarct size or serum troponin levels. Surprisingly, we found the predominant phenotype in Hif2aloxP/loxP Myosin-Cre+ mice. Indeed, mice with induced Hif2a deletion in cardiac myocytes experienced dramatic increases in myocardial injury when compared to Hif1aloxP/loxP Myosin-Cre+ mice or Myosin-Cre+ controls (Fig. 1e–g). Together, these studies suggest a role for myocyte-specific Hif2-alpha in cardioprotection during ischemia and reperfusion injury.

**Identification of Areg as Hif2-alpha target during IRI.** After having shown that mice with induced Hif2-alpha deletion in cardiac myocytes experience increased myocardial injury, we investigated a transcriptional mechanism that could elicit Hif2a-dependent cardioprotection. Previous studies implied Hif1-alpha in cardioprotection from ischemia. In contrast, a functional role of Hif2-alpha in cardioprotection is largely unknown. Since HIFs mediate adaptive responses through the induction of target genes, we performed a microarray study comparing transcript levels of the post-ischemic myocardium from Myosin-Cre+ or Hif2aloxP/loxP Myosin-Cre+ mice. Consistent with a role of myocyte-specific Hif2-alpha as a transcriptional inducer, we identified a set of genes that increased in the post-ischemic myocardium of Myosin-Cre+ but not in Hif2a-loxP/loxP Myosin-Cre+ mice. Among transcript levels upregulated in an Hif2a-dependent fashion, the strongest differentially regulated transcript was the epithelial growth factor Amphiregulin (Areg). Among transcript levels upregulated in an Hif2a-dependent fashion, the strongest differentially regulated transcript was the epithelial growth factor Amphiregulin (Areg). From the microarray study, we examined Areg transcript and protein levels in the area-at-risk (AAR) of Hif1aloxP/loxP Myosin-Cre+, Hif2aloxP/loxP Myosin-Cre+, or Myosin-Cre+ mice. Consistent with our findings in the microarray, we observed that Areg transcript and protein levels significantly increased in Myosin-Cre- and mice with cardiomyocyte-specific Hif1a deficiency. In contrast, the induction of Areg in post-ischemic myocardial tissues was significantly attenuated in Hif2aloxP/loxP Myosin-Cre+ mice (Fig. 2c–e; Supplementary Fig. 2). Together with previous studies from the cancer field suggesting that Hif2-alpha binds to the Areg promoter and induces its transcription, our findings indicate that Hif2-alpha transcriptionally induces Areg during murine myocardial IRI.

**HIF2-alpha regulates AREG expression in human cardiac myocytes.** Based on the findings that Hif2-alpha regulated Areg transcript and protein levels in a murine in vivo model of myocardial ischemia and reperfusion injury, we performed studies in cultured human cardiomyocytes to demonstrate that the observed changes in murine Areg levels also occur in human myocardial tissues. Thus, we exposed human cardiac myocytes (HCM) to hypoxia and measured AREG transcript and protein...
Fig. 1 Contribution of myocyte-specific hypoxia-inducible factor (HIF) isoforms Hif1a or Hif2a to cardioprotection. a Schematic of breeding approach to generate mice with induced myocyte-specific HIF deletions used in subsequent studies. Hif1aloxP/loxP or Hif2aloxP/loxP mice were crossed with Cre-recombinase mice under the control of Myosin-heavy chain promoter (Myosin-Cre+); these mice express Cre-recombinase under the control of a tamoxifen-inducer. Control animals (Myosin-Cre+), Hif1aloxP/loxP Myosin-Cre+ or Hif2aloxP/loxP Myosin-Cre+ received a daily dose of 1 mg i.p. Tamoxifen five consecutive days to induce the Cre-recombinase activity. After 7 days, animals underwent experimental protocol (60 min of in situ myocardial ischemia followed by 120 min of reperfusion). b HIF1-alpha or HIF2-alpha immunoblot analysis from homogenized myocardial tissue, harvested from male Myosin-Cre+ and Hif1aloxP/loxP Myosin-Cre+ or Hif2aloxP/loxP Myosin-Cre+ mice, matched in age and weight. Mice underwent a thoracotomy with no further treatment(-I) or 60 min of myocardial ischemia (+I) followed by 120 min reperfusion; β-Actin (ACTb) served as a loading control. One representative blot out of three experiments is shown. c, d Quantification by densitometry of the HIF-immunoblot results relative to ACTb. Data are expressed as mean fold change ±SD normalized to untreated myocardial tissue from Myosin-Cre+ compared by one-way ANOVA followed by Bonferroni’s multiple comparison test (n = 3 per group; c: F5,12 = 6.74, p = 0.0033; d: F5,12 = 21.85, p < 0.0001). e Infarct sizes ±SD in Myosin-Cre+, Hif1aloxP/loxP Myosin-Cre+ or Hif2aloxP/loxP Myosin-Cre+ mice, presented as the percentage to the area-at-risk after 60 min of ischemia, followed by 120 min of reperfusion (Myosin-Cre+ n = 5; Hif1aloxP/loxP Myosin-Cre+ n = 5; Hif2aloxP/loxP Myosin-Cre+ n = 4); per group mean ± SD; compared by one-way ANOVA followed by Bonferroni’s multiple comparison test; F2,11 = 7.901; p = 0.0075). f Representative infarct staining from Myosin-Cre+, Hif1aloxP/loxP Myosin-Cre+ or Hif2aloxP/loxP Myosin-Cre+. g Troponin serum levels after 60 min ischemia, followed by 120 min of reperfusion in Myosin-Cre+, Hif1aloxP/loxP Myosin-Cre+ or Hif2aloxP/loxP Myosin-Cre+ (Myosin-Cre+ n = 5, Hif1aloxP/loxP Myosin-Cre+ n = 5, and Hif2aloxP/loxP Myosin-Cre+ n = 4 per group; presented as mean ± SD; compared by one-way ANOVA followed by Bonferroni’s multiple comparison test; F2,11 = 19.14, p = 0.0003)
levels. To address a functional role for HIF2-alpha in AREG induction, we generated HCM cell lines with stable lentiviral-mediated shRNA-mediated repression of HIF1-alpha or HIF2-alpha. Indeed, lentiviral-mediated shRNA transduction in HCM repressed HIF1-alpha or HIF2-alpha transcript levels at baseline (Fig. 3a, b). To examine if the lentiviral-mediated shRNA repression of HIF1-alpha or HIF2-alpha also attenuated HIF protein stabilization during hypoxia, we exposed control-transduced HCMs, or HCMs transduced with lentiviral shRNA targeting HIF1-alpha or HIF2-alpha to ambient hypoxia (1% oxygen for 16h). While exposure of control cells to ambient hypoxia stabilized HIF1-alpha and HIF2-alpha protein, these responses were abolished in HCM cell lines with corresponding shRNA-mediated HIF repression (Fig. 3c–e). Together, these
studies indicate that these human myocyte cell lines can be used to study differential roles for HIF1-alpha- or HIF2-alpha-dependent gene induction during hypoxia. Indeed, induction of AREG transcript and protein levels remained intact in HCM transduced with control or HIF1-alpha-repressing shRNA, but were entirely abolished following shRNA-mediated repression of HIF2-alpha (Fig. 3f–h; Supplementary Fig. 3). Taken together, these findings demonstrate that HIF2-alpha is a transcriptional driver of AREG in HCM.

AREG levels increase in patients with ischemic heart disease. After having shown that Areg levels increased in a murine myocardial ischemia and reperfusion injury model or in HCM exposed to hypoxia, we next performed proof-of-principle studies to examine AREG levels in cardiac tissues obtained from healthy controls or patients with ischemic heart disease (IHD). We received these cardiac tissues from a human biobank that stores human heart samples14. Cardiac tissues from IHD patients were retrieved from explanted hearts during cardiac transplantation.

Fig. 3 Functional role of HIF2A in the transcriptional regulation of amphiregulin (AREG) in human cardiac myocytes. a–e Human cardiac myocytes (HCM) underwent control, HIF1A-specific or HIF2A-specific short hairpin RNA (shRNA) lentiviral transfection to suppress transcription of hypoxia-inducible factors HIF1A or HIF2A. a, b shRNA-transfected HCM were exposed to ambient hypoxia (1% oxygen) for 16 h and analysis of transcript changes by RT-PCR of HIF1A or HIF2A, respectively. Transcriptional changes were calculated relative to an internal housekeeping gene (Actin-b). Data are expressed as mean fold change ± SD compared to normoxic cells (n = 6 per group). c Immunoblot for HIF1A or HIF2A from shRNA-transfected normoxic or hypoxic HCM. β-Actin (ACTb) served as a loading control. d, e Quantification by densitometry of the HIF1-alpha or HIF2-alpha immunoblot results relative to ACTb. Data are expressed as mean fold change ± SD normalized to sh Control compared by one-way ANOVA followed by Bonferroni’s multiple comparison test (n = 3 per group; F5,12 = 14.48, p = 0.0002; F5,11 = 6.726, p = 0.0042). f HCM transfected with shRNA directed against HIF1A, HIF2A or control shRNA were exposed to ambient hypoxia (1% oxygen) for 16 h or were maintained under normoxic conditions (21% oxygen). Subsequently, total RNA was isolated and probed by RT-PCR for transcriptional changes of amphiregulin (AREG). Data are expressed as mean fold change ± SD compared to normoxic cells (n = 3 per group). Transcriptional changes were calculated relative to an internal housekeeping gene (ACTb). Data compared by one-way ANOVA followed by Bonferroni’s multiple comparison test (F5,12 = 14.33; p = 0.0001). g HCM transfected with shRNA directed against HIF1A, HIF2A, or control shRNA, total protein was isolated and immunoblotted for AREG. β-Actin (ACTb) served as a loading control. One representative blot out of three experiments is shown. h Quantification by densitometry of the HIF immunoblot results relative to ACTb. Data are expressed as mean fold change ± SD normalized to normoxic cells (sh Control) and compared by one-way ANOVA followed by Bonferroni’s multiple comparison test (n = 3 per group; F5,12 = 11, 58; p = 0.0003)
Cardiac tissues that served as healthy controls were taken from donor hearts that were deemed sufficient as a cardiac allograft, but could not be used for transplantation due to logistic reasons. Consistent with a functional role for myocardial ischemia in AREG induction, AREG protein levels were elevated in patients with IHD compared to healthy controls (Fig. 4a, b; Supplementary Fig. 4). Taken together, these proof-of-principle studies indicate that AREG expression increased in cardiac tissues from patients experiencing myocardial ischemia.

**Areg deficiency is associated with enlarged infarct sizes.** After having shown that murine and human myocardial AREG levels increased during conditions of limited oxygen availability via transcriptional control by HIF2-alpha, we addressed the functional role of AREG during IRI. Since mice with induced Hif2a deletion in cardiac myocytes experienced larger myocardial injury, we anticipated a cardioprotective role of Areg. To address this hypothesis, we performed studies in previously described mice gene-targeted for Areg. Exposure of Areg<sup>−/−</sup> mice to 60 min of myocardial ischemia followed by 120 min of reperfusion revealed larger infarct sizes and elevated levels of the myocardial necrosis marker troponin I (Fig. 5a–c). Moreover, reconstitution of Areg<sup>−/−</sup> mice via intravascular infusion of murine recombinant Areg reduced ischemic myocardial tissue injury, including smaller infarct sizes (Fig. 5d, e) and attenuated release of troponin I (Fig. 5f). Together, these findings suggest that genetic deletion of Areg increases myocardial tissue injury following murine ischemia and reperfusion injury, while reconstitution with 10 µg recombinant Areg can rescue the phenotype of Areg<sup>−/−</sup> mice.

**Blockade of ErbB1 signaling increases myocardial injury.** Since genetic deletion of Areg profoundly increased myocardial injury, we performed pharmacologic studies in wild-type mice targeting...
the Areg receptor ErbB1. Indeed, cardiac tissues express receptors for epidermal growth factors (ErbB receptors), which play important roles in cardiac development. ErbB1 is expressed on cardiac myocytes, but undetectable on vascular endothelia and previous studies demonstrate that AREG solely binds and signals through the ErbB1. Based on these studies, we hypothesized that AREG elicits its cardioprotective effects via activation of the ErbB1 receptor. To address this hypothesis, we performed myocardial ischemia and reperfusion injury in the presence of the pharmacologic ErbB1 receptor antagonist AG1478. Mice received 20 mmol/kg of AG1478 or vehicle 30 min prior to myocardial ischemia, given as slow infusion via a catheter placed into the carotid artery. Subsequently, mice underwent 60 min of myocardial ischemia and 120 min of reperfusion. Consistent with a functional role for AREG in cardioprotection, treatment with the ErbB1 receptor antagonist AG1478 increased myocardial infarct sizes and significantly elevated serum troponin levels. For this purpose, we treated mice with recombinant Areg increases myocardial Areg protein levels in the myocardium, and provides a rational for the therapeutic effects observed following treatment with recombinant Areg.

**Inhibition of ErbB1-ligand binding increases infarct sizes.** As next step, we sought to confirm the above findings with the pharmacologic ErbB1 inhibitor AG1478 utilizing a more specific approach for the ErbB1 receptor. For this purpose, we used the recombinant, monoclonal antibody cetuximab, which binds solely to the extracellular domain of the ErbB1. Binding of cetuximab to ErbB1 completely inhibits binding of the natural ligand and activation of the receptor signaling cascade. For the purpose of this study, mice received 20 mg/kg of cetuximab or vehicle 15 min prior to myocardial ischemia, given as slow infusion via a carotid artery catheter. Subsequently, mice were submitted to 60 min of myocardial ischemia followed by 120 min of reperfusion. Consistent with the effect of pharmacological inhibition of ErbB1 utilizing AG1478, treatment with the anti-ErbB1 antibody cetuximab was associated with increased myocardial infarct sizes and significantly elevated serum troponin levels (Fig. 6a–c). Together these findings indicate that exogenous administration of recombinant murine Areg dampens murine IRI. Based on our studies in mice with induced Hif2a deletion in cardiac myocytes or studies in mice with global Areg deletion, we hypothesized that treatment with recombinant murine Areg could provide cardioprotection from ischemia and reperfusion injury. We used a treatment regime consistent of 10 μg recombinant Areg given as a slow infusion via a catheter placed into the carotid artery 15 min prior to the onset of myocardial ischemia. Control mice received a similar infusion with vehicle. Subsequent exposure to 60 min of myocardial ischemia followed by 120 min of reperfusion was associated with significantly reduced myocardial infarct sizes and troponin I serum levels compared to vehicle treated mice (Fig. 7a–c; Supplementary Fig. 5).

Next, we investigated whether intraarterial administration of recombinant murine Areg increases myocardial Areg protein levels. For this purpose, we treated mice with recombinant Areg and submitted them to our model of myocardial ischemia and reperfusion injury. As controls we used Areg−/+ or Areg−/− animals receiving vehicle infusion. After 120 min of reperfusion, we identified the AAR by Evans blue counterstain technique and immunoblotted for murine Areg protein. Myocardial ischemia and reperfusion alone increased myocardial Areg levels (Fig. 7d). Based on these findings, we hypothesized that AREG elicits its cardioprotective effects via activation of the ErbB1 receptor. To address this hypothesis, we performed myocardial ischemia and reperfusion injury in the presence of the pharmacologic ErbB1 receptor antagonist AG1478. Mice received 20 mmol/kg of AG1478 or vehicle 30 min prior to myocardial ischemia, given as slow infusion via a catheter placed into the carotid artery. Subsequently, mice underwent 60 min of myocardial ischemia and 120 min of reperfusion. Consistent with a functional role for AREG in cardioprotection, treatment with the ErbB1 receptor antagonist AG1478 increased myocardial infarct sizes and significantly elevated serum troponin levels. For this purpose, we treated mice with recombinant Areg increases myocardial Areg protein levels in the myocardium, and provides a rational for the therapeutic effects observed following treatment with recombinant Areg.
Areg treatment reconstitutes Hif2a-deficient mice. Next, we investigated whether reconstitution with recombinant Areg rescues the phenotype of mice with cardiomyocyte-specific Hif2a deficiency. For this purpose, we treated Hif2aloxP/loxP Myosin-Cre+ mice with 10 µg recombinant Areg given as a slow infusion via a catheter placed into the carotid artery 15 min prior to the onset of myocardial ischemia. Myocardial injury and serum troponin levels after subsequent exposure to 60 min ischemia and 120 min of reperfusion was significantly reduced in comparison to control mice (Fig. 8a–c).

Based on the above studies showing that Areg deficiency is associated with reduced activation of the survival kinase Akt after myocardial ischemia and reperfusion, we harvested the AAR form Hif2adloxP/loxP Myosin-Cre+ and immunoblotted the isolated protein for total levels Akt and phosphorylated Akt. Consistent with our findings in Areg+/− mice, we observed that Hif2a deficiency in cardiomyocytes is associated with attenuated Akt-phosphorylation in response to ischemia and reperfusion (Fig. 8d, e; Supplementary Fig. 6). In line with previous studies showing cardioprotection via Akt-phosphorylation, these findings indicate the possibility that Hif2-stabilization could mediate cardioprotection via increased activation of Akt through elevating cardiac Areg signaling. Taken together, the above studies indicate that treatment with recombinant Areg is associated with normalized susceptibility of Hif2aloxP/loxP Myosin-Cre+ mice to ischemic myocardial injury and provide further evidence for a Hif2-alpha-dependent Areg induction in cardioprotection.

Discussion
In the present studies, we address the functional roles of HIFs in mediating cardioprotective responses through the induction of specific gene products. As mice with global deletion of HIFs are not viable, we generated mice with inducible deletion of Hif1a or Hif2a in cardiac myocytes. Ischemic tissue injury was similar in Myosin-Cre+ and Hif1a(loxp/loxp)Myosin-Cre+ mice, while we uncovered a profound phenotype in Hif2a(loxp/loxp)-Myosin-Cre+ mice, including an almost 64% increase of infarct sizes and dramatically elevated serum troponin levels. These data indicate a surprising role for myocyte-specific Hif2a in cardioprotection from ischemia. In order to identify potential target genes that could mediate the cardioprotective role of Hif2a, we performed a microarray study comparing ischemic cardiac tissues from controls or mice with cardiac myocyte-specific deletion of Hif2a. The dominant read-out of this array and confirmatory studies identified an Hif2a-specific induction of the epidermal growth factor Areg during myocardial ischemia. Indeed, AREG expression was also elevated in cardiac tissues samples of patients with ischemic heart disease. In addition, pharmacologic studies utilizing inhibitors of AREG signaling (AG1478 or cetuximab treatment) showed increased myocardial injury. Moreover, myocardial tissue injury increased in mice with genetic deletion of Areg, while treatment of wild-type mice with recombinant Areg attenuated myocardial tissue injury. Finally, reconstitution of Hif2a(loxp/loxp)-Myosin-Cre+ mice with recombinant Areg rescued their phenotype. Based on these findings we conclude that HIF2-
alpha coordinates the induction of AREG in cardiac myocytes, and thereby conveys potent cardioprotection from myocardial ischemia and reperfusion injury.

In line with the present studies, others have shown a functional role of HIFs in cardioprotection during ischemia and reperfusion injury. For example, inhibition of HIF-degradation provides strong protection from ischemia and reperfusion injury by various mechanisms. One study examined the functional role of prolyl-hydroxylase enzymes (PHD), which control the stabilization of HIFs. In this study the authors demonstrated that PHD-deficient mice experienced higher levels of Hif1-alpha and concomitant cardioprotection from ischemia and reperfusion injury. Furthermore, loss of function of PHD2 protects from ischemia and reperfusion injury and improves cardiac function weeks after ischemia due to an increase of Hif1-alpha and Hif2-alpha.

Cardioprotective strategies, such as remote preconditioning and ischemic preconditioning, also rely on a stabilization of HIF1-alpha. Interestingly, in the present study we show that though HIF1-alpha activity is induced by a single ischemia reperfusion episode (without preconditioning), this activation is not enough to promote cardioprotection. Therefore, HIF1-alpha only confers cardioprotection in ischemic preconditioning settings but not after a single episode of ischemia and reperfusion. This could be related to temporal or tissue-specific issues. For example, it is conceivable that HIF1-alpha stabilization to provide cardioprotection may only be efficient when it occurs prior to the insult. Alternatively, it is conceivable that HIF1-alpha stabilization may occur in the whole heart—particularly the myocyte compartment—however, HIF1-alpha-dependent cardioprotection through ischemic preconditioning may involve different tissue compartments of the heart—such as the myeloid compartment or endothelial cell. Interestingly, a recent study found that vascular-endothelial Hif1alpha mediates the acute phase of cardioprotection by ischemic preconditioning, whereas our previous study suggest that HIF1-alpha is essential for the protective effects of ischemic preconditioning. In contrast to this, ischemic preconditioning was preserved in mice with cardiomyocyte-specific deletion of HIF2-alpha. This suggests that the axis HIF2-alpha-AREG does not play a role in ischemic preconditioning. Pharmacologic HIF activators can be used to imitate the cardioprotective responses elicited by ischemic preconditioning. In contrast to this, HIF1-alpha in cardiomyocytes does not influence myocardial injury in ischemia and reperfusion as shown in the present study. This indicates that myocyte-mediated cardioprotection through HIFs predominantly involves HIF2-alpha. Thus, the present findings suggest that myocyte-specific HIF2-alpha increases myocardial ischemia tolerance, which has not been previously reported. Along these lines, studies in other organs than the heart have implicated HIF2-alpha in organ protection from ischemia and reperfusion injury, for example during ischemia and reperfusion injury of the kidney.

Our findings implicate myocyte-specific Hif2a in cardioprotection from ischemia and reperfusion injury, whereas previous studies have found a role for Hif1a in mediating cardioprotection elicited by ischemic preconditioning. Indeed, based on several previous studies, it appears that HIF1-alpha is essential for cardiac ischemic preconditioning. At first look, these findings appear to be in contrast with the present studies showing a cardioprotective role for HIF2-alpha as well. However, different protocols (ischemic preconditioning versus ischemia and reperfusion injury) and different tissue compartments (e.g. vascular compartment versus the myocyte compartment) likely account for functional differences of the HIF isoforms HIF1-alpha and HIF2-alpha. Specifically, a previous study from the laboratory of Dr. Semenza demonstrated that mice with heterozygote deletion of Hif1alpha are not protected by ischemic

Fig. 8 Reconstitution of cardiomyocyte-specific Hif2a-deficient mice with recombinant amphiregulin. **a-c** Hif2aloloxP/loxPMyosin-Cre+ of similar age, gender, and weight as control mice were exposed to 60 min of myocardial ischemia, followed by 120 min of reperfusion; infarct sizes were measured by double staining with Evan’s blue and triphenyltetrazolium chloride and serum samples were collected. All Infarct sizes are presented as the percentage of infarcted tissue in relation to the area-at-risk. Serum troponin levels were determined by ELISA. Note that data in (a-c) in the "no treatment group" are used in part in Fig. 1c-e to display and analyze IR injury from similar experimental conditions. **a** Infarct sizes of Hif2aloloxP/loxPMyosin-Cre+ mice after 60 min of ischemia and 120 min reperfusion that were pre-treated with 10 μg recombinant murine AREG administered over a 15 min period via an indwelling arterial catheter or received no pharmacologic intervention. Data presented as the percentage of infarcted area in relation to area-at-risk. Statistical significance assessed by two-sided, unpaired Student’s t-test (no treatment n = 6; 10 μg AREG n = 9, data presented as mean ± SD). **b** Representative infarct staining of Hif2aloloxP/loxPMyosin-Cre+ that were pre-treated with 10 μg recombinant murine AREG administered over a 15 min period via an indwelling arterial catheter or received no pharmacologic intervention. **c** Troponin I [ng/ml] and Troponin [ng/ml] in the tissue compartments of the heart—particularly the myocyte compartment—however, HIF1-alpha-dependent cardioprotection through ischemic preconditioning may involve different tissue compartments of the heart—such as the myeloid compartment or endothelial cell. Interestingly, a recent study found that vascular-endothelial Hif1alpha mediates the acute phase of cardioprotection by ischemic preconditioning, whereas our previous study suggest that HIF1-alpha is essential for the protective effects of ischemic preconditioning. In contrast to this, ischemic preconditioning was preserved in mice with cardiomyocyte-specific deletion of HIF2-alpha. This suggests that the axis HIF2-alpha-AREG does not play a role in ischemic preconditioning. Pharmacologic HIF activators can be used to imitate the cardioprotective responses elicited by ischemic preconditioning. In contrast to this, HIF1-alpha in cardiomyocytes does not influence myocardial injury in ischemia and reperfusion as shown in the present study. This indicates that myocyte-mediated cardioprotection through HIFs predominantly involves HIF2-alpha. Thus, the present findings suggest that myocyte-specific HIF2-alpha increases myocardial ischemia tolerance, which has not been previously reported. Along these lines, studies in other organs than the heart have implicated HIF2-alpha in organ protection from ischemia and reperfusion injury, for example during ischemia and reperfusion injury of the kidney.

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preconditioning. Similarly, other studies indicate that Hif1α repression with siRNA prevents cardioprotection by ischemic preconditioning. However, the role of HIF2α in cardioprotection has previously been unclear. To address the functional role of Hif2α during myocardial ischemia and reperfusion injury, we used genetic models and found that myocyte-specific Hif2α is critical for the cardioprotection during ischemia and reperfusion injury. To address the functional role of Hif2α in ischemic preconditioning we performed additional studies. Here we found that cardioprotection by ischemic preconditioning is intact in control mice (Myosin-Cre+ mice) or Hif2αloxP/loxPMyosin-Cre+ mice, whereas cardioprotection by ischemic preconditioning is abolished in Hif2aloxP/loxPMyosin-Cre+ mice (Supplementary Fig. 7A-C).

Together, with previous studies, these findings indicate a somewhat specific role for HIF1-α in mediating cardioprotective effects of ischemic preconditioning.

Previous studies on the role of HIF1-αalpha found that the cardioprotective effects of ischemic preconditioning can be pharmacologically recapitulated utilizing the HIF activator DMOG. Importantly, the PHD inhibitor DMOG will result in the stabilization of both HIF isoforms—HIF1-αalpha and HIF2-αalpha—in different cardiac tissue compartments, including the vasculature and cardiac myocytes. To answer the question if DMOG-elicted cardioprotection also involves HIF2-αalpha, we performed additional studies. Here, we found that the cardioprotective effect of DMOG is intact in control mice (Myosin-Cre+ mice) but attenuated in mice with myocyte-specific deletion of Hif2α (Hif2αloxP/loxPMyosin-Cre+ mice; Supplementary Figure 8A, B). Similarly, in mice with siRNA-mediated Hif1α repression, DMOG-dependent cardioprotection was attenuated, as we had reported previously.

Together, these findings indicate that DMOG likely mediates cardioprotection via stabilization of both HIF isoforms—HIF1-αalpha and HIF2-αalpha. Previous studies suggest that HIF1-αalpha-dependent cardioprotection involves purinergic signaling events, particularly through the A2B adenosine receptor, whereas the current studies implicate AREG signaling as a mediator of HIF2-αalpha-dependent cardioprotection. This notion is also supported by the current findings that Hif2αloxP/loxPMyosin-Cre+ mice can be rescued via treatment with recombinant AREG.

The present finding that HIF2α regulates AREG functionally is in line with previous studies investigating the transcriptional pathway of AREG induction during hypoxic conditions. For example, a previous microarray analysis of colon-derived epithelial cells revealed a hypoxia-dependent increase in AREG expression. Similarly, the authors of this study found that AREG expression was also induced in tissues from mice exposed to whole animal hypoxia. Interestingly, and consistent with the present findings from myocardial ischemia and reperfusion injury, this study revealed that AREG induction was independent of the classic transcriptional response mediated via HIF1-αalpha, but rather indicated an evolutionarily conserved cAMP response element (CRE) that constitutively binds the CRE binding protein (CREB) in AREG induction during hypoxia. More recent studies from the cancer field provide direct evidence for a functional role of HIF2α in the induction of AREG expression during conditions of hypoxia via direct binding of HIF2α to the AREG promoter, and thereby increasing its transcriptional activity. These studies found that the PHD-HIF2α-AREG pathway influences breast cancer progression and suggest PHD2 as a potential tumor suppressor in breast cancer. However, the functional role of HIF2α-dependent induction of AREG in myocardial ischemia and reperfusion injury has not been previously examined. Indeed, our findings implicate the PHD-HIF2α-AREG pathway in mediating increased ischemia tolerance of the myocardium.

The present studies provide pharmacologic evidence that AREG-mediated cardioprotection involves the growth factor receptor ERBB1 receptor. Specific inhibition for the AREG receptor ERBB1 produced a similar phenotype in mice as genetic deletion of Areg. Consistent with these findings, previous studies had demonstrated that AREG binds to the ERBB1 receptor at a low affinity, thereby causing constant activation of ERBB1. This distinguishes AREG from other endogenous ligands for the ERBB1 receptor. For example, EGF binds to ERBB1 with high affinity, causing a strong and rapid activation, but it also causes receptor internalization and subsequent degradation, thereby terminating its signaling events. Moreover, several potential mechanisms could influence how the HIF2A-AREG-ErbB1 signaling cascade provides cardioprotection. Previous studies have found a link between ErbB1 signaling and the inhibition of apoptosis via activation of “survival kinases.” For example the ErbB1/ErbB2 heterodimer was found to activate AKT, which in turn blocks p53—a master regulator of apoptosis. In the present study, AREG expression during myocardial ischemia, exogenous AREG administration of Hif2a expression, and HIF2α-alpha stabilization all increased the activated form of AKT. This suggests that AKT activation in cardiomyocytes upon ischemia-reperfusion is executed by HIF2-αalpha and AREG. Indeed, previous studies found a dominant role of AKT activation in ischemic preconditioning. Since Hif2aloxP/loxPMyosin-Cre+ undergo ischemic preconditioning, whereas Hif2aloxP/loxPMyosin-Cre+ do not, we consider that AKT activation in cardiomyocytes during ischemia preconditioning is driven by HIF1-αalpha. Independent of AKT activation, ERBB1 reduces apoptosis via inhibition of caspase-3 and caspase-9 activity. However, other studies found that apoptosis contributes to myocardial injury after days or maybe weeks. In the acute setting, such as used in the present study, necrosis mainly determines infarct sizes and the influence of apoptosis to myocardial injury is considered rather limited. Thus, we believe that in our study, the anti-apoptotic effect of AREG-ERBB1 signaling only has a minor impact on infarct sizes. Other studies have indicated that AREG signaling dampens acute inflammation, such as occurs in the context of myocardial ischemia and reperfusion injury. For example in a model of skeletal muscle injury, regulatory T cells (Treg) strongly upregulate AREG, while treatment with recombinant AREG enhances muscle repair after injury via suppressive Treg. Other studies suggest that ERBB1 receptor activation promotes the glycolytic capacity, and thereby provide optimization of cellular metabolism during conditions of limited oxygen availability—such as occurs during myocardial ischemia.

In patients with IHD we found a strong induction of AREG protein levels. This is consistent with a previous microarray study showing a 16-fold increase of AREG transcript levels and a 3-fold increase in protein expression in patients undergoing coronary bypass surgery. In view of the present findings, the AREG transcript and protein increase during bypass surgery is likely caused by stabilization of HIF2α-alpha protein levels, and could reflect an endogenous protective pathway. Indeed, in the present studies, treatment with recombinant AREG provides robust cardioprotection from ischemia and reperfusion injury (see Fig. 7). Together, these findings suggest that pharmacologic HIF activators (particularly for HIF2α) or treatment with recombinant AREG could be used to prophylactically treat cardiac surgery patients to enhance myocardial ischemia tolerance, and thereby improve outcomes of these major surgical interventions. Similarly, patients at risk for myocardial injury could receive such a treatment approach.

Taken together, the present studies identify a previously un-identified role for myocyte-specific HIF2α-alpha in protection...
from myocardial ischemia and reperfusion injury. Extensions of these findings suggest HIF2A-dependent induction of AREG in cardioprotection from ischemia, and implicate pharmacologic strategies that stabilize HIFs or promote AREG signaling in cardioprotection from ischemia and reperfusion injury.

Methods

Cell culture and hypoxia. HCM were purchased from ScienCell Inc. (Carlsbad, CA). During the experiments, we rehydrated lentiviruses or stably transfected cells in culture medium supplemented with 0.2% Tween 20 and 4% bovine serum albumin. The antibody to β-actin (clone AC-15; catalog-# 4970) and phosphorylated Akt (clone D25E6; catalog-# 13038). The anti-AREG antibody that we used was purchased from R&D Systems (Minneapolis, MN), and was raised in rabbits against recombinant human AREG followed by 5 min of reperfusion, before 60 min of ischemia and 120 min of reperfusion. We used untreated littermates of the same genotype as the recipient due to logistic or other reasons. In case of the nonfailing donor hearts, the family/next of kin gave permission for organ/tissue donation for purposes of transplantation and research. Ischemic cardiac tissue was obtained from the left ventricle of patients with ischemic cardiomyopathy, in whom the hearts were explanted during the process of orthotopic heart transplantation.

Transcriptional analysis. Primer set (sense sequence and antisense sequence, respectively) for murine Amphiregulin was as following: Areg (Qiagen, Cat# QT00069587); in case of human cell samples the following primer sets were used: AREG (Qiagen, Cat# QT02450469); HIF1A (Qiagen, Cat# QT00083664); HIF2A (Qiagen, Cat# QT00069587). Each target sequence was amplified in human cardiac tissues. Collection and use of these samples was approved by the "University of Colorado–Division of Cardiologyp Biobank". Control cardiac tissues were derived from the left ventricle of healthy hearts considered for transplantation, but could not be transplanted into a recipient due to logistic or other reasons. In case of the nonfailing donor hearts, the family/next of kin gave permission for organ/tissue donation for purposes of transplantation and research. Ischemic cardiac tissue was obtained from the left ventricle of patients with ischemic cardiomyopathy, in whom the hearts were explanted during the process of orthotopic heart transplantation.

Immunoblotting experiments. To measure Hif1α, Hif2α, Areg, total AKT, or phosphorylated AKT protein content in the post-ischemic murine myocardium, we euthanized the animals after 60 min of myocardial ischemia and 120 min reperfusion. Subsequently, we flushed the circulation via an arterial catheter with ice-cold normal saline solution, identified the myocardial tissue that underwent ischemia and immediately shock frozen this section of the heart in liquid nitrogen. For immunoblotting studies, murine or human tissues samples were homogenized and lysed for 10 min in ice-cold lysis buffer (150 mM NaCl, 25 mM Tris, pH 8.0, 5 mM EDTA, 2% Triton X-100, and 10% mammalian tissue protease inhibitor cocktail; Sigma-Aldrich). For immunoblotting studies in HCM, we lysed the cells in 1% Triton X-100 containing extraction buffer at 14°C in 5 μg of Protease Inhibitor Cocktail (Catalog # 76000) and added 1 ng of Phalloidin (Catalog # A22287). Samples were solubilized in reducing Laemmli sample cocktail and were then boiled for 5 min. Samples were subsequently resolved on a 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in phosphate-buffered saline (PBS) supplemented with 0.2% Tween 20 and 4% Bovine serum albumin. The antibody to detect human Hif1A was purchased BD transduction laboratories (clone 54/HIF-1α; catalog-# 610958). From Novus Biologics we purchased antibodies to detect human Hif1A (clone H1A1p676; catalog# NB105-105) or Hif2α (clone ep190b; catalog# NB100-132). Antibodies were purchased from Santa Cruz Biotechnology to blot for human AREG or murine Areg (clone G-4; catalog# sc-74501). From Cell Signal Technology, we purchased an antibody to detect total Akt (clone 40D4; catalog# 2920) and phosphorylated Akt (clone D25S6; catalog # 13038). The anti-actin antibody (clone JLA20; catalog# CP01) was purchased from EMD Millipore. The membranes were incubated with the primary antibody in 10 μg/ml (dilution 1:1000-1:5000) for 1 h and then washed with PBS. The membranes were then incubated in 1:5000 goat anti-mouse horseradish peroxidase. The wash was repeated, and proteins were detected by enhanced formalin for fixation overnight. One day after the experiment, the heart slices were put between a histology slide, with a clamp on each side and photographed with a digital camera. Then, AR and the infarct size were determined by planimetry using the ImageJ (National Institutes of Health). The infarct sizes were determined, using the following formula: infarct size = size of infarct/size area-at-risk × 100. We have previously described the details of this technique.

Knockdown of HIF1A and HIF2A in vitro. Stable cells cultures with decreased HIF1A and HIF2A expression were generated by lentiviral-mediated shRNA expression. Sigma’s MISSION pLKO.1 lentiviral vectors targeting HIF1A had shRNA sequences of CCGGCCAGTTATGATTGTGAAGTTACTCGAGTAACCAGATCTCCTCATGGTTTTT (TRCN0000003807). For controls, non-targeting shRNA sequence of CCGGCCAGTTATGATTGTGAAGTTACTCGAGTAACGAGATCTCCTCATGGTTTTT was used. HEK293T (American Type Culture Collection) cells were co-transfected with pLKO.1 vectors and packaging plasmids to produce lentivirus. Filtered supernatants and 8 μg/ml of Polybrene were used for infection of HCM and cells were selected with puromycin dihydrochloride (Sigma) (2 μg/ml).

Mice. All animal procedures were performed in a AAALAC-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals approved by the University of Colorado Denver Institutional Animal Care and Use Committee. We used male mice with an age of 8- to 16-week-old mice in all studies. All experiments fulfilled the NIH guidelines for use of live animals. To generate cardiac myocytes specific-deletion, Mysos-Cre+ (B6.FVB(129)Tg(Mybht cre/Ese1)), Hif1αfl/fl (B6.129-Hif1atm3Royj), and Hif2αfl/fl (Epsad1tm1Mcs/l) were purchased from Jackson Laboratory (Bar Harbor, ME) and crossed. To induce Cre-recombinase activity, mice underwent tamoxifen treatment for five consecutive days with 1 mg i.p. per day. Tamoxifen was dissolved in sterile peanut oil and administered in a volume of 100 μl. Between the last tamoxifen injection and experimentation were at least 7 days for recovery. Areg−/− (B6;129-Aregtm1Mmcr/Mmcr) mice were purchased from Mutant Mouse Regional Resources Centers (Chapel Hill, NC). Areg+/− mice were maintained using heterozygous breeding strategy (Areg+/−) to bypass lactation difficulties in younger females. As controls, we used mice with the same genetic background as Areg−/− (B6;129) mice. Genotyping in all strains used was performed by GeneTyper Inc. (NY, USA).

Murine model of myocardial ischemia. Mice underwent in situ myocardial ischemia and reperfusion injury as described previously. In short, we used sodium pentobarbital to induce and maintain anesthesia. For induction we administered sodium pentobarbital in a dose of 70 mg/kg i.p., to maintain anesthesia in a dose of 10 mg/kg/h i.p. The mice rested on a temperature-controlled heated table (RT, Effenberg, Munich, Germany) and were mechanically ventilated with a pressure-controlled ventilation mode (ventilator settings: inspiratory pressure 10 mbar, frequency 110 breaths/min, positive end-expiratory pressure of 5 mbar, FiO2 0.4). After thoracotomy, we visualized the left coronary artery (LCA), and connected the weights to each end of the suture 10 mbar. The LCA changed from bright red to pale and an ST-elevation on the connected EKG (from red to dark violet). In addition, the coloration of the myocardium supplied by the LCA changed from bright red to pale and an ST-elevation on the connected EKG was visible. After 60 min of ischemia, the weights were taken off and reperfusion occurred. This was followed by reversal by reperfusion at 14°C to a tissue described above. In a subset of experiments, mice underwent ischemic preconditioning as described previously. In short, ischemic preconditioning repre-
chemiluminescence. Densitometry was performed using ImageJ 1.51i as described. Full gel scans of all western blots presented in the figure can be found in the supplementary figures (Supplementary Figs. 1–6).

Recombinant murine Areg treatment. Carrier-free, recombinant mouse Amphiregulin protein (Areg) was purchased from R&D Systems (Minneapolis, MN). In short, Amphiregulin was dissolved in 0.9% NaCl solution and 10 µg were administered over 15 min by a syringe pump connected to an indwelling carotid artery catheter. When the injection was complete, we began the in situ myocardial ischemia.

AG1478 treatment. The ErbB1 inhibitor AG1478 was purchased from Tocris (Bristol, United Kingdom). AG1478 was dissolved in 100 mM Captisol (Cydex, Overland Park, KS) at a concentration of 5 mM as described. Prior myocardial ischemia, we administered 200 mmol/l/kg AG1478 over a 15 min period via the carotid artery catheter connected to a syringe pump. Finally, we continued with our myocardial ischemia model.

Cetuximab treatment. Cetuximab (Erbitux®) was purchased from Bristol-Myers Squibb. This monoclonal antibody is specifically directed against the extracellular domain of ErbB1, thereby completely blocking signaling through this receptor.

Immediately prior to myocardial ischemia, we diluted cetuximab in a sterile, isotonically adjusted sodium chloride solution and administered 20 mg/kg over a 15 min period via the carotid artery catheter connected to a syringe pump. Finally, we continued with our myocardial ischemia and reperfusion injury as described above.

Dimethylxaloylglycine treatment. Dimethylxaloylglycine (DMOG) was purchased from Sigma-Aldrich. DMOG permeates the cells and inhibits this prolyl-4-hydroxylase, which upregulates HIFs. As described previously, mice received 1 mg DMOG via a single intraperitoneal injection 4 h prior to myocardial ischemia and reperfusion injury—as described above.

Data analysis. Based on previous studies, we performed an a priori sample size analysis for infant sizes (standard deviation, SD, 5%) and serum troponin measurements (SD 21 ng/ml)57. We consider a difference biologically relevant, when the p-value is less than 0.05 to be statistically significant. For multiple comparisons, we used the unpaired Student’s t-test (StatMate® and Prism® software). Statistical analysis was performed using the Student’s t-test (StatMate® and Prism® software). Statistical analysis was performed using the Student’s t-test (StatMate® and Prism® software). Statistical analysis was performed using the Student’s t-test (StatMate® and Prism® software). Statistical analysis was performed using the Student’s t-test (StatMate® and Prism® software). Statistical analysis was performed using the Student’s t-test (StatMate® and Prism® software).
36. Ryan, H. E., Lo, J. & Johnson, R. S. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *EMBO J.* 17, 3005–3015 (1998).

37. Du, M. et al. Renalase is a novel target gene of hypoxia-inducible factor-1 in protection against cardiac ischaemia-reperfusion injury. *Cardiovasc. Res.* 105, 182–191 (2015).

38. Ong, S. G. et al. HIF-1 reduces ischaemia-reperfusion injury in the heart by targeting the mitochondrial permeability transition pore. *Cardiovasc. Res.* 104, 534–545 (2014).

39. Adiru, R. S. et al. Disruption of hypoxia-inducible transcription factor-prolyl hydroxylase domain-1 (PHD-1/-) attenuates evx vivo myocardial ischemia/reperfusion injury through hypoxia-inducible factor-1alpha transcription factor and its target genes in mice. *Antioxid. Redox Signal.* 15, 1789–1797 (2011).

40. Horstman, J. et al. Hearts of hypoxia-inducible factor prolyl 4-hydroxylase-2 hypomorphic mice show protection against acute ischemia-reperfusion injury. *J. Biol. Chem.* 285, 13646–13657 (2010).

41. Holscher, M. et al. Cardiomyocyte-specific prolyl 4-hydroxylase domain 2 knock out protects from acute myocardial ischemic injury. *J. Biol. Chem.* 286, 11185–11194 (2011).

42. Sarkar, K. et al. Hypoxia-inducible factor 1 transcriptional activity in endothelial cells is required for acute phase cardioprotection induced by ischemic preconditioning. *Proc. Natl. Acad. Sci. USA* 109, 10504–10509 (2012).

43. Steinh, D. P. et al. Non-canonical HIF-2alpha function drives autonomous breast cancer cell growth via an AREG-EGFR/ErbB4 autocrine loop. *Oncogene* 31, 2283–2297 (2012).

44. Carvalho, S. et al. An antibody to amphiregulin, an abundant growth factor in fluids, inhibits ovarian tumors. *Oncogene*. https://doi.org/10.1038/onc.2015.93 (2015).

45. Sorkin, A. & Goh, L. K. Endocytosis and intracellular trafficking of ErbBs. *Exp. Cell Res.* 231, 3093–3106 (2006).

46. Henson, E. S. & Gibson, S. B. Surviving cell death through epidermal growth factor receptor (EGFR) signaling. *Cell Death Differ.* 9, 1139–1148 (2002).

47. Zhou, B. P. et al. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat. Cell Biol.* 3, 973–982 (2001).

48. Rossello, X., Riquelme, J. A., Davidson, S. M. & Yellon, D. M. Role of PI3K in myocardial ischaemic preconditioning: mapping pro-survival cascades at the trigger phase and at reperfusion. *J. Cell Mol. Med.* https://doi.org/10.1111/jcmm.13394 (2017).

49. Peng, X. H. et al. Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1alpha signal pathways increases resistance to apoptosis by up-regulating survivin gene expression. *J. Biol. Chem.* 281, 25903–25914 (2006).

50. Krijnen, P. A. et al. Apoptosis in myocardial ischaemia and infarction. *J. Clin. Pathol.* 55, 801–811 (2002).

51. McCully, J. D., Wakiyama, H., Hsieh, Y. J., Jones, M. & Levitsky, S. Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury. *Am. J. Physiol. Heart Circ. Physiol.* 286, H1923–H1935 (2004).

52. Zaiss, D. M. et al. Amphiregulin enhances regulatory T cell-suppressive function via the epidermal growth factor receptor. *Immunity* 37, 275–284 (2013).

53. Zaiss, D. M., Gause, W. C., Osborne, L. C. & Artis, D. Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. *Immunity* 42, 216–226 (2015).

54. Burzin, D. et al. A special population of regulatory T cells potentiates muscle regeneration. *Cell 155*, 1282–1295 (2013).

55. Makinoshima, H. et al. Epidermal growth factor receptor (EGFR) signaling regulates global metabolic pathways in EGFR-mutated lung adenocarcinoma. *J. Biol. Chem.* 289, 20813–20823 (2014).

56. Voisine, P. et al. Differences in gene expression profiles of diabetic and non-diabetic patients undergoing cardiopulmonary bypass and cardioplegic arrest. *Circulation* 110, I1280–I1286 (2004).

57. Koeppen, M. et al. Adora2b signaling on bone marrow derived cells dampens myocardial ischemia-reperfusion injury. *Anesthesiology* 116, 1245–1257 (2012).

58. Eckle, T., Koeppen, M. & Eltzhig, H. Use of a hanging weight system for coronary artery occlusion in mice. *J. Vis. Exp.* 19, 2526 (2011).

59. Vassileva, I. et al. Systematic evaluation of a novel model for cardiac ischemic preconditioning in mice. *Am. J. Physiol. Heart Circ. Physiol.* 291, H2533–H2540 (2006).

60. Pfaff, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45 (2001).

61. Gassmann, M., Grenacher, B., Rohde, B. & Vogel, J. Quantifying western blots: pitfalls of densitometry. *Electrophoresis* 30, 1845–1855 (2009).

62. Johns, T. G. et al. Antitumor efficacy of cytotoxic drugs and the monoclonal antibody 806 is enhanced by the EGF receptor inhibitor AG1478. *Proc. Natl. Acad. Sci. USA* 100, 15871–15876 (2003).

Acknowledgements

The authors would like to thank Megan Weiland, Stephanie Bonney, and Kristan Magee for their technical assistance and Sean Colgan for providing expert opinion and feedback. Furthermore, we would like to thank The Colorado Clinical & Translational Sciences Institute (CCTSI) for providing and maintaining the BEDCap database of de-identified tissue samples (NIH/NCRR CCTSI Grant Number UL1 RR025780). The present study is supported by National Institute of Health Grants R01 DK097075, R01–HL092188, R01–HL098294, PO1–HL114457, and R01–HL119837 to H.K.E.; National Heart, Lung, and Blood Institute (NIH-NHLBI) Grant 1R01HL102267 and R01HL112247–01 to T.E. and Deutsche Forschungsgemeinschaft (DFG) research fellowship to M.K.

Author contributions

M.K. designed the study, performed and designed experiments, analyzed and interpreted data, and wrote the manuscript; J.W.L. performed experiments, analyzed data, and edited the manuscript; S.W.S., and K.S.B. performed experiments, and analyzed data; S.K. helped with the study design; I.V.Y. supported the design of microarray analysis, performed the microarray studies and statistical analysis, and assisted with data analysis and interpretation; P.M.B. directs the University of Colorado–Division of Cardiobiokeid and provided important research tools; T.E. assisted with study design and data analysis and edited the manuscript; H.K.E. designed the study, interpreted data, and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-03105-2.

Competing interests: The authors declare no competing financial interests.

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